



Dengue Virus Non-Structural Protein 5 as a Versatile, Multi-Functional Effector in Host–Pathogen Interactions

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Dengue is emerging as one of the most prevalent mosquito-borne viral diseases of humans. The 11kb RNA genome of the dengue virus encodes three structural proteins (envelope, pre-membrane, capsid) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5), all of which are translated as a single polyprotein that is subsequently cleaved by viral and host cellular proteases at specific sites. Non-structural protein 5 (NS5) is the largest of the non-structural proteins, functioning as both an RNA-dependent RNA polymerase (RdRp) that replicates the viral RNA and an RNA methyltransferase enzyme (MTase) that protects the viral genome by RNA capping, facilitating polyprotein translation. Within the human host, NS5 interacts with several proteins such as those in the JAK-STAT pathway, thereby interfering with anti-viral interferon signalling. This mini-review presents annotated, consolidated lists of known and potential NS5 interactors in the human host as determined by experimental and computational approaches respectively. The most significant protein interactors and the biological pathways they participate in are also highlighted and their implications discussed, along with the specific serotype of dengue virus as appropriate. This information can potentially stimulate and inform further research efforts towards providing an integrative understanding of the mechanisms by which NS5 manipulates the human-virus interface in general and the innate and adaptive immune responses in particular.

Keywords: Flavivirus, NS5, moonlighting proteins, signaling pathways, protein–protein interactions (PPIs), antiviral immunity, apoptosis, spliceosome

INTRODUCTION

Dengue is a global epidemic resulting in over 100 million clinical cases globally each year with symptoms ranging from fever to hemorrhage and/or shock that can be fatal, especially among children (Guzman et al., 2010; Bhatt et al., 2013). The disease is caused by four distinct dengue virus (DENV) serotypes (DENV-1, 2, 3, 4). DENV is a positive-strand RNA virus that belongs to the genus Flavivirus, family Flaviviridae. The genome encodes three structural (Env, PreM, Capsid) and

seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. Of these NS1 interacts with NS4A/B and promotes viral replication (Chen et al., 2018; Płaszczycza et al., 2019), NS3 performs helicase and protease functions (Swarbrick et al., 2017), NS4A induces autophagy (McLean et al., 2011), and NS4B facilitates dissociation of NS3 helicase from viral RNA (Umareddy et al., 2006).

NS5 is the largest and the most conserved DENV protein. It serves two important functions: one is the RNA-dependent RNA polymerase (RdRp) activity that is required for viral replication (Iglesias et al., 2011). The second is RNA methyltransferase (MTase) activity important for RNA capping during polyprotein translation (Liu et al., 2010; Klema et al., 2016). Additionally, NS5 forms an RNA replicase complex with NS3 in the endoplasmic reticulum during viral replication. After replication, NS5 dissociates from NS3 and translocates to the nucleus (Kapoor et al., 1995). So far, nuclear translocation has been reported for DENV-2 and -3 serotypes (Brooks et al., 2002; Hannemann et al., 2013). Yeast two-hybrid (Y2H) studies suggest that nuclear translocation may occur because the nuclear import receptor importin- β competes with DENV-NS3 for binding with NS5 (Johansson et al., 2001). While the nuclear accumulation of NS5 does not seem to be essential for viral replication (Kumar et al., 2013), it appears to be linked to an increase in the production of the cytokine IL-8 that has been historically correlated with severe dengue (Medin et al., 2005).

Given that NS5 is important for viral replication and serves as a major target for cytotoxic T cell responses (Duangchinda et al., 2010; Alves et al., 2016), there has been much interest to target it for vaccine development and anti-viral interventions. Mutational studies on the NS5-MTase domain identified several residues that are likely to be critical in viral replication (Kroschewski et al., 2008). 2'-O-methylation of the viral RNA is crucial for the dampening of host immune responses at the early stages of the viral life cycle. Abrogation of the 2'-O-MTase by changing a single amino-acid (E216A) results in an earlier activation of anti-viral responses exemplified by RIG-I (a sensor of foreign RNA), IL-8 (a pro-inflammatory cytokine), and IFIT2 (an interferon-induced protein that inhibits translation) leading to viral attenuation (Chang et al., 2016). Several inhibitors of MTase and RdRp activities have been identified by large-scale *in vitro* screening (reviewed by (Lim et al., 2015)). Additionally, NS5 interacts with host proteins such as STAT2 that are critical for type I interferon (IFN-I) signaling and innate responses and inhibits host anti-viral responses (reviewed recently by (Ashour et al., 2009; El Sahili and Lescar, 2017)). In addition to such well-studied instances, recent high-throughput studies in a variety of experimental systems, as well as bioinformatic analyses, suggest that NS5 interacts with a diverse spectrum of host proteins (Rawlinson et al., 2006; El Sahili and Lescar, 2017; Amemiya et al., 2019). The goal of this review is to provide the interested researcher with a consolidated, annotated list of known and potential NS5-interacting human proteins obtained from multiple studies, highlight significant candidate interactors and situate them in specific biological contexts wherever possible. Additionally, information on the serotype of the viral strain

(DENV1–4) used in the cited studies have been retained and highlighted wherever appropriate.

COMPILATION OF NS5-INTERACTING HOST PROTEINS FROM THE LITERATURE

While some of the NS5 interacting host proteins such as STAT2 are well-known, and extensively reviewed (Ashour et al., 2009; El Sahili and Lescar, 2017) the goal of our efforts here is to compile a comprehensive list of NS5 interacting host proteins. We approached this by compilation of NS5 interacting human proteins a) discovered by experimental pull-down studies reported in the literature; b) curated in databases (bioinformatics and Y2H studies). We briefly elaborate on each of these approaches followed by a list of NS5 interacting proteins compiled through these approaches. Finally, we comment on the gaps in our understanding of the role of these interactions and directions that future research in the field could take.

Pull-Down Studies

Typically, pull-down studies have used cell lines that are infected with defined DENV serotypes and/or strains or transfected by DENV-NS5 protein. While this approach has the advantage of direct evaluation of protein-protein interactions (PPIs) the result may be influenced by the cell line used, and the serotype/strain used for infection/transfection. One study infected HEK 293T and Huh7 cells with strep-tagged full-length DENV-2 (strain 16681) and determined 53 binding partners (De Maio et al., 2016). Another study transfected HEK 293T cells with NS5 of DENV-2 (strain 16681) followed by affinity purification-mass spectrometry (AP-MS) (Shah et al., 2018), and the data so generated were analysed using MiST (mass spectrometry interaction statistics (Verschuere et al., 2015) and CompPASS (Comparison of Multiple Protein Alignments with Assessment of Statistical Significance (Sadreyev and Grishin, 2003)). This resulted in the identification of 26 NS5-interacting host proteins. Another study by Carpp et al. (2014) identified 53 interactors of NS5 using HEK293-T cell line. As the addition of affinity tags to the coding sequences of NS5 and NS3 prevented the production of recombinant virions, they used the I-DIRT (isotopic differentiation of interactions as random or targeted) immunoaffinity purification method (Tackett et al., 2005). Cell lines grown in the normal medium were transfected with GFP-tagged NS3/NS5 followed by DENV-2 infection. Cell lines growing in media containing isotopically labeled arginine and lysine ($^{13}\text{C}_6$, $^{15}\text{N}_4$) were mock-transfected followed by DENV2 infection. After lysis of both samples, equal amounts of the extracts were mixed. This approach distinguishes between pre-lysis and post-lysis interactions by identifying non-specific post-lysis interactions due to the increased proportion of heavy relative to light isotopes (Carpp et al., 2014). In a fourth pull-down study (Poyomtip et al., 2016), a full-length DENV-2 construct (strain 16681) with tandem affinity purification (TAP)-tagged NS5 containing a poly-histidine and FLAG tags (inserted following N173 in MTase domain of NS5) was

propagated in BHK21 cells followed by infection in Huh-7 cells. The NS5 complexes were isolated *via* FLAG-IP and analyzed by mass spectroscopy. This study revealed 97 NS5 interactors, prominent among them being heterogeneous nuclear ribonucleoproteins (hnRNPs) and proteins involved in lipid metabolism (Poyomtip et al., 2016).

Information From Databases and Yeast Two-Hybrid Studies

We used P-HIPSTer (pathogen-host interactome prediction using structure similarity; <http://phipster.org>) which is a database of computationally predicted PPIs compiled for a set of 1,001 fully sequenced human-infecting viruses. The predictions are based on protein structural similarity and homology modeling, exploiting both sequence and structure-based information to infer interactions between pathogen and human proteins (Lasso et al., 2019). This database employs the extensively validated Pre-PPI (predicting protein-protein interactions) algorithm for its predictions. Additionally, we also used DenvInt (<https://denvint.000webhostapp.com/>) which is a dengue-specific database of serotype-related experimental evidence of PPIs based entirely on experimental evidence (Dey and Mukhopadhyay, 2017). It curates data from Y2H, bacterial two-hybrid, pull-down, and co-localization experiments (Khadka et al., 2011; Le Breton et al., 2011; Mairiang et al., 2013). This database indicates that of all DENV proteins, NS5 interacts with the largest number of human proteins (152).

Based on databases and published studies, we have compiled a total of 377 proteins that are known/predicted to interact with DENV-NS5 protein. **Figure 1A** depicts the number of interacting proteins identified by each of the above-stated methods of discovery. **Supplementary Table 1** provides an extensive annotated list of these different NS5 interactors along with the serotype and method by which these are deduced. The minimal overlap of the NS5 interactors deduced by these different approaches may be due to the differences in overall methodologies. Pull-down studies use specific cell lines, viruses, or viral strains as explained above. Overexpression of target proteins in cell lines through transfection does not mimic the actual viral infection scenario, pull-down studies can lead to the precipitation of protein complexes, whose components may not all directly interact with the target protein. Extensive washing steps involved in this protocol may lead to dissociation of weak or transitory interactors. Yeast two-hybrid, though a rapid technique for large scale screening of PPIs, does not truly reflect the sub-cellular localization of the expressed protein or the abundance of the interacting proteins inside the cell. However, though bioinformatics analysis has the advantage of taking into consideration many viral variants and the conserved amino acids among them, which is usually not feasible in experimental systems that rely on a limited set of viral strains, it can produce potentially false-positive results.

Using the available data from the combination of approaches described above, we determined the biological pathways that these interactors are potentially involved with using the KEGG database, Gene Ontology (GO) analysis, and WEB-based GENE Set AnaLysis Toolkit (Liao et al., 2019) available at [http://www.](http://www.webgestalt.org/)

[webgestalt.org/](http://www.webgestalt.org/). **Supplementary Figure 1** provides top biological processes, cellular components, and functions of these NS5 interactors. **Supplementary Table 2** provides an extensive list of the top pathways with a false discovery rate < 0.05. **Figure 1B** outlines some of the major pathways that are enriched for NS5 interacting host proteins pertaining to JAK-STAT signaling pathway, spliceosome, cell cycle, protein processing in ER, necroptosis, and protein synthesis. These are further elaborated in the section below.

CRITICAL COMMENTS ON THE GROWING LIST OF NS5-INTERACTING HUMAN PROTEINS

The most studied NS5 interactor is STAT2. Su et al. reported that SUMOylation of DENV-NS5 is vital for suppressing STAT2-mediated IFN responses (Su et al., 2016). Excellent reviews are available on this subject and thus we are not elaborating on this aspect further (El Sahili and Lescar, 2017). Interestingly, the expanding list of NS5 interactors started revealing several other proteins that are involved in JAK-STAT signaling as outlined in **Figure 1B**, some of which are deduced by pull-down studies (STAT2, MTOR), some by Y2H studies (PIAS1, PIAS3, IFNAR2, TYK2, IFNGR1, IFNGR2), and the others by bioinformatics approaches (STAT1, STAT3, GRB2, EP300) (See **Figure 1B**). It is interesting to note that dengue NS5 not only interacts with IFN- α/β Receptor Subunit 2 (IFNAR2) but also interacts with interferon-gamma receptors 1 and 2 (IFNGR1, IFNGR2). This raises the possibility that NS5, in addition to interfering with the JAK-STAT signaling pathway (Best, 2017), may also interfere with the action of Type I IFN's or IFN γ , which are the key innate and adaptive anti-viral cytokine respectively. Notably, a case-control study that sequenced the DENV-1 NS5 gene in 31 patients of varying disease severity found that polymorphisms corresponding to amino-acids 124 and 166 (I124M and G166S respectively) correlated with increased disease severity in what was designated as viral "clade 2" relative to "clade1" by the researchers. Computational analysis of these amino acid variants indicated that this effect was probably due to the stronger interaction of clade 2 NS5 with the type-I interferon receptor and Janus kinase-1 (JAK-1), eventually suppressing JAK-STAT signaling (Delgado-Enciso et al., 2018) thereby dampening key pathways of the innate immune response. Further studies are needed to understand which domain of NS5 interacts with these different proteins, and what the direct and indirect effects of these interactions are.

It is interesting to note that the list of NS5 interactors constitutes a large number of proteins involved in the spliceosome machinery. Pre-mRNA splicing is a critical mechanism of gene regulation in eukaryotic cells since a majority of protein-encoding transcripts are alternatively spliced (Lee and Rio, 2015). As mRNA splicing is altered in various pathological conditions, it is a potential target for therapeutic intervention using small molecules (Effenberger et al., 2017). De Maio et al. (2016) showed NS5 binds to

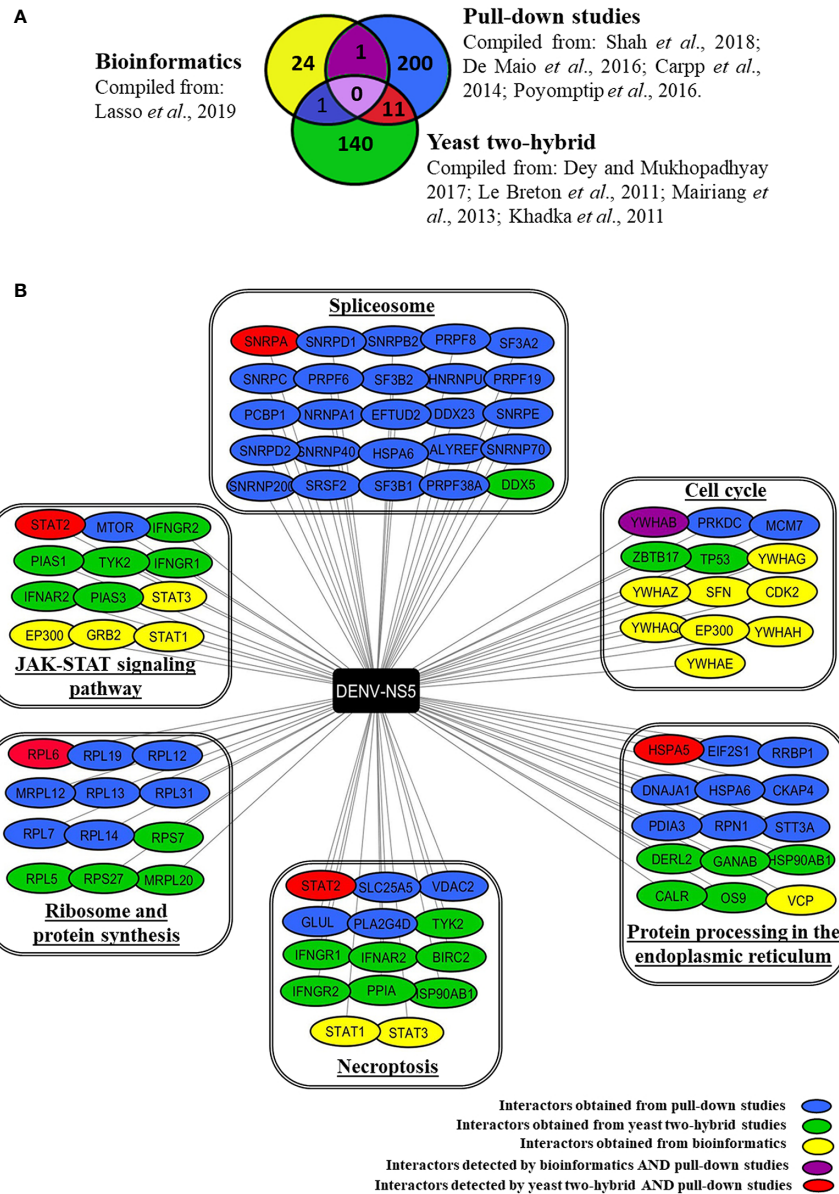


FIGURE 1 | Human interacting partners of DENV-NS5 curated from various experimental studies and databases. **(A)** The Venn diagram indicates the number of DENV-NS5 interacting proteins that are shared with and/or unique to PPI studies in the literature viz., yeast-two-hybrid studies, pull-down studies, and bioinformatics. Yeast-two-hybrid data were curated from the DenvInt database, bioinformatics-based data was obtained from P-HIPSTER, and pull-down data has been derived from published data sources. All cited sources and extended data are compiled and listed in **Supplementary Table 1**. **(B)** Some of the NS5 interactors involvement in key KEGG pathways as obtained using the WEB-based GENE SeT AnaLysis Toolkit. The interactors are grouped in boxes based on the key pathways that they are involved in as obtained from KEGG. The proteins are color-coded according to the method used for their identification. A list of all significant pathways with a false discovery rate (FDR) < 0.05 is given in **Supplementary Table 2**. The complete results of the GO filtering are shown in **Supplementary Figure 1**. SPTAN1 is the only protein detected by both bioinformatics and yeast two-hybrid experiments but has not been shown here because it was associated with a false discovery rate > 0.05 which is the threshold for our compilation.

spliceosome complexes and reduces the efficiency of pre-mRNA processing. Using proteomic analysis and functional experiments, this study demonstrated that NS5 interacts with CD2BP2 and DDX23 from the U5 small nuclear ribonucleoprotein (snRNP) particle to modify the inclusion/exclusion ratio of alternative splicing events, altering the

mRNA isoform abundance of known antiviral factors such as CFTR, EDI, and Bclx (De Maio *et al.*, 2016). DENV-NS5 targets nuclear RNA-binding protein 10 (RBM10) for proteasomal degradation. RBM10 regulates alternative splicing, favoring anti-viral mRNA isoforms of proteins such as spermidine/spermine-N1-acetyltransferase (SAT1) (Pozzi *et al.*, 2020). Its

degradation favors pro-viral isoforms, aiding viral replication; however, it is unknown whether this is a direct interaction or not. Interestingly, in this regard, it is interesting to note that NS5 reduces the splicing efficiency of endogenous RIG-I mRNA, and also increases the expression of dominant-negative forms of IKK ϵ during DENV infection, all leading to maintenance of the pro-viral conditions in the cell (De Maio et al., 2016). The NS5 protein of ZIKA and JEV has also been shown to interact with spliceosome-associated proteins (Kovanich et al., 2019). Considering these, it is proposed that NS5 interaction with the spliceosome machinery could be an immune suppression strategy (De Maio et al., 2016). Some recent studies have shed new light on other NS5-interacting human proteins. For example, a ChIP assay study of DENV-2 NS5-transfected HEK293 cells found increased binding of NF- κ B on the RANTES promoter than in cells mock-transfected with the empty vector (Khunchai et al., 2015). Elevated RANTES expression in NS5 transfected HEK-293 was validated at both mRNA and protein levels using real-time PCR and ELISA respectively (Khunchai et al., 2015).

NS5 interacts with a host protein, death domain associated protein 6 (Daxx) competitively, which dissociates the Daxx-NF- κ B complex. This leads to an increased availability of NF- κ B to bind with RANTES promoter and increases RANTES expression (Khunchai et al., 2012). This is very interesting given the observation that NS5 upregulates RANTES which is a key cytokine produced in severe dengue cases (Khunchai et al., 2015; Soo et al., 2017). However, a different study showed that NS5 transfection of HEK293 cells led to upregulation of IL-8 *via* activation of CAAT/enhancer-binding protein (c/EBP) (Medin et al., 2005). Further studies are needed to understand how NS5 transfection influences NF- κ B given that NF- κ B is a pleiotropic factor that can affect multiple biological processes such as cytokine production, transcription, translation, and apoptosis. In this regard, it is interesting to note that many of the apoptosis-related proteins (e.g., BIRC2; SPTAN1; TUBAL3, etc.) are also shown to interact with dengue NS5 (**Supplementary Table 1**).

Interestingly, NS5 interacts with several proteins that are typically associated with lipid metabolism (fatty acid synthase, hydroxysteroid (17 β) dehydrogenase 12, pyruvate carboxylase, ATP citrate lyase). This indicates that NS5 may have a direct role in influencing lipid metabolism (Heaton and Randall, 2010; Carpp et al., 2014; Poyomtip et al., 2016). Further understanding of the role of NS5 in these pathways is important given that lipid metabolism is necessary for viral replication (Melo et al., 2018).

Some of the recent emerging studies are beginning to indicate that NS5 has a causal link in autophagy *via* influencing a host deubiquitinase protein, USP42 expression *via* increased microRNA, miR-590 (Mishra et al., 2019) and TRAF-6 (Pu et al., 2017). However, the interacting partners of NS5 involved in these processes are yet to be identified.

An interesting line of studies in the recent past suggests that NS5 also interacts with promyelocytic leukemia-nuclear bodies (PML-NBs) that are typically involved in several cellular processes including antiviral response (Lallemand-Breitenbach and de Thé, 2010; Khunchai et al., 2012; Giovannoni et al., 2015).

These various lines of evidence indicate that besides the well-known dampening of the initial anti-viral response, NS5 can interact with several other host proteins to influence other aspects of host cell physiology as well. The precise effect of these NS5-host protein interactions on the overall survival and propagation of the virus as well as on the host innate and adaptive immune responses remains to be determined.

FUTURE PROSPECTS

DENV-NS5 interactors participate in a variety of biological processes, most importantly JAK-STAT signalling, RNA processing, cell cycle progression, necroptosis, protein synthesis, and protein processing in the ER among others. DENV-NS5 is an attractive target for drugs and small molecules to inhibit viral replication (Rawlinson et al., 2006; Lim et al., 2015; Shimizu et al., 2019; Troost and Smit, 2020). RNA interference (RNAi)-based approaches have been explored for therapeutic potential against a variety of viral infections, including dengue [reviewed in (Stein and Shi, 2008; Arbuthnot, 2010; Levanova and Poranen, 2018)]. Validating the top hits among the listed NS5-interactors by RNAi in human cell lines and observing the effect of such inhibition of specific host proteins on viral viability or pathogenesis could rapidly identify promising host proteins for disease management. Stepwise investigation of the utility of knocking down interactor-protein levels *via* RNAi and/or deploying interactor decoys to hamper the NS5-interaction with specific host proteins suggest themselves as potential avenues for further clinical research. Some of the NS5-interactors that modulate immune functions or lipid metabolism may serve as potential targets (Canard, 2011). The choice of host protein(s) would be critical, and those involved in more specialized pathways like necroptosis or cytokine production may be preferred over those involved in essential processes like protein synthesis or RNA processing to minimize collateral damage to the host. In case of dengue, RNAi approaches have obtained promising results by targeting TNF- α in cell culture and mice (Subramanya et al., 2010). Furthermore, cell line-based RNAi studies targeting Hsp60 (Padwad et al., 2009), proteins involved in membrane trafficking (Ang et al., 2010) and protein processing in the ER (Savidis et al., 2016), and the IFN- λ receptor 1 (Hsu et al., 2016) indicate that an appropriate choice of host protein, can favorably influence the course of viral infection and disease pathogenesis. Since most of the experimental data on NS5-interacting host proteins available to date are for DENV-2, it would help to learn about serotype-specific differences to fine-tune drug usage. Further investigation of NS5-host protein interactions and their outcomes *vis-à-vis* viral infection and disease pathogenesis can potentially open novel avenues for effective viral therapy and/or clinical management.

AUTHOR CONTRIBUTIONS

KM-K, AC, and RS contributed to the conception and design of the review. PB organized the database and performed the analysis.

PB and GS wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2021.574067/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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