

A Review of NS Protein Roles in Dengue Virus Transmission and their Inhibitory Factors

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Abstract: *The Dengue virus is an RNA virus which lacks an effective vaccine. Its transmission is due to the virion's 3 structural and 7 nonstructural proteins. The nonstructural proteins each play a specific role in impacting the interferon - mediated viral defense system, viral replication, initial viral delivery, and host body symptoms. By inhibiting these proteins, we can prevent viral replication within the host cell through the Dengue virion. The review highlights the protein functions to provide a clear scope for researchers to build vaccination trials off of, especially the protein specifics instead of a broad perspective, and develop questions for virus inhibition.*

Keywords: Microbiology; Virology; Dengue Virus; NS Proteins; Viral Replication

1. Introduction / Literature Survey

Dengue fever is an exponentially growing virus, with cases initially starting at five hundred thousand clinical diagnoses in 2000, and rising to 4.2 million in 2019, purely clinically diagnosed, reported cases. ⁴ The risk of infection for the virus is present in around 130 countries globally, but most cases have been distributed in Asian countries. The geography regarding disease transmission is an essential factor in the impact of Dengue fever, greatly due to constant circulation within specific regions, like Asia, and sudden explosive outbreaks as displayed by the American area 3.1 million cases in 2019. ⁴ The unpredictable changes in geography regarding disease transmission have led to it being an essential basis for scientific inquiry since the early 2000s. ⁴ Due to these erratic spikes in transmission, Dengue fever has significantly been studied to develop viral vaccinations. However, most vaccinations have yet to effectively work due to the damaging side effects outweighing the positive impact of all distributed and studied vaccines.

Regarding studying vaccination, the transmission of Dengue fever is a central component as vaccination aims to inhibit the transmission of any disease. The mosquitoes of the *Aedes aegypti* and occasional *Ae. albopictus*, which are specifically female, as members of the Flaviviridae family, pose an initial threat to humans acquiring the virus. ¹ The transmission cycle of Dengue fever initially starts off with humans having the specific disease, and mosquitoes take in blood from the experiencing human viremia. ¹ The virus binds to receptors on the cell surface of the midgut epithelium in the mosquito and sufficiently replicates its viral RNA in the mosquito. ¹ Dengue fever contains viral RNA, referred to as a positive - sense RNA that can be directly translated into proteins within the host cell. ² The viral genome encodes ten genes which are translated as long, single polypeptides cut into ten proteins. The virus replication and infection are also specifically completed in the salivary glands of the mosquito, making them vessels of transmission, transporting virions within their salivary glands, from the blood of one human to another, transmitting the viral RNA of the Dengue fever to the new human through the biting and the use of salivary glands. ⁸ Envelope

proteins on the Dengue fever virion use endocytosis to enter the human cell and cause a conformational change of the cell as entering. ¹⁰ As a structure, Dengue fever has RNA that is encapsulated in capsid and a membrane of M and E proteins in a spherical formation, allowing for the envelope to have entry. ²

Once the virion has entered the cell, it combines with the endosomal membrane. Then it is released into the cytoplasm, and the viral particle then releases the genome as it comes apart. The viral RNA is translated, as positive - sense, into the ten proteins from the chains of polypeptides, being able to replicate inside of the cells. ² As this occurs near the endoplasmic reticulum, where protein synthesis happens, the newly developed RNA is released from the endoplasmic reticulum. The proteins are then transported to the Golgi apparatus for distribution across the infected cell and from the infected cell. ¹⁰ They then mature in order to be transported out of the cell, in an infectious form, to continue the process of replicating in or infecting other cells. ²

For the replication to occur of the viral RNA and the interaction with the endoplasmic reticulum, the NS proteins come into play, all six of them. The NS proteins come from the polypeptide and are the proteins from 7 of the ten proteins encoded in the viral genome. ² They play a crucial role in the replication cycle of the Dengue virus when entering a host cell. These proteins are also greatly involved in inhibiting the natural protection of our immune system by playing a role in inhibiting the IFN mediated viral defense system. ¹⁰ The IFN mediated viral defense system aids host cells in producing proteins to prevent viral replication, however as the Dengue Fever NS proteins play a role in inhibiting this system, the key component of our immune response.

Viruses replicate by making sure their RNA is translated in the cell into a certain number of proteins, which then aid in the replication process through the synthesis from the endoplasmic reticulum and the spread of the replicated virus in the Golgi apparatus. ² Without the seven encoded proteins, the Dengue virus would not be able to replicate its RNA in a host cell, so in order to develop a sufficient vaccine to inhibit the transmission of the virus, the seven

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proteins should be some of the key components addressed.¹⁰ Researchers have aimed much of their vaccine development regarding viral replication and the NS proteins. However, a lack of compiled information has increased the time and lack of information regarding vaccine development, resulting in less vaccination regarding the lesser - known NS proteins, all of which are crucial to the RNA replication process.

A multitude of vaccines has been developed for Dengue fever over the years. However, most have not been passed, and even when passed, they were rescinded due to the side effects nullifying the effectiveness of the treatment.⁶ Previous vaccine candidates have included vaccines like Dengavaxia, which have involved a live virus or a virus that is not live being injected into the body in order to increase immunity and inhibit replication. Some have included some of the ten encoded proteins, and many of them have included the use of the NS proteins. They have also included the capsid proteins due to their importance in replication or invasion. However, the trend viewed in the potential, but unused in clinical version, vaccination was that they focused on the capsid proteins, signaling pathways, and purely the highly studied NS1 protein.⁶ Most vaccine candidates did not take into account, for example, the NS5 protein candidate, a key to endoplasmic reticulum replication and inhibition of the interferon defense system.

Regardless of the type of Dengue fever type, as they only differ symptomatically in terms of joint pain and retro - orbital pain, understanding the specific roles of each type of NS protein, the viral replication, immune response, and invasion can aid in the development of future vaccine candidates. There is still potential discovery regarding the NS4 proteins, NS3 proteins, NS2 proteins, more specifically, but also to the more well - known NS proteins like the NS1.³ Future treatments can start to rely on these NS proteins as well due to more developed research being presented because, as seen by the previous vaccine candidates, vaccines focus on the most understood aspect of a disease, such as signaling pathways or the capsid proteins. By adhering to such trends, understanding the NS proteins more in - depth can shift the focus onto those components as well in vaccine trials. This research study is going to explore what is understood about the NS proteins in dengue virus replication and what remains to be discovered. A better understanding of the NS proteins can aid in the treatment development for the dengue virus by identifying the specific roles in replication and preventing the immune response that they play.

2. Discussion

2.1 NS1 Protein

Dengue fever has seven nonstructural proteins that are important for replication, one of them being the NS1 protein which is all collectively vital. The NS1 protein, specifically, is a 48 - kDa glycoprotein that has been found in a range of other flaviviruses, and it is especially involved in the viral RNA replication occurring during Dengue fever transmission. While it is generally thought that the NS1 protein plays a role in viral replication, the precise nature of this role is unknown, though it has been shown to interact

with the NS4A and NS4B proteins which both do play a role in viral replication. NS1 also plays a key part in inhibiting the IF - mediated viral defense system of natural immune system response in order to further the transmission of the virus.³ Furthermore, the NS1 proteins play a role in vascular leakage, coagulopathy, and thrombocytopenia. When viral replication occurs, the proteins need to be transported through the Golgi apparatus and the Endoplasmic reticulum³. The NS1 protein has been found to be involved in the important modification of viral proteins that occurs in the lumen of the ER and Golgi. Due to these numerous discovered functions of the NS1 protein, it has been widely targeted for Dengue fever treatment strategies, specifically in terms of inhibiting the protein function through binding and inhibiting the effects that the NS1 protein has in different components of the virus impact.³

The initial structure of the NS1 protein is that of a monomer, which after post - translational modification, form homodimers pertaining to the membranes of different components of the cell and the cell itself. Even though the exact mechanisms of the NS1 protein have yet to be discovered, it has a connection to the glycosyl - phosphatidylinositol and lipid rafts.³ The NS1 protein is also noted to be the only NS protein that is secreted through infected cells continuously. The structure of the secreted protein is a hexamer, much like a high - density lipoprotein. The high lipid content is an important part of the protein's secretion due to it aiding in attaching to the cell membrane and interacting with glycosaminoglycans. The structure of the protein is important to coagulation as rich lipid content interferes with the coagulation cascade, and the accumulation of the proteins has been found to play a part in the disease's critical phase. It has even been found that the concentration of NS1 secretion has correlated with disease severity, which is a prospect that is important to look into when understanding possible treatment options.³ Due to its important role in secretion, the NS1 protein has also been found to protect the virion from complement - dependent lysis due to promoting C4 degradation.²⁴ The NS1 proteins have a significant impact due to their secretion and structure, which can influence future innovation regarding disease inhibition. An example of such would be the relationship between NS1 secretion and disease severity. For high - risk patients, preventing this accumulation can prevent high disease severity for an easier recovery. One way to do so could be through the use of sugars, as they are known for preventing the secretion of glycoproteins. Another possible treatment option to explore would be finding a binding protein using molecular drug data banks to find a molecule to bind to the NS1 protein in order to inhibit further involvement and thus keeping away the great impact the NS1 protein has on secretion and replication. A recent study has applied this in terms of peptide synthesis by involving four random peptides conjugated with N - terminal fluorescent tag and C - terminal cell against the production of the virus itself by binding to the NS1 protein.²⁴ However, this is not applicable to the serotypes and does not completely reduce the production of the disease in a host's body. Overall the structure of the protein is important for being analyzed in order to further understand possible vaccination options for the production of the DENV virus in a body.

The IFN - mediated - viral defense system is the immune response of a host that protects from the replication of viral RNA. The IFN - mediated - viral defense systems contain interferon, which are proteins used to signal nearby cells to strengthen their antiviral defenses. The NS1 protein is one of several nonstructural proteins that inhibits interferon synthesis, which helps the virus overcome a host cell's defense and start the replication process. As the NS1 protein also interferes with the complement system, it is clearly a prevalent factor in the immune invasion of the virus. By finding an inhibiting molecule to the NS1 protein, the host immune response can be advanced as a supplement because the interferons would no longer be reduced in production and signaling due to one of the interfering proteins.

Another prevalent impact of the NS1 protein is that it contributes to disease severity not only by accumulation from continuous secretion but also by inducing interleukin (IL - 10) through monocytes.²⁵ The IL - 10 has been found to suppress dengue - specific T cell response, and pathogenesis is increased by the NS1 protein as it promotes the production of the IL - 10.⁵ Through this impact on the immunoprotective role of the T - cells, the NS1 proteins play another minor, complementary role in the immune system.

Dengue fever plays a role in the pathogenesis of the virus as presented by the impact on IL - 10, but also due to its impact on vascular leakage. Vascular leakage in many Dengue fever patients is reversible and quick, which is explained by autophagy - mediated junction disruption.²⁵ A connecting component that could be further explored is the NS1 - induced macrophage migration inhibitory factor secretion (MIF) involved in the NS1 related autophagy in the infected cells.²⁵ The MIF concentration is highly related to disease severity and endothelial hyperpermeability. In addition to affecting endothelial junctions, NS1 causes vascular leakage by inducing endothelial glycocalyx degradation mediated by heparanase - 1.⁵ MIF is also involved in NS1 related HPA - 1 and MMP - 9 secretion and degradation of the endothelial glycocalyx.³ All of these contribute to the immune response and vascular leakage caused by the NS1 protein, separate from the IFN - mediated - viral defense system. These can be further investigated due to their involvement in physiological processes and modulating homeostasis/inflammatory responses. Prospective studies include further analyzing the structure of the MIF and supplementing the endothelial glycocalyx as a treatment factor with previously utilized drugs like Endocalyx.

Another potential exploration factor would be to further understand how the NS1 is involved in thrombocytopenia, as it is known that the NS1 protein has an impact on platelet activation and aggregation, a key part in coagulopathy as well. The NS1 and LPS can activate immune cells with the use of TLR4.³ As seen, the NS1 is predicted to induce platelet activation and enhance aggregation, possibly leading to the over - destruction of platelets during dengue infection.³ This component also correlates to vascular leakage, another great impact of the NS1 protein on the host function.

The function of the NS1 protein is not really specified to one job, creating this idea of ambiguity with its function in replication as well, but a higher understanding of its impact

on disease severity, complement activation, and immune response. Many previous prospective treatments that have aimed to find a molecule to bind to the NS1 protein have not really functioned well either in host response or barely playing a part in preventing disease transmission. This could be understood by the idea that there is not much known about the role the NS1 protein plays in viral RNA replication, so specific strategies cannot be created, and this may also indicate that the role itself is not significant and can be supported by other key replication proteins like the NS5 protein. In order to aid vaccine development and treatment, it is important to further study the viral replication that is contributed to by the NS1 protein but also starts to focus treatment strategies on not only binding to the protein but also pertaining to its impact on complement activation and immune response. Many of the other proteins like the RNA - dependent - RNA polymerase of NS5 play greater roles in replication, so by inhibiting the NS1 protein, the other proteins may have made up for the protein in terms of the RNA. However, the NS1 protein plays a clear, important role in other factors and severe implications of Dengue fever. Prospective treatment could include exploring sugars for preventing secretion and understanding more about vascular leakage for treatment and prevention of highly severe Dengue fever cases.

2.2 NS2A Protein

The nonstructural protein 2A of the Dengue virus is a key part of viral replication, specifically interfering with immune response and virion assembly. It is also known to behave as a viroporin in the DENV - 2 serotype and as a 22 - 25 kDa hydrophobic transmembrane protein that is a part of viral replication, viral assembly, viral release, inhibiting the interferon, and interfering with the JAK - STAT pathways. The NS2A protein is known to associate with the Endoplasmic reticulum. However, only recently have studies revealed the topological specifics of the protein interactions that result in the NS2A's function of virion assembly and release.¹⁶ The protein is also known for creating protein - protein and membrane - protein interactions.²⁸ However, most of its functions within the viral cycle and its active regions are widely unknown.²⁰ The NS2A protein is also known as a highly hydrophobic protein, and the membrane is necessary in order for the protein to perform its functions. Much of the recent research has defined that the NS2A protein has ten regions with significant hydrophobic and interfacial values alone. Along with that study and the interactions of these regions or residues with peptides, it was found that the peptide 25 molecules were a large component in understanding the needed interaction between the NS2A protein and the membrane for viral replication.¹⁶ Recently, along with membrane interaction discoveries, it was found that NS2A protein had a correlation to "NLRP3 inflammasome activation, and further apoptosis - associated speck - like protein containing caspase recruitment domain (ASC) oligomerization, and IL - 1 β secretion through caspase - 1 activation".²² It was even found that the NS2A protein coordinates nucleocapsid and virus formation. Overall the NS2A does play a combined role in membrane - protein interaction, virion assembly, immune response, specific pathway interaction, nucleocapsid interaction, and more. However, it is still unclear the exact underlying

mechanisms that cause it to impact viral replication and much more of these functions.

The topological analysis of the NS2A protein has really developed an understanding of the NS2A protein and the membrane, specifically virion assembly and maturation. A previous study analyzed how the NS2A protein on the ER membrane would interact, which showed how the first transmembrane segment of the protein consisted of two helices separated by a helix breaker mediated by the R84 and P85 amino acids.²⁸ It was also found that the R84 amino acid was an important part of viral assembly and release and viral synthesis. This finding should be further explored by analyzing the structure of the ligand for possible inhibitory molecules such as Arg - 84 previously used in an E. coli study. The structural study also revealed that transmembrane segment 2 interacts with the ER function in virion replication, specifically on the side of the lumen, which could be further modeled for druggable molecules.¹⁶ Another transmembrane segment, number 3, was actually also found to be greatly impactful in terms of assembly and maturation of the virion due to it containing two charged residues of K82 and R84. The R84 residue was especially significant as when it was mutated in the experiment, viral synthesis and viral production were reduced, in turn reducing the efficiency of the assembly and release of the virion.¹⁶ Specifically, the mutant form of R84A displayed no infectious virion cells, again associated with viral assembly and maturation.

The Dengue virus contains many nonstructural proteins and three structural proteins which need to be cleaved for the assembly of the virus. A study found that the NS2A protein plays a great part in nucleocapsid formation, virus formation, recruiting viral RNA, bringing protease to the site of assembly of the virus, and recruiting structural proteins. The final 285 nucleotides of viral 3' UTR work as a signal that "hires" for the purpose of packaging, which then binds to the cytosolic loop of NS2A.¹⁶ The actions help NS2A to gain nascent RNA to bring the virion assembly site to the replication complex. NS2A also transports the C - prM - E polyprotein and NS2B - NS3 protease to the assembly site of the virion through engaging with prM, NS3, and E, leading to the particular C - M - E cleavage. The developed C protein assembles into genomic RNA to form nucleocapsid, followed by the envelopment of prM and E and virion formation. These are the molecular mechanisms that NS2A brings in terms of virion formation, and it can be understood by further examining the cytosolic loop, 285 nucleotides that are mentioned, the 3' UTR color really impacts virion formation, and the entire protein function itself.

The NS2A protein is also characterized as viroporin, specifically in Dengue virus serotype 2, which is due to its ability to permeabilize many different membrane models by forming membrane channels and self oligomerization. The NS2 protein was also found to be a cofactor of viral protease and participated in the degradation of cGAS, which is a cytosolic DNA sensing pathway that is a central component of interferon immune response.²⁸ There are many roles that the NS2 protein plays in a specific manner. Without the real mechanisms of its main role, still, an important discovery to be made. However, one of those minor roles includes

interfering with the JAS - STAT pathways, involved in processes such as immunity, cell division, cell death and tumor formation, and the interferon - mediated viral defense system, especially due to inducing an inflammatory process.¹⁶ The NS2A protein played a part in this inflammation through a significant increase in the expression of NLRP3, ASC oligomerization, caspase - 1 activation, and IL - 1 β secretion in the cell supernatant after priming with LPS.¹⁶ Furthermore, it was demonstrated that IL - 1 β release during DENV infection relays mainly in the assembly of the inflammasome NLRP3 and Caspase - 1 activation. Most basically, it meant that due to the expression of the NLRP3, there was also a demonstration of interleukin involving disease pathogenesis. It was also suggested that NS2A may be involved in intracellular Ca⁺⁺ homeostasis and/or mitochondrial disruption, thereby boosting the activation of the NLRP3 inflammasome that leads to the overproduction of IL - 1 β . The IL - 1 β is a cytokine protein, again reemphasizing the relationship with the NLRP3 with inflammation and inherently the interferon immune system as well. By focusing on the aspect of Caspase - 1 activation that could also impact the interferon response caused by the NS2A protein, the one proposed idea would be to examine further the implications of the Pysin - only proteins as they are known to regulate Caspase - 1 activation through inhibition of inflammasome.

Another component of the NS2A protein would be a peptide part of the protein itself called the peptide dens25 due to its part in membrane model systems, derived from further examining the regions of the NS2A protein. The initial influence of the peptide was found when it was presented that peptide dens25 sectioned with region b and the 18–29 peptides surround a broad region where the understood transmembrane segments of NS2A residue.¹⁶ Peptide dens25 has a particularly great leakage value, impacts the thermotropic behavior of DMPC and DMPG, and has been found to attach to the membrane surface. The peptide is also reliant on negatively charged phospholipids and total membrane surface charge, which affects the stability of the membrane, resulting in CF release, which is reliant on lipid composition and the lipid/peptide molar ratio. Peptide dens25 also impacts membranes at different levels, from the surface of the membrane down to the hydrophobic core. Its location should be at or near the membrane interface, impacting lipid fluidity and having an orientation named in - plane. Moreover, its interfacial properties suggest that this region could act similar to that of a pre - transmembrane domain sectioning into and interacting with the membrane. This being dependent on the membrane composition and/or other proteins which would impact the topology of the membrane.

Overall, the NS2A protein has many structural topologies and components and numerous specific interactions with different proteins, membranes, and virion release. However, the specific mechanisms of viral replication and involvement with the interferon system have yet to be discovered, making it one of the lesser understood nonstructural proteins. However, the idea of a cofactor of viral protease and it acting along with the NS3 protein in the immune response and cytokines displays that there are many dependent components of the protein in how it promotes other key

protein involved in functions like the RNA dependent RNA polymerase, which is the NS5 protein. Many studies have not aimed to find an inhibitory molecule for this protein or utilized it during drug development, but due to how much is known about it can be used as a supplement to these other proteins that keep them sustaining even during vaccine administration and inhibitory molecules, so it is important to conduct further research about this protein to learn more about its viral replication implications.

2.3 NS2B/NS3 Protein

The Dengue virus protease is formed of NS2B and NS3 and is essential to viral replication as it is responsible for the cleavage of 8 of the 13 polyprotein cleavage sites. The cleavage is the main component of the maturation of the virion and is caused by the hand-in-hand function of these two proteins, which form the NS2B - NS3 pro complex that activates the protease function of the NS3 protein. A protease is an enzyme that catalyzes the hydrolysis of proteins and peptides, inherently making it important for the cleavage of polyproteins.

In terms of protease function, both the NS2B and the NS3 work together as NS3 has an active protease domain, while the NS2B has a hydrophilic region that is necessary for the activation of the NS3 protease domain. For the NS3 protein, the N-terminal 167 amino acid residues contained the active protease domain.¹⁹ The NS2B protein, on the other hand, has three hydrophobic regions on the sides of a conserved hydrophilic domain of 40 amino acid residues, approximately proving this relationship between the two proteins. The hydrophobic regions of the NS2B have been found to be disposable for protease activity. However, they are needed for cotranslational membrane insertion of the full NS2B and proper activation of the NS3 protease domain.²¹ A component of the NS3 structure that could be further analyzed would be the C-terminal region of the flavivirus NS3 protease domain, as it contains components that are part of multiple nucleoside triphosphates (NTPase) and the DEXH family of RNA helicases.¹⁹ The RNA helicase activity has resulted in the stimulation of many viral GTPases in many viruses, including Hepatitis C and bovine viral diarrhoea virus p80.¹⁹ This could be further researched to find if there are any viral inhibitors specific to the other viruses that could be applicable to inhibiting this protein, treating the effects of this protein, or helping to supplement immune response against the viral implication of NS2B/NS3.

Another structural component that could be further explored includes that the negatively charged NS2B protein is positioned on a positively charged cleft on the NS3 protein. The subunit interaction plays a critical factor in finding small molecule and peptide inhibitors that stop the proper function of both the proteins via this positioning. One previous study focused on the RC - 1, which is a cationic peptide that has been used previously to inhibit such interaction in diseases like HIV, H5N1 avian influenza, Herpes Simplex Virus, and more. In the study, it was found that the RC - 1 does, in fact, significantly reduce the viral replication of the Dengue fever virion by interrupting this interaction by binding to the positively charged cleft of

NS2B.²¹ Due to the low toxic activities and lack of pro-inflammatory response, the RC - 1 proves to be a significant viral inhibitor of the NS2B and NS3 protein. For future application, the RC - 1 peptide could be researched to see if it impacts viral replication further, but finding if it inhibits any other of the nonstructural proteins or even the capsid proteins, which are all essential to Dengue fever transmission.

The NS3 protein, specifically, has also been found to have a great interaction with many human proteins, including the TRIP 11, ERC1, and the ANP32B. The analysis of these proteins has been found to help predict the interaction between Dengue fever and human transmission due to it being an important part of human cell infection. The TRIP11 protein plays a role in the function of the Golgi apparatus as it is a Golgi microtubule-associated protein, which can explain the component of viral replication in the Golgi apparatus for the virion as a result of the NS3 protein interactions. The ERC1 protein regulates neurotransmitter release and promotes cell migration/release, which is another important role the NS3 protein plays in allowing the virion to transport across cells in order to further infect the host cells. The ANP32B mediates the export of unspliced or partially spliced viral mRNA via interactions which is another complementary role NS3 impacts during in order to allow viral replication.¹³ The interactions between these proteins can, in the future, be further analyzed to see if there is a way to inhibit these small interactions and to see how vital a role they play in viral replication and if there are treatments that can focus on these interactions in order to assist the immune system.

The NS3 protein has not only multiple interactions with human proteins when viral replication occurs but also two nonstructural proteins, which include the NS2B and NS5 proteins. The NS5 protein is an RNA-dependent-RNA polymerase (see below). The interaction between NS3 and NS5 plays a vital role in the balanced synthesis of positive and negative-strand RNA for viral replication. The NS3 protein interacts with the RdRp domain of the NS5 protein, specifically the LYS - 330 residue.¹⁸ It was found that the different NS3 - NS5 interaction - defective mutants can impair infectious virus production, viral protein synthesis, and RNA replication to varying degrees, which is likely to be dependent on the importance of the amino acids that are involved in NS3 - NS5 interaction. By further researching the amino acids that are involved in the interaction, researchers can also inhibit the function in viral replication through small molecules. Previously, many small molecules have been researched to inhibit the NS2B and NS3 interaction, such as the RC - 1, but researchers can also focus on the NS3 and NS5 interaction in order to further find out if viral inhibitors of this interaction can really hinder viral replication.¹⁸

There have been multiple viral inhibitors found for the NS2B and NS3 interaction. However, not many have been found for the NS5 interaction. The RC - 1 could be applicable in this case to see if it also inhibits NS3 and NS5 interaction as it plays a role in the NS2B and NS3 interaction. Other viral inhibitors for the NS2B and NS3 interaction have included curcumin which has stopped viral

maturation and infectivity.²⁷ The viral inhibitors also include Protein - based Allosteric inhibitors, which were found through a molecular docking site for the allosteric binding site and are listed as structures in Table 1 (Table 1).²⁷ All of these molecules could be further applied to see if they play any role in inhibiting other NS2B or even the significant NS3 interaction, both with the host cells and the NS5 protein (Table 1). This could be found using a molecular docking test or by testing the compound themselves, individually on the actual Dengue virus virion and human cells, through a purified protein interaction study. Overall, due to the nature of the NS3 protein to interact with many other proteins and a small component of viral replication, the structures found in docking studies can be applied to multiple components of its function as a protein for both human protein interaction, other Dengue - specific proteins, and the transmission from the mosquito to a human host cell.

2.4 NS4A/NS4B Protein

The NS4A and NS4B proteins of the Dengue virus are involved in assisting in ER - associated replication and the integration of the Dengue proteins. The proteins are highly hydrophobic transmembrane proteins responsible for the membrane arrangements leading to the formation of the viral replication complex. The NS4A protein is also known for inducing autophagy in epithelial cells, which protects the host cell from death and the NS4B protein is a negative modulator of the NS3 helicase formation, which also interferes with STAT 1, an important transcription factor.⁹ The NS4A protein interacts with the NS4B protein in infected host cells, and the cells express the NS4A and NS4B protein together when there is a lack of other viral proteins. There have been multiple active regions and residues found for the NS4A and the NS4B protein in recent studies as they are critical in the replication process of the virus and in the protein - membrane interactions of the DENV, however many of their specific molecular mechanisms are unknown, like the other highly hydrophobic protein, NS2A.¹⁷

The NS4A protein is a highly hydrophobic protein that contains an initial sequence of residues 1 - 49 and does not interact with membranes, and is found to play a role as a cofactor of the NS3 protein. As the NS4A is essential to NS3 function, the codependent relationship displays how in order to inhibit one or the other, both need to be suppressed by a druggable compound/molecule instead of just individually. The NS4A protein, along with the other viral proteins necessary for replication, promotes membrane rearrangement for viral replication due to the protein being found in cytoplasmic foci associated with the endoplasmic reticulum and in reticular structures. This role is further backed up by the NS4A - 2k cleavage site, residues 123 to 130, and a C - terminal fragment named 2k that acts as the signal sequence for translocation of the NS4B protein into the ER lumen as it shows the role the NS4A plays in membrane interaction in coherence with the NS4B protein.⁹ The 2K regulates NS4A's function in modulating the ER membrane through distinct mechanisms. Another function of the NS4A was that it was found to play a role in partaking in the induced autophagy of epithelial cells, which protects the

host cells from cell death. The first 48 amino acids of NS4A were also reported to form an amphipathic helix that mediates oligomerization. Both the NS4A and the NS4B induce the unfolded protein response in the host and inhibit interferon signaling.¹⁷ The NS4A protein as a whole plays the main role as a cofactor of the NS3 protein and as a binding protein to the NS4B protein in order to assist both of the proteins in further carrying out their roles. However, it has additional minor roles in autophagy and membrane rearrangement essential to viral replication.

The NS4B protein is another one of the highly hydrophobic proteins present in the Dengue virus and contains two hydrophobic regions associated with the endoplasmic reticulum on the lumen side and contains 3 C - terminal transmembrane domains.⁹ Additionally, the NS4B proteins are known to have an association with interfering with the phosphorylation of the STAT1, important for transcription, which blocks the IFN - α/β induced signal transduction cascade.¹⁷ In relation to the NS3 protein, the NS4B protein is known to be a negative modulator of the NS3 helicase function, which is dependent on the confirmation of the NS4B.⁹ In a potential treatment method, focusing on this protein interaction can aid in inhibiting the NS4B role in the interior immune response and in viral replication. One previous study found that a single point mutation could interfere with the interaction between the NS3 and the NS4B protein, which could also be a potential factor to study with the interaction of the NS3 and the NS4A protein. The NS4A and the NS4B proteins are also known to work with each other for anti - host response and viral replication.²⁰ The NS4B also contains regions that are essential to IFN antagonism for the viral life cycle.²⁰ The protein is also involved in protein - protein and lipid - protein interactions, which can fluctuate between multiple different conformational states, different from the other fusion proteins.²⁶ The NS4B protein overall plays a great role in protein interaction, anti - host response, and viral replication.

Due to the presence of many interfacial and hydrophobic regions in both the NS4A and NS4B proteins, it is known that both the proteins oscillate between metastable and stable conformation, one mechanism of forming the replication complex. The DENV rearranges internal cell membranes to establish specific sites of replication, critical for the virus life cycle, which is the main role of the two proteins, and are the only two proteins that play such a role. Due to their significant impact in replication, together, it is important to find an inhibiting molecule to both the proteins to sustainably eliminate this role of creating specific replication sites.²⁹ Additionally, it is also known that the NS4B protein is similar to that of the protein found in Hepatitis - C, which could be further analyzed for druggable molecules. Compound 14a is a compound that is a viral inhibitor of the NS4B, which could be further modeled to see if it also inhibits NS4A functions as well. Similar to Hepatitis - C, the NS4B protein involved in the Dengue virus is also reported to have NTPase activity, playing another minor role in viral assembly. It is also known that both the NS4A and the NS4B protein are the core proteins involved in Hepatitis - C viral replication, which could also apply to the NS4 proteins of the Dengue virus. There have not been many studies that have released viral protein inhibitors for these two proteins

due to the lack of knowledge developed in the field of these highly hydrophobic DENV proteins. However, by applying these possible relationships, there could be a possibility that the lesser - known molecular mechanisms of the NS4 proteins could help to develop a vaccine by inhibiting both of the protein functions.

2.5 NS5 Protein

NS5 protein is the most conserved protein in the Dengue virus and is found to be 102 kDa when expressed. The NS5 protein in the Dengue Virus plays a vital role in viral replication, specifically acting as an RNA - dependent RNA polymerase and as an inhibitor of the interferon (IFN) mediated viral defense system. The NS5 protein, additionally, commonly works together with the NS3 protein as central enzymes in the complex of replication. Together they are the keys to the catalytic activities present in genome replication and 5'UTR RNA capping, assembling the ribosome prior to initial capping⁷. Another component of the NS5 protein is that it contains a structure similar to other NS5 proteins across many different flaviviruses. As the NS5 protein is an RNA - dependent RNA polymerase, it is also linked to the MTase domain, which is an important part of capping the viral RNA and methylation activity which protects the viral RNA from degradation by the host cell.⁷ The protein is also known for inducing interleukin - 8 secretion and transcription.¹⁵ Overall, the NS5 protein plays a central role as an RNA - dependent RNA polymerase and in inhibiting immune response. However, in relation to other domains and proteins, it plays smaller roles in further progressing viral replication.

The central role of an RNA - dependent RNA polymerase is to catalyze the replication of RNA, which is the role played by the NS5 protein. When a host is infected with the Dengue virus, a positive sense of viral RNA is released into the cytoplasm. The NS5 protein then transcribes it as a negative sense RNA strand and then utilizes the negative strand to further synthesize a greater excess of positive - sense viral RNA.⁷ The produced viral RNA is then used to express the polyprotein, a chain of small proteins with versatile roles, through ribosomes of the host cell and encapsulation of new viral components.¹² The structure and folding of the NS5 protein are related to polymerase activity in certain aspects of replication as well. These collective movements would result in an increase in the volume of the RNA tunnel and enable the translocation of the nascent dsRNA, similar to that in the HCV virus. The conformational changes are thought to be controlled by amino - acid motifs located in the connections between the fingers and the thumb subdomains.¹⁴ Such a structural change in order to allow for particular aspects of replication makes it a component of NS5 interaction that could be further studied in order to find if potential viral inhibitors of the protein in the HCV virus could be further applied to Dengue virus as well. Additionally, the RNA dependent RNA polymerase has a particular rate of its function, and structural change also plays a role in the aspect due to the four mechanisms of the RdRp in terms of structure and conformation: the resting mode, which is the closed conformation regarding the priming loop, the initiation state were the primary phosphodiester bond is creating, the priming loops here is

utilized a way for stabilization, and the conformational change which opens the dsRNA exit tunnel.¹² The conformational change is understood to be the rate - limiting step for the RNA polymerase activity. Finally, the protein is encased in an open conformation, which allows for proper RNA polymerization.

The NS5 protein is also crucial to 5' UTR RNA capping and methylation activity. The 5' UTR RNA capping component is when the specific region of 5'cap is a nucleotide on the 5' end of some primary transcript like messenger RNA, aiding in initial translation.⁷ Another role of the NS5 protein is that it contains a Methyltransferase domain (MTase), which is responsible for the certain capping mechanism that is a part of the protein. The MTase is responsible for capping the newly formed genomic RNA. The Viral capping resembles the 5' capping of mRNA in the eukaryotic cell.¹¹ It prevents cell degradation and increases interaction with the ribosome, key for translation. Capping defects decrease viral replication and lead to developing viruses that are more susceptible to the provided immune response, as they cause higher interferon signaling and response in antibodies. The MTase domain capping activity aids the virus to escape from such host cell sensors. Methylation plays a great role in the virus being able to avoid the immune response, and higher IFN sensitivity was found to be resulted from defects in 2' - O - methylation on the second to last A nucleotide of the genome repeats, as it allows the viral RNA to escape being found by IFN induced protein with tetratricopeptide.¹¹ The structure of the methyl domain is also a component that is important for producing an inhibitory molecule for the NS5 protein. Some of the basic structural components include a C - terminal side, an N - terminal side, a third helix domain with an α - helix and two β strands, and a magnesium ion is also present in the RNA - bound structure.¹¹ The magnesium ion is particularly interesting due to its function of coordinating the phosphates and helps to position the substrate for methyl transfer.¹¹ This structural component could further be analyzed to prevent the methylation component of the NS5 protein. Overall, methylation activity is one of the key components of the NS5 protein due to it coming hand in hand with both RNA translation and mediating the IFN immune response.

Another role of the NS5 protein is its guanylyltransferase activity, which displays the relationship between the NS3 protein and the NS5 protein, specifically for the same function of RNA capping. An enzyme - GMP covalent complex was found on the NS5 protein indicating the relationship, and the 5' - triphosphatase activity of the C - terminal helicase domain of NS3 to result in a 5' end of diphosphate to the RNA shows the involvement of the NS3 protein as well.¹¹ The guanylyltransferase activity of the MTase domain of NS5 is increased by the presence of NS3, probably through the molecular interaction regarding the region surrounding the linker domain of NS5 and the subdomain 3 of the NS3 helicase domain.¹¹ Focusing on this domain could also aid in inhibiting both the molecules or even keeping this dual function from occurring. The NS5 protein is also able to bind to prolidase, a peptidase that promotes cell surface expression of the IFN - I receptor.¹² This interaction prevents the maturation of the receptor, and as a result, its proper insertion in the plasma membrane and

the transduction of the IFN - I signaling pathway, inhibiting the normal activation of the primary immune defenses upon viral infection. ⁷ This shows the capacity of flaviviruses to inhibit the interferon immune response at various nodes along the signaling pathway. NS5 protein was also found to be a Small Ubiquitin - like Modifier (SUMO) related both in vivo and in vitro, and a SUMOylation site was identified in the N - terminal domain of the protein. ⁷ This post - translational modification seems to increase the stability of the protein, and its reduction yields a less effective replication of DENV. ¹² It was also found that SUMOylation of NS5 is required for NS5 mediated suppression of IFN response. Another small function of the NS5 protein was that it plays a role in the elevated chemokine levels induced by the Dengue virus as it induced interleukin - 8 secretion and expression through CAAT/enhancer - binding protein and by inducing inflammation as well. ¹⁵ In order to target chemokine level when treating a patient with Dengue fever, which is a component of disease pathogenesis, the CAAT binding protein should be further analyzed as a part of the NS5 for inhibiting such an effect. ⁷ There are multiple smaller components of the NS5 protein, many of them connecting to larger components of methylation and inhibiting IFN immune response, but some also play a part in the progression of pathogenesis.

The NS5 protein is central to methylation activity, inhibiting IFN response and viral replication. However, it also plays a smaller role in disease pathogenesis. The many structural components and domains that also interact with other proteins should be focused on when inhibiting the various functions of the molecule in a specific manner. However, in order to inhibit overall function, the methyltransferase domain is displayed to be a prime spot for multiple functions of the NS5 protein. Additionally, the NS5 protein was found to be similar to the HCV protein, which could be further related to see if any inhibitory molecules or treatment related to the NS5 proteins of the HCV virus could be applicable to the Dengue virus specifically the NS5 protein, as well. Overall the NS5 protein is the largest and one of the better known nonstructural proteins that play a significant role in viral replication, immune response, disease progression, and disease transmission for the Dengue virus.

3. Conclusion

The viral genome of Dengue fever contains a long polypeptide chain that encodes ten genes which are responsible for ten proteins important to viral replication, including the 7 NS proteins. The specific ten proteins are the capsid (C), membrane protein (M), an envelope protein (E), and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The NS5 protein is known as the RNA - dependent RNA polymerase that is required for RNA replication as the protein acts as an enzyme that assembles the viral RNA. The NS1, NS2A, NS4A, and NS4B have not been greatly explored yet. However, they are important to inhibiting immune response and pathogenesis of the virus. In regards to immune response, the NS2A, NS4A, or NS4B can increase replication of the IFN sensitive virus, which along with other interferon - related factors, can inhibit the IFN mediated viral defense system naturally present in a host's body for preventing the replication of viral RNA.

The last two proteins, NS2B and NS3 form a protease involved in breaking up the polyprotein containing all the NS proteins into their individual parts for carrying out their particular roles. The proteins do play important parts in interacting with each other and the cycle of transmission between mosquitoes and the human body⁸. Each of the protein also plays a more specific role in multiple aspects of disease progression as described above, but they all work together in mediated the IFN immune response and in the specific part of viral replication that takes place in the endoplasmic reticulum and is related to the Golgi apparatus when infecting a host cell. Overall the NS proteins are deeply involved in the process of viral RNA replication, virion transmission inside of a host cell, protein synthesis in favor of dengue fever, and prevention of the IFN - mediated defense system.

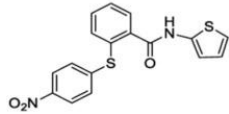
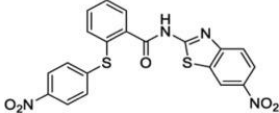
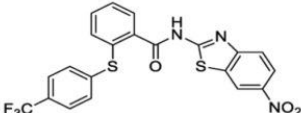
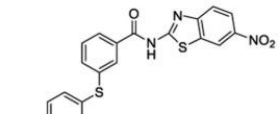
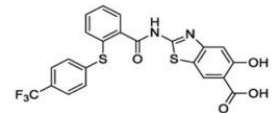
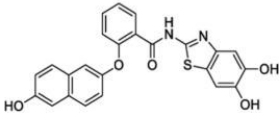
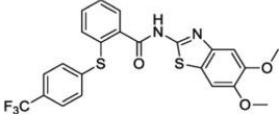
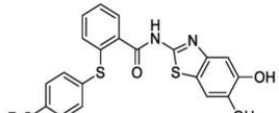
I propose that they work in a manner, with first the NS2B and the NS3 proteins working together to form a protease which breaks down the polyprotein with the individual NS proteins in order to separate the particular proteins in order to execute their individual roles. The C, E, and prM capsid proteins then open the host body cell for the nonstructural proteins to enter. Then, after the breaking down of the polyproteins, they all collectively play their individual roles together in order to inhibit the interferon - mediated viral defense system that occurs in the host body; in this role, the NS4A and NS4B proteins would be most critical. After that occurs, the NS5 protein would then act as an RNA - dependent RNA polymerase, assembling the viral RNA for transmission within the host body as well. Finally, the NS1 protein would play an assumed role of aiding in the endoplasmic reticulum and Golgi apparatus for the replicated RNA to be transported to other host cells as well. All of the proposed manners in which the proteins work together are displayed in Figure 1 as well (Figure 1).

4. Future Scope

There is a clear pathway that these proteins could be related by. Even with other micro - roles, they all play together as well. As a result, it is important to focus on their aspects in viral transmission in order to develop a better treatment. As they all play a part in either allowing the viral RNA to complete its function or inhibiting the IFN immune response in our body, by targeting them, the transmission of the Dengue Virus can be inhibited. Possible treatment options that could still be explored include finding a binding molecule or a combination of binding molecules that would bind to multiple or even a single one of the NS proteins, inherently inhibiting their function in transmission. Additionally, another option would be to create a type of drug that would focus on the pathways they influence, like regarding hemorrhage, in order to prevent the spread of proteins in the asymptomatic method. Also, by looking at other retroviruses, which share similar NS proteins, possible treatments or drugs that have worked for them can also be tested on the NS protein present in the Dengue virus as well. With a clear and developed understanding of the proteins, there are a wide variety of treatment options that can be tested that could prevent future ineffective vaccinations as well, and the knowledge of these proteins is ever - expanding, so by looking into their small impacts on the

body could really be a big change in inhibiting the overall transmission of the Dengue Virus.

Table 1: The table above displays found inhibitors for the ns2b/ns3 proteins.

| Compound no. | Structure | Result for compound ^a : | | | | |
|--------------|---|--|--|--------------------------------|--|--|
| | | 1, DENV-2 (IC ₅₀ [μM]) ^b | 2, DENV-3 (IC ₅₀ [μM]) ^b | 3 (toxicity [μM]) ^c | 4 (EC ₅₀ [μM]) ^d | 5 (IC ₅₀ [μM]) ^e |
| 1 |  | 98 ± 4 | 31.8 ± 4.5 | 30 | 3.5 ± 0.3 | 15.6 ± 3.4 |
| 2 |  | 34 ± 5 | 5.4 ± 2.9 | <1 | ND ^f | ND |
| 3 |  | 22 ± 1 | 21 ± 4 | 1 | 0.1 ± 0.0 | 0.2 ± 0.0 |
| 4 |  | 26 ± 1 | ND | 3 | 0.3 ± 0.1 | 0.7 ± 0.1 |
| 5 |  | 66 ± 3 | 12.3 ± 2.2 | 10 | 0.9 ± 0.1 | 2.3 ± 0.7 |
| 6 |  | 4.2 ± 0.44 | 0.99 ± 0.1 | 10 | 0.8 ± 0.2 | 3.2 ± 1.2 |
| 7 |  | 10% inhibition at 50 μM | NI ^g | 30 | 2.5 ± 0.1 | 9.3 ± 2.5 |
| 8 |  | 3.6 ± 0.11 | 9.1 ± 1.02 | 3 | >3 | >3 |

Note. Reprinted from “Novel Dengue Virus NS2B/NS3 Protease Inhibitors, ” by H. Wu, 2014, *Antimicrob Agents Chemother*, 59 (2)

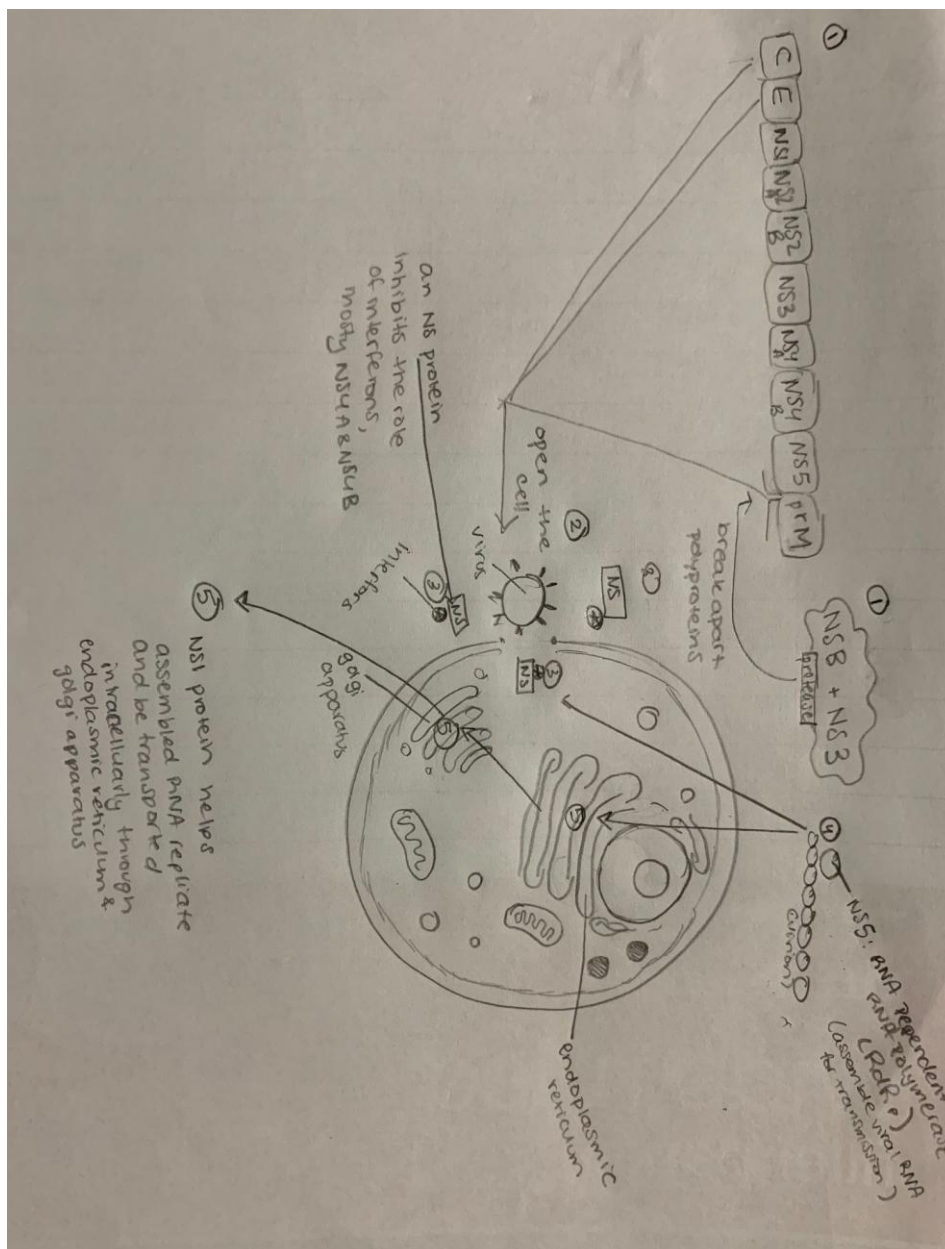


Figure 1: Hypothesized NS Protein Function

The proposed function of the nonstructural and capsid proteins present in the dengue virus transmission process; descriptions and directions for the processes are also provided above, with labelling of key location to take note of.

5. Acknowledgements

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