Chapter 19

An Overview of Current Approaches Toward the Treatment and Prevention of West Nile Virus Infection

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Abstract

The persistence of West Nile virus (WNV) infections throughout the USA since its inception in 1999 and its continuous spread throughout the globe calls for an urgent need of effective treatments and prevention measures. Although the licensing of several WNV vaccines for veterinary use provides a proof of concept, similar efforts on the development of an effective vaccine for humans remain still unsuccessful. Increased understanding of biology and pathogenesis of WNV together with recent technological advancements have raised hope that an effective WNV vaccine may be available in the near future. In addition, rapid progress in the structural and functional characterization of WNV and other flaviviral proteins have provided a solid base for the design and development of several classes of inhibitors as potential WNV therapeutics. Moreover, the therapeutic monoclonal antibodies demonstrate an excellent efficacy against WNV in animal models and represent a promising class of WNV therapeutics. However, there are some challenges as to the design and development of a safe and efficient WNV vaccine or therapeutic. In this chapter, we discuss the current approaches, progress, and challenges toward the development of WNV vaccines, therapeutic antibodies, and antiviral drugs.

Key words West Nile virus, Vaccine, Antiviral drug, Therapeutic antibody

1 Human WNV Diseases and the Need for Antiviral Drug or Vaccine

West Nile virus (WNV), a neurotropic RNA virus belonging to the FLAVIVIRIDAE family, is generally transmitted to human by infected mosquito bites, primarily by Culex species [1, 2]. However, WNV can also be transmitted through other less frequent routes, including a transfusion of blood and blood components [3, 4], organ transplantation [5], breastfeeding [6], and congenital infections [7]. After an infected mosquito bite, WNV replicates in keratinocytes and skin-residential dendritic cells (Langerhans cells), and the latter cells carry the viruses to draining lymph nodes to cause viremia [8, 9]. Subsequently, WNV disseminates to the peripheral organs, such as spleen and liver, and possibly to spinal cord and brain. Human WNV infection
may cause injury and death of neurons with various clinical manifestations, such as encephalitis, meningitis, flaccid paralysis, persistent neurologic sequelae, and possibly death, particularly in the elderly and immunocompromised individuals [1, 10, 11]. WNV strongly activates host immune responses, which play important roles in controlling viremia, viral dissemination to the central nervous system (CNS), and recovery from the disease [11]. However, the mechanism of WNV pathogenesis, including its tropism to neurons, CNS invasion, and viral or host factors that contribute to imbalance between viral pathology and host immunity still remain poorly understood.

Although WNV was first discovered in Uganda in 1937, it had been considered as a minor public health concern until its first appearance in the USA in 1999 [12]. Since then, it has dramatically spread to all the continental states of the USA and became an endemic disease throughout North America within a few years [13–16]. In the USA alone, there have been over 40,000 reported cases of WNV between 1999 and 2014, of which ~45% were classified as neuroinvasive and claimed lives of nearly two thousand people [17]. However, the actual WNV burden is likely much higher than previously thought because only about 20% of infected individual develop a clinical WNV disease [13]. It has been estimated that over three million individuals have been infected with WNV in the USA, of which about 780,000 had a symptomatic disease [18]. WNV also has potential to develop unusual clinical manifestations [19–21] and may involve in renal diseases [22, 23], myasthenia gravis [24], and myocarditis [25], suggesting that the range and severity of WNV disease may be even worse than previously believed. Importantly, increasing numbers of WNV outbreaks during the last 15 years have been associated with greater number of neuroinvasive cases and a higher rate of fatalities [16, 17]. However, no vaccine or antiviral therapeutic is currently available, which limits current treatments to only supportive care measures, such as intravenous fluids, antipyretics, respiratory support, and prevention of secondary infections. Considering the worldwide distribution of this virus and evidence of its potential to change in pathogenicity and transmission [26–30], there is an urgent need to develop safe and effective antiviral drugs or vaccines against WNV infection [31]. Intensive research during past decades has made significant progress in the design and development of several treatment and prevention methods for WNV infection (reviewed by [32–34]). Here, we discuss the current approaches and recent progress toward the development of vaccines, therapeutic antibodies, and antiviral drugs against WNV infection in humans.
2 WNV Structure and Therapeutic Targets

WNV is a spherical virus with 50 nm in diameter, which comprises an icosahedral nucleocapsid surrounded by a lipid envelope [35]. The virus contains a single-stranded, capped, and plus-sensed RNA genome of approximately 11 kb in size. The viral genome encodes a polyprotein precursor, which undergoes posttranslational processing by cellular and viral proteases to generate three structural proteins (capsid [C], premembrane [PrM], and envelope [E]), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins form virion structure, whereas the non-structural proteins play essential roles in the replication of viral genome, assembly of virion, and viral pathogenesis [36–38]. Thus, the structural and non-structural proteins of WNV may be potential targets for developing vaccines and antiviral therapeutics (Table 1).

In a WNV virion structure, C protein encapsulates viral genomic RNA to form a nucleocapsid that is enveloped by a lipid bilayer into which trimmers of prM-E heterodimers form the spike-like projections. Among these structural proteins, E protein mediates crucial roles in binding to cellular receptors, membrane fusion, and entry of WNV into host cells, making it a key target for the development of vaccines, neutralizing antibodies, and entry inhibitors. Crystal structure analysis has confirmed that E protein folds into three structurally distinct ectodomains (EDs) termed EDI, EDII and EDIII [39–43]. Among these, the EDIII consists of the major neutralizing epitopes and is an antigen of choice to elicit production of neutralizing antibodies [43–46]. Based on the structural characterization of antiflaviviral monoclonal antibodies from both human and nonhuman primate, it appears that the epitopes of flaviviral E protein are more complex and diverse than previously thought [47–50]. In addition, mapping of B-cell and T-cell epitopes has led to the identification of many immunodominant epitopes in both structural and nonstructural proteins of WNV [51, 52].

Among the non-structural proteins, NS3 and NS5 are best-characterized, multifunctional proteins, both of which contain enzymatic activities that are essential for viral replication [53–55]. Such enzymatic functions of NS3 and NS5 have received considerable attention as potential targets for antiviral drug development [34, 54]. The NS3 protein contains two distinct functional domains. The N-terminal domain of NS3 (184 amino acid residues) has serine protease activity that requires a polypeptide cofactor NS2B for activation [54, 56, 57]. Recent X-ray crystallographic studies have shown that the conformation of β-loop of NS2B controls the substrate binding by NS2B/NS3 protease [58, 59]. In contrast, the C-terminal domain of NS3 functions as an RNA helicase,
| Targets | Structural features | Functions | Targeting approaches |
|---------|---------------------|-----------|---------------------|
| Envelope (E) | Contains a central domain I, a extended finger-like domain II, a immunoglobulin-like domain III and a hydrophobic pocket between domain I and II; domain II contains a fusion loop; contains the major epitopes for B and T cells | Mediates virus binding to host cellular receptors and membrane fusion | Drug targeting to block viral entry, disrupt membrane fusion, and produce neutralizing monoclonal antibodies |
| Capsid (C) | Alpha-helical structural protein | Encapsidates viral RNA, induces host cell apoptosis [326], and to disrupt nucleosome formation | Internal deletion in capsid gene results in deficient replication and reduced pathogenicity [113] |
| Membrane (PrM) | Forms heterodimer with E protein | Virion assembly and fusion modulation [38] | Antigen for production of antibodies |
| NS1 | Secreted glycoprotein, contains epitopes for antibody production | Immune evasion activities [37] | Potent antigen for antibody production; ablation of NS1 glycosylation attenuates WNV |
| NS2A | Transmembrane protein, associates with endoplasmic reticulum membrane; component of replication complex | Virion assembly/maturation; antagonizes host immune responses | Alanine to proline substitution at position 30 of NS2A attenuates viral virulence [36] |
| NS2B | Consists of a 40-amino acid hydrophobic region, transmembrane protein, component of replication complex | The 40-amino acid hydrophobic region serves as cofactor for NS3 | NS2B cofactor activity can be targeted by inhibitors |
| NS3 | Multifunctional protein containing two functional domains; contains a shallow ATP binding pocket and an additional domain that is not present in human helicases | Serine protease (N-terminal); Helicase (C-terminal) | Substrate-based inhibitors can target NS3 protease; small-molecule inhibitors may target helicase domain |
| NS4B | Transmembrane protein, component of replication complex | Inhibits NS3 ATPase activity | NS4B forms ATP-binding site that may be targeted by a drug |
| NS4B | Transmembrane protein, component of replication complex | Participates in viral replication and immune evasion | Selected mutations [365] in NS4B attenuate WNV |
| NS5 | Multifunctional protein containing two functional domains | Methyltransferase and guanylyltransferase (N-terminal); RNA-dependent RNA polymerase (C-terminal) | NS5 functions can be targeted by various inhibitors |
nucleoside triphosphatase, and RNA triphosphatase \[60, 61\]. Although the ATPase and helicase activities of NS3 function independently, NS4A protein has been suggested to regulate both of these activities \[62\]. Besides its role in cleaving the viral protein, the protease activity of NS3/NS2B may also contribute to host cell apoptosis and neuropathogenesis by cleaving host proteins \[63\]. Similarly, NS5 is another multifunctional protein containing N-terminal methyltransferase/guanyltransferase, and C-terminal RNA-dependent RNA polymerase (RdRp) activity \[64, 65\]. The N-terminal methyltransferase and guanyltransferase activities of NS5 are essential for the formation of a cap structure in viral mRNA \[66\]. Thus, the functions of NS5 are crucial for both protection of viral genome and efficient translation of viral polyprotein. The N-terminal domain of NS5 contains multiple residues that can be phosphorylated by host protein kinases \[67\]. Besides its function in viral replication, NS5 also plays a role in viral pathogenesis by antagonizing host’s interferon response \[28\]. Other nonstructural proteins NS2A, NS2B, NS4A, and NS4B form the scaffold for the viral replication complex and also have roles in the replication of viral genome and host immune evasion \[68–70\]. Mutations in NS4B protein may attenuate WNV and other flaviviruses \[68, 69\]. In addition, a recent successful clinical trial of a hepatitis C virus NS5A inhibitor suggests that targeting non-structural proteins may be an ideal strategy to develop therapeutics against other flaviviruses, including WNV \[71\].

3 Current Approaches and Progress in WNV Vaccine Development

WNV infection induces potent activation of host immune responses that is critical for controlling viremia, viral dissemination into the CNS, and recovery from WNV diseases \[1, 11\]. Studies of WNV pathogenesis in animal models have demonstrated that humoral responses (antiviral antibodies) are essential in limiting viremia and neuroinvasive diseases \[72, 73\]. Thus, development of a vaccine that produces high titer of neutralizing antibodies would offer efficient protection against WNV infection \[44\]. Several epitopes for both B and T cells have been characterized in WNV proteins \[52, 74–78\]. In particular, the C-terminal EDIII of E protein that contains critical neutralizing epitopes is the major target for neutralizing antibodies against WNV infection \[40, 44, 78, 79\]. In addition to humoral immune response, cell mediated immunity by CD4+ and CD8+ T cells play critical roles in recovery from WNV infections \[80–83\]. Thus, the efficient generation of vaccine-induced immunity against WNV may also require activating and shaping of multiple effectors of adaptive immune response by early innate signaling pathways \[84–86\].
Development of an effective vaccine requires multiple steps from design and development to rigorous evaluation of both safety and efficacy (Fig. 1). Since the biology of vaccine-induced immunity and principles in vaccine development and testing have been extensively discussed before, we will not discuss these topics here. Immunization of the laboratory animals and subsequent challenge of those animals with a pathogen under controlled conditions is the common method for early evaluation of vaccine effectiveness. Various factors such as route of administration, immunogen dose, and type of adjuvant greatly influence the effectiveness of a vaccine candidate. Mouse models of WNV infection partially mimic the clinical course of WNV disease in humans, which not only help with the understanding of WNV pathogenesis but also facilitate the development and testing of WNV vaccines. In animal studies of vaccine efficacy, infected animals are observed for mortality and monitored for survival, pathology, seroconversion, immune responses, and vaccine safety. Plaque reduction neutralization test (PRNT) is a gold standard method to assess whether a candidate WNV vaccine induces neutralizing antibodies in both animals and humans, for which standard guidelines and methods are available [87]. Neutralizing antibody titer is correlated with protection against disease for other licensed flavivirus vaccines and is considered a key marker to assess vaccine efficacy [88]. For example, the protective threshold for a Japanese encephalitis virus vaccine is correlated with the titers of neutralizing antibodies, with a serum PRNT\textsubscript{50} \geq 1:10 considered protective [88]. Although WNV lineage 1 strains are commonly involved in human disease and were used in vaccine efficacy testing, the recent emergence of pathogenic lineage 2 strains in Europe has raised additional concerns in WNV vaccine efficacy studies, because WNV vaccine candidates based on lineage 1 strains may not protect against the lineage 2 strains. After promising results from animal studies, human WNV vaccines are further evaluated in terms of protection against natural challenge, as well as their safety and immunogenicity during series of clinical trials (phases I–III).

### 3.1 Vaccine Developments and Testing

![Fig. 1 Overview of vaccine development process](image)

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### 3.2 Vaccine Development Approaches

Both traditional and modern approaches have been used for the development of WNV vaccines, and the most common approaches are listed below.
(a) **DNA-based vaccine**: By using the power of modern genetic tools, viral protein(s) can be expressed in a suitable vector to develop DNA vaccines against WNV infection \([89, 90]\). However, this strategy is sometimes hampered by poor immunogenicity and potential safety concerns, such as integration of foreign DNA into the host genome.

(b) **Chimeric/recombinant vaccine**: This approach relies on the replacement of gene(s) of the established viral vaccine strain by equivalent WNV genes. Several live attenuated vaccines and viral vectors may be used as a backbone for developing recombinant WNV vaccine candidates, such as yellow fever virus (YFV) vaccine (YFV-17D) \([91, 92]\), attenuated DENV serotype 4 (DENV4) \([93, 94]\), HIV-based lentivirus vector vaccine \([95]\), Schwarz strain of attenuated measles virus \([96]\), vesicular stomatitis virus (VSV) vaccine vector \([97, 98]\), and adenovirus A \([98]\).

(c) **Live-attenuated vaccine**: Attenuation of WNV can be achieved by classic cell culture passage or animal passage and targeted genetic mutations. However, several potential challenges including residual pathogenicity, reversion to virulent strain, relative short self-life, and the demand of a safe biological production system need to be overcome.

(d) **Inactivated (killed) vaccine**: Chemical inactivation of live viruses may be used to develop inactivated or killed virus particles. The limitations are the possibility of incomplete inactivation, short-lived immunity, and the requirement of multiple doses for efficient immunization.

(e) **Subunit or recombinant protein vaccine**: This approach uses soluble recombinant protein(s) or protein expressed in virus-like particle (VLP) platform as a vaccine candidate. Success of this approach relies on optimum immunogenicity of a vaccine, as the protein vaccines generally require multiple boosts with strong adjuvants to provide acceptable efficacy.

Although no vaccine is currently available against WNV infection in human, several WNV equine vaccines are available (Table 2). Several human vaccines are under development, and some are in clinical trials (Table 3). Their current status and approaches used for development are discussed below.

**West Nile Innovator® DNA**: West Nile Innovator® DNA is the first licensed DNA vaccine for veterinary use following a successful demonstration of vaccine induced B and T cell-based immunity after immunization of mice and horses with DNA vaccines expressing \(prM\) and \(E\) genes of WNV \([89]\) or the domain III (DIII) region of \(E\) gene \([99]\). This approach has been widely used do develop various DNA vaccines against WNV. One of the first DNA vaccines introduced in phase...
| Vaccine name               | Company                      | Vaccine approach | Design/features                                      | Status                               | References                      |
|----------------------------|------------------------------|------------------|------------------------------------------------------|--------------------------------------|---------------------------------|
| West Nile-Innovator® DNA   | Fort Dodge Animal Health/Pfizer | DNA              | Plasmid DNA encoding WNV prM-E                       | Licensed (discontinued)             | Davis et al. [89]               |
| Vetera™ West Nile vaccine  | Boehringer Ingelheim         | Killed           | Whole virus                                          | Commercialized                      |                                 |
| West Nile-Innovator®       | Pfizer                       | Formalin inactivated | Whole virus                                        | Commercialized                      | Ng et al. [127]                 |
| RecombiTek®                | Merial                       | Recombinant vaccine | WNV prM-E in canarypox virus                         | Commercialized                      | Karaca et al. (2005)            |
| PreveNile®                 | Intervet                      | Recombinant vaccine | WNV prM-E in yellow fever vaccine (17D) backbone    | Licensed in 2006 (recalled in 2010 after severe reaction) |                                 |
| Vaccine name (company)         | Vaccine approach | Design/features                                      | Status of development                                      | Reference(s)                        |
|-------------------------------|------------------|------------------------------------------------------|------------------------------------------------------------|-------------------------------------|
| Chimeri-Vax-WN (Acambis, Sanofi-Pasteur) | Recombinant      | WNV prM-E and E replacing capsid and non-structural protein of yellow fever vaccine strain (17D) | Phase I clinical trial completed, Phase II trial ongoing    | Biedenbender et al. [366]            |
| WN-DEN4                       | Recombinant      | WNV prM gene in a backbone of attenuated DEN-4       | Phase I clinical trial completed, Phase II trial ongoing    | Pletnev et al. [94]                 |
| WN-80E                        | Subunit protein  | Recombinant E protein lacking transmembrane domain   | Phase I clinical trial completed                             | Lieberman et al. [136, 147]         |
| WNVDNA017-00-VP (VRC in collaboration with Vical) | Plasmid based DNA vaccine | Plasmid DNA vector that express WNV-NY99 prM-Env under a cytomegalovirus promoter | Phase I clinical trial completed                             | Martin et al. [90]; Ledgerwood et al. [100] |
| HydroVax-001 (OHSU, NIH funded) | Inactivated      | Chemical inactivation by H\textsubscript{2}O\textsubscript{2} | Phase I clinical trial ongoing                              | http://www.nih.gov/news/health/jul2015/niaid-06.htm |
I clinical trial was based on a circular plasmid DNA vector incorporating a cytomegalovirus (CMV) promoter to express the WNV-NY99 prM and E coding sequences in downstream of a modified JEV signal sequence (VRC-WNVDNA017-00-VP) [90]. Although no serious adverse effects were reported, its low immunogenicity hampered further development [90]. In an effort to improve immunogenicity of this vaccine, an additional regulatory element from human T-cell leukemia virus type 1 (HTLV-1) was incorporated in conjunction with the previously used CMV promoter [100] but without significant success when tested in the clinical trial [100]. Several studies have also tested carrier-conjugation and different inoculation routes [101, 102] to improve immunogenicity and efficient delivery of DNA vaccines. For example, a DNA vaccine expressing full length of truncated WNV E gene derivatives conjugated to the P28 region of C3d (a complement protein) induced strong IgG titers and efficient protection of mice when vaccinated by gene gun method [103]. In another study, a plasmid DNA vector expressing the ectodomain of WNV E protein into linear polyethyleneimine (LPEI) nanoparticles covalently bound to mannose was developed. However, this conjugation failed to generate sufficient E-protein specific humoral responses, despite the boosting of the vaccinated mice with recombinant E protein induced a significant increase in neutralizing antibodies [104].

Large deletions of capsid gene in the flaviviral RNAs result in a failure to produce infectious virions but retain the ability to replicate viral RNA genome and express prM and E proteins [105, 106]. This novel property has been used to develop several plasmids DNA (pDNA) vectors that after transfection produce single-round infectious particles (SRIPs), which in turn produce virus-like particles (VLP) containing viral surface proteins without viral genome. This strategy has been used to develop several candidate DNA vaccines against flavivirus by expressing E and prM proteins in a plasmid vector and forming VLPs. Using this approach, several plasmid-DNA constructs were developed as candidate DNA vaccines against WNV. This type of DNA vaccines encode for single-round infectious particles expressing E/prM [107–109], a full-length cDNA copy of attenuated WNV Kunjin strain [110], or ectodomain of E protein [111].

3.3.2 Live Attenuated Vaccine

Cell culture or animal passage used to be conventional methods to develop live-attenuated vaccines. The advents of genetic manipulation techniques make it feasible to introduce targeted mutations into the viral genome and attenuate viruses. Using this approach, a WNV vaccine (RepliVAX WN) developed by an internal deletion of a region in capsid gene [112, 113] has been shown to induce neutralizing antibodies and protective immune responses in mice.
RepliVAX WN strongly activates B cell population secreting anti-NS1 IgG antibody and induces prolonged activation of memory CD8$^+$, CD4$^+$, and NS1-specific plasma cells. Innate immune signaling pathways, such as TLR3 and MyD88-dependent signaling pathway, are involved in strong activation of B cell response, development of germinal center, generation of long-lived plasma cells, and production of antibodies following immunization with RepliVax WN vaccine. In addition, another live attenuated WNV vaccine developed by generating mutations in glycosylation sites of E and NS1 proteins induces neutralizing antibodies and protective immune responses in mice. Similarly, approaches of introduction of mutations in NS4B, NS2A, or E gene that were previously characterized to attenuate JEV-SA-14142 have also been used to develop attenuated WNV vaccine candidates.

Using this approach, a recombinant live attenuated WNV vaccine for veterinary use was developed and licensed in 2004 by Merial (RecombiTEK). This vaccine expresses WNV prM and E proteins in a canarypox virus backbone. Using a similar approach, a chimeric vaccine (ChimeriVax-WN02) has been developed by replacing prM and E genes in YFV vaccine strain (YFV-17D) with WNV-NY99 prM and E genes. ChimeriVax-WN02 was the first recombinant WNV vaccine candidate tested in clinical trial. Introduction of three mutations responsible for attenuation of JEV (SA14-14-2) in equivalent positions of WNV E gene further attenuated ChimeriVax-WN02. Similarly, DENV4 vaccine candidate (rDEN4Δ30), attenuated through a 30-nucleotide deletion in the 3′ untranslated region (UTR) of the viral genome, was further engineered to express WNV-NY99 prM and E. After preclinical evaluation in mice, geese, and monkeys, rDEN4Δ30 showed strong immunogenicity in the clinical trial. In addition, a chimeric DENV2 vaccine candidate expressing the WNV NY99 prM and E proteins has been shown to protect mice from infection with WNV NY99 strain. Another recombinant WNV vaccine based on influenza vaccine (FLU-NA-DIII) was developed by cloning DIII of WNV E into the N-terminal region of neuraminidase of influenza virus. This vaccine candidate induced WNV-specific neutralizing IgG and protected mice against lethal WNV infection. Similarly, a recombinant adenoviral vaccine vector (CAdVax-WNVII) expressing all three structural proteins (C, prM, and E) along with NS1 of WNV induced neutralizing antibodies in mice. Several other recombinant WNV vaccines have also been developed by expressing WNV protein in the backbones of attenuated measles virus, vesicular stomatitis virus, and herpes virus-1.
The most common approach to develop non-replicating inactivated viral vaccines is to inactivate entire virus particles by using chemicals. A formalin-inactivated WNV vaccine based on WNV-NY99 strain was the first successful veterinary vaccine (marketed by Pfizer as West Nile Innovator®) licensed in 2003 [127]. Another veterinary WNV vaccine using killed virus was also licensed by USDA (marketed by Boehringer Ingelheim as Vetera™ WNV). Recently, an inactivated WNV vaccine (WN-VAX) based on WNV NY99 protects mice against lethal WNV infection and exhibits immunogenicity in monkeys [128]. In addition to WNV NY99, formalin inactivation of WNV IR598 strain induces neutralizing antibody and protects immunized geese [129]. As an alternative to traditional formalin-based vaccines, a novel hydrogen peroxide (H$_2$O$_2$) inactivation approach has been recently used to produce a whole-virus vaccine against WNV [130, 131]. Mice immunized with H$_2$O$_2$-inactivated WNV vaccine candidate developed high serum neutralizing titers, and offered complete protection of vaccinated mice against lethal WNV challenge [130]. One of such H$_2$O$_2$ inactivated vaccine (HydroVax-001) has been recently introduced into phase I clinical trial. Although inactivation of virulent WNV virus strain has been successfully achieved by chemical-inactivation method [127, 129], use of a naturally attenuated Kunjin strain of WNV [132, 133], or chemically synthesized virus by cDNA system [134] as starting material has also been proposed.

Several studies demonstrated that soluble recombinant protein or VLP based approach could serve to develop WNV vaccines [135–139]. VLP are specialized subviral particles that lack of viral genome and solely contain viral structural proteins [140, 141] or express viral proteins on envelope membranes [142, 143]. Different vectors and production system were evaluated for development of various subunit vaccines against WNV. For example, a recombinant truncated form of WNV E protein produced in *Escherichia coli* induced neutralizing antibodies and protected mice from lethal WNV challenge [46, 144]. In addition, a recombinant truncated WNV E protein produced in the SF+ insect cells via baculovirus infection induced neutralizing antibodies and protected mice and hamsters from WNV infection [145]. Recombinant baculovirus was also used to express WNV prM and E proteins in mammalian cells under the CMV promoter, with or without vesicular stomatitis virus glycoprotein (Bac-G-prM/E). Such vaccines induced robust immune responses when inoculated in mice and produced both neutralizing antibodies and inflammatory cytokines [146]. In a recently proposed novel approach, known as pseudotyping, a retrovirus Gag polyprotein forms a VLP scaffold to display the ectodomain of human membrane glycoprotein (CD16) that was fused to the high affinity IgE receptor gamma chain (RIgE).
Using this retrovirus-based VLPs platform, a WNV vaccine was generated by replacement of the CD16 ectodomain in CD16-RIgE glycoprotein with EDIII of WNV, which induced neutralizing antibodies in mice [139].

A recombinant E protein of WNV-NY99 produced in Drosophila S2 cells (WNV-80E, developed by Hawaii Biotech) is the only WNV subunit vaccine candidate that has been tested in phase I clinical trial. Although preclinical studies revealed WNV-specific neutralizing antibody responses in vaccinated animals [136, 147, 148], the immunogenicity of this vaccine in humans was low. To increase immunogenicity, conjugation of recombinant proteins with nanoparticles or pathogen-associated molecular patterns (PAMPs) as carrier/adjuvant have been tested. For instance, a recombinant WNV E protein administered with unmethylated CpG oligonucleotide adjuvant or loaded onto CpG-modified nanoparticles strongly activated dendritic cells and lymphocytes and elicited Th1-dominant immune responses by producing high titers of IgG2a and IgG2b in immunized mice [149, 150]. Similarly, mice injected with DIII of WNV E conjugated with bacterial flagellin (STF2Δ.EIII) [137] or VLP derived from bacteriophage AP205 engineered to express DIII of WNV E (DIII-C-AP205) [138] also significantly increased neutralizing antibody production and protected the immunized mice.

Despite the intensive efforts in development of WNV vaccines, only a few reached the clinical trial stages. Of those in clinical trials, most candidate vaccines fail to demonstrate efficient immunity and safety. Development of new tools for antigen screening, expanded understanding of immunological correlates of vaccine-induced immunity, and discovery of novel adjuvants for vaccine delivery may facilitate the design and the development of WNV vaccines. For example, knowledge of genomic information and bioinformatics has been used for in silico identification of candidate antigens and development of vaccines by a novel method called “reverse vaccinology” [151, 152]. This comprehensive tool can quickly identify all potential antigens coded in the genome and may be used to develop a novel viral vaccine [153]. Development of vaccine against group B streptococci proved the potential of this approach [151]. Similarly, a “structural approach” that improves antigenicity of vaccines by rational designing has been developed by utilizing the knowledge of immunology, structural biology, and bioinformatics [154]. In addition, increased understanding of immunogenetics and role of environmental and host factors that determine the variation of vaccine immunity may offer new approaches to design a more effective vaccine against WNV infection in humans.

3.4 Potential Novel Approaches for WNV Vaccine Development

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4 Antibody-Based Therapy: A Promising WNV Therapeutic

Therapeutic monoclonal antibodies (mAbs) or hyperimmune sera have been successfully used for prophylaxis of a number of infectious and noninfectious diseases, including WNV infection. In recent years, the number of mAbs in preclinical development and clinical trials has been increased significantly [155]. So far, nearly 50 mAbs have been approved for therapeutics by US FDA, including a humanized mAb Synagis (palivizumab) for preventive use against respiratory syncytial virus (RSV) infection in neonates and immunocompromised individuals [156–158]. Many mAbs have been developed against viruses, such as SARS-CoV, influenza, HIV-1, and other (re)emerging viruses including WNV [155, 159–163]. Some of them showed excellent therapeutic potential for clinical use in humans.

WNV infection induces a potent humoral immune response, which is essential in controlling viremia and limiting WNV dissemination to the CNS [72, 73]. Hyperimmune sera, γ-globulin, or affinity-purified antibodies harvested from WNV-infected humans and animals protect both wild-type and immunocompromised mice from WNV challenge in laboratory conditions [164–166]. In addition, WNV patients who received antibodies from the WNV seropositive donors recovered from WNV infection [167–171]. These studies not only encouraged the efforts toward the development of human or humanized monoclonal antibodies against WNV, but also led to the discovery of several potent monoclonal antibodies that showed efficient protection of mice and hamsters from WNV infection [172–175]. Among these, a humanized anti-WNV mAb (Hu-E16) that binds to a highly conserved epitope in WNV E protein blocks viral fusion and provide post-exposure therapeutic potential [172]. This antibody is currently being assessed for its potential use as a WNV therapeutic antibody [176]. The phase I clinical trial showed that another humanized recombinant antibody targeting E protein of WNV (known as MGAWN1) has a good safety and tolerance profile in healthy humans [177], however, the phase II trial to assess its efficacy in WNV infection failed due to poor enrollment of participants. Besides the development of whole antibody, recombinant fusion proteins are also generated from single-chain antibody fragment of the variable region. Such antibody fragments that target E protein may be potential candidates for immunoprophylaxis and therapy of WNV infections. A recombinant human single-chain variable region antibody fragments (Fv-Fc) fusion protein has a protective role against WNV infection in mice [178].

One of the potential limitations of this approach is antibody-dependent enhancement (ADE), a phenomenon by which infection of some viruses is enhanced by virus-reactive antibodies resulting in more efficient virus entry through Fc receptor-mediated
pathways. This phenomenon plays a role in the pathology of severe dengue infection and has also been observed in WNV in vitro [179]. Although the role of ADE in WNV disease is unclear, the development of a therapeutic antibody against WNV should address this potential issue. Another limitation of antibody-based therapeutics is high production cost, which limits mAb scalability. Producing therapeutic proteins, including antibodies in plants, may be a promising solution. Feasibility of this approach has been affirmed by the successful production of anti-WNV monoclonal antibody Hu-E16 in plants (Nicotiana benthamiana) (MAb-pE16) [180, 181]. The plant-derived MAb-pE16 confers a potent neutralizing activity in vitro without ADE, efficiently binds to complement and Fc receptors, and protects mice against lethal WNV-challenge with similar potency as their mammalian-cell counterparts [180, 182, 183].

Antibodies employ several mechanisms to control WNV and other viral infections, including blockage of viral entry, Fc-dependent viral clearance, complement-mediated viral lysis, and antibody-dependent cytotoxicity of infected cells. Most of the current researches in the development of therapeutic antibodies against WNV are designed and tested for efficient neutralization potential [184, 185]. Increased understandings of the biology of antibody Fc regions, in particular, the roles of glycan in Fc mediated functions may facilitate the design and development of high-quality antibody through glycoengineering [186, 187]. Such engineering of antibody Fc region may be used to overcome ADE, modulate pharmacokinetics, and enhance Fc mediated effector functions, such as enhancement of antibody-dependent cell mediated cytotoxicity (ADCC), complement binding, and phagocytosis [188].

Recent technological advancement not only in development, production, and purification but also in ease of achieving desirable quality, efficacy, and safety required for the FDA approval makes monoclonal antibodies a promising therapeutic option. Thus, monoclonal antibodies may prove useful for WNV prophylaxis and therapy particularly in the elderly and immunocompromised individuals with limited ability to respond to a vaccine. To meet its therapeutic goal, a controlled clinical trial of therapeutic antibody should ensure its prophylactic and therapeutic efficacy along with optimal dose and timing of administration across the range of patient groups.

5 Moving Towards Anti-WNV Drug Discovery: Recent Approaches and Future Directions

Development of effective therapeutics have been successful in treating many viral diseases including influenza, HIV-1, hepatitis C virus (HCV), and hepatitis B virus (HBV). WNV causes transient viremia in human and animal models that is associated with
its dissemination to brain and development of more severe disease [73, 189–192], suggesting that reducing viral loads by an antiviral drug during the early phase of infection may offer efficient control of WNV or lessen the chances of progression to neuroinvasive diseases. In addition, antiviral drugs are particularly useful for the elderly and the immunocompromised patients who may fail to develop efficient vaccine-induced immunity. Recent progress in the structural characterization of WNV and other flaviviruses broadens the understanding of WNV biology and provides a foundation for the development of small molecule inhibitors for WNV therapeutics [34, 193]. In addition, better understanding of the pathogenesis of WNV and other flaviviruses has offered new opportunities for designing many different classes of promising antiviral therapeutics by targeting both viral replication and the host cell metabolism.

5.1 Approaches for Drug Discovery

The development of an antiviral drug is a multistep process that takes years before it reaches the market. A general overview of a drug development process is outlined in Fig. 2. Hit-to-lead is an initial stage in a drug discovery, where small molecule hits are screened and further evaluated to identify promising lead compounds with a therapeutic potential. Recent progress in the development of multiple approaches for designing, screening, identification, and validation of hit compound (reviewed by [194–199]) have witnessed growing interests in the field of drug development. Significant progress in structural and functional characterization of both structural and nonstructural proteins of WNV and other flaviviruses has facilitated identification of therapeutic targets and hit-to-lead screening. For example, characterization of pseudo-atomic structure of mature and immature WNV [35], atomic resolution structure of WNV and other flaviviral protein by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy [39, 41, 48, 200–203], and structural characterization of binding of a neutralizing monoclonal antibody to E protein of WNV [48] have greatly increased our understanding of both structural and functional aspects of potential therapeutic targets. Two approaches have been commonly used for small molecule inhibitor screening include target-based approach and cell-based approach. For target-based screening, several methods can be applied, including enzyme activity-based screening, fragment-based screening, affinity-based screening, structure-based rational
designing, and in silico docking [197, 198, 204–207]. In contrast, cell-based approaches use viral infection and replication-dependent assays to identify inhibitors [208]. Each of these approaches presents their unique sets of merits and challenges. For instance, it is generally difficult to identify a target and also achieve specificity by cell-based assay because such identified inhibitors may potentially affect multiple steps of viral infection cycles and may target both viral and host proteins. Although inhibitors identified by cell-based assay may prove useful as antiviral drug candidates, these compounds could also act nonspcifically; thus further elucidation of their mechanism of action is required. The target-based approaches are highly efficient in screening process, however, an inhibitor screened by such method may require further modification for effective cellular permeability and validation for its antiviral activity, selectivity and toxicity by using cell-based assays. In the final stages, lead compounds are selected for in vitro and in vivo pharmacokinetic profiling, including efficacy, plasma stability (half-life), exposure, bioavailability, and preclinical toxicity before planning a clinical trial.

5.2 Current Status of Anti-WNV Drug Development

The most common strategies for antiviral drug development include blocking virus attachment or entry into host cells and inhibiting viral replication, either by targeting on viral components or host cells metabolism. There has been a significant progress in development and testing of potential antivirals against flaviviruses including WNV (reviewed by [34, 209, 210]). Several natural and synthetic compounds, antiviral peptides and siRNAs have been identified to target both structural and nonstructural proteins of WNV and evaluated for their potential therapeutic roles. Other approaches include targeting host cell metabolism and physiology and modulating host immune system by using antiviral cytokines as potential therapeutics against WNV. Current status and strategy used to develop antiviral drugs targeting WNV and other flaviviruses are described below.

5.2.1 Natural and Synthetic Compounds as Small Molecule Inhibitors of WNV

Several natural and synthetic compounds have been identified to target both structural and non-structural proteins of flaviviruses. Most of these compounds are designed to target DENV and some of them also show antiviral activities against WNV and other flaviviruses. As these compounds are diverse in their chemistry, they are discussed below based on their modes of action.

Viral Entry/Fusion Inhibitors

The E protein of WNV and other flaviviruses play key roles in viral entry into host cells by mediating viral attachment to host cell receptors and subsequent membrane fusion [35, 41, 211, 212]. After binding to host cell receptors, WNV enters into cells through a clathrin-independent endocytosis process followed by a low-pH-dependent viral uncoating in the endosome to release viral genome
into the cytoplasm for replication [213]. Inhibitors that disrupt the interaction of E protein with cell receptors or inhibit membrane fusion would be a potential antiviral against WNV. Two successful HIV drug Maraviroc (CCR5 antagonist) and Enfuvirtide (a peptide inhibitor) that respectively block viral entry [214] and membrane fusion [215] attest to this antiviral strategy. The flaviviral E glycoprotein contains several functional sites such as a hydrophobic pocket, the receptor-binding domain and stem domain that may be targeted by inhibitors. Among these, the hydrophobic ligand-binding pocket in a hinge region between domain I and II of E protein plays an important role in low-pH-mediated membrane fusion process and is a unique target for developing small-molecule inhibitors against flaviviruses [216, 217]. Various screening approaches were used to identify inhibitors against DENV and other flaviviruses that bind into this hydrophobic pocket and interfere with the conformational changes of E protein [207, 218–221]. Most of these inhibitors were designed and tested against DENV, some of which were reported to exhibit antiviral activities against WNV [207, 220]. However, these compounds failed in the further drug development due to their undesirable properties, such as low solubility and cytotoxicity.

The domain III of E glycoprotein that mediates receptor-binding can be potentially targeted by developing inhibitors that can disrupt the viral attachment to host cell receptors. Neutralizing antibodies against E protein have proven the potential of this strategy. Several compounds have also been shown to interfere with the binding of flaviviruses to host cell receptors [222–226]. However, lack of understanding of cell receptor for WNV has hampered the success of this approach. Identification of cellular receptors for WNV and understanding of virus–receptor interaction may provide new opportunities to identify small molecule inhibitors that interrupt the binding of WNV to host cell receptors.

Viral proteases are essential for WNV life cycle for they cleave the viral polyprotein precursors into functional proteins. Successful development and licensing of protease inhibitor against HIV-1 [227, 228] and HCV [229, 230] provides the proof of concept and feasibility for similar targeting of proteases of other viruses. The N-terminal domain of flaviviral NS3 (amino acids 1–169) has serine protease activity whereas a hydrophobic region of NS2B protein serves as cofactor to activate the enzymatic activity of NS3 [53, 231, 232]. The NS3 protease of WNV processes the viral polyprotein precursor into structural and non-structural proteins and disruption of this activity is lethal for WNV [54, 57]. In addition, WNV NS2B/NS3 protease can also cleave host proteins and may contribute to neuropathogenesis [63]. Recent progress in the expression of stable NS2B/NS3 and identification of the high-affinity substrate for this viral enzyme has promoted large-scale
screening of protease inhibitors for WNV and other flaviviruses. A wide range of assays, such as conventional enzyme-substrate based detection, HPLC, ELISA, and high-throughput fluorescence-based detection methods have been developed for screening of viral protease inhibitors [233–236]. Thus, NS3 protease is an attractive target for the development of antiviral against WNV and other flaviviruses (reviewed by [237]).

Except for aprotinin, a pancreatic trypsin inhibitor, most of the classical inhibitors of serine protease do not inhibit flaviviral NS2B/NS3 protease activity [238, 239]. Although aprotinin is a potent inhibitor of flaviviral protease, this compound was withdrawn from the market in 2008 due to safety issues [240]. To screen and identify small molecule inhibitors of flavivirus protease, both high throughput screening and structure-based drug designing have been used. These strategies are based on the identification of allosteric inhibitors that target the interface of NS2B-NS3 protease, or the active site of NS3 protease. The former strategy may overcome nonspecificity of the latter due to the largely conserved active sites of the human and viral serine proteinases. A number of inhibitors for WNV NS2B/NS3 protease have been identified by in silico docking or high-throughput screen using in vitro enzyme activity-based assays [34, 59, 206, 241–249]. However, most of these compounds failed to demonstrate potent antiviral activity in cell culture. Although a few compounds identified by these approaches show anti-WNV activity in cell-based assays [62, 206, 242], none of the inhibitors has progressed beyond the hit optimization stage. Discovery of NS2B/NS3 protease inhibitors has been hampered largely due to the difficulties in obtaining co-crystal structures of inhibitor-protease complexes. Moreover, because of the weak binding affinity of NS2B/NS3 active site due to its flat and charged nature, the design of potent small molecule inhibitors by structure-based method becomes difficult [53, 242, 250, 251].

The helicases are enzymes that unwind nucleic acid by using energy derived from hydrolysis of NTP. The C-terminal domain of NS3 of WNV contains helicase/nuclease activities and plays important roles in virulence and pathogenesis [252–254]. High throughput assays that measure helicase activity by monitoring helicase-catalyzed strand separation in real-time by using radioactive or fluorescent-labeled oligonucleotides have been developed to screen helicase inhibitors [255–258].

Several small molecule inhibitors targeting helicase of HCV and HIV-1 have been developed [259]. By using the substrate-based assay, a few compounds have been identified and evaluated in vitro against NTPases/helicases of WNV and other flaviviruses [260–262]. However, inhibitory effects of WNV helicase by these compounds are specific to either DNA or RNA substrate. For example, a compound named 4,5,6,7-tetrabromobenzotriazole

NS3 Helicase/Nuclease
Inhibitors
(TBBT), a halogenated benzotriazole, inhibits NS3 helicase, but not NTPase activity [260]. A series of ring-expanded nucleoside/nucleotide analogs (RENs) also inhibit NTPases/helicases activities of flaviviruses, including WNV, HCV, and JEV [263, 264], however, these compounds did not show any promising anti-WNV activity in cell culture. A nucleoside analog imidazo[4,5-d]pyridazine nucleosides [265], and a broad-spectrum antiparasitic drug named ivermectin [266] inhibit NS3 helicase and also show anti-WNV activity in cell culture.

The RNA-dependent RNA polymerase (RdRp) activity of C-terminal NS5 protein of WNV and other flaviviruses is an attractive target for developing antiviral agents [55, 267–269]. Two approaches used to target WNV RdRp include nucleoside inhibitors (NIs) or non-nucleosides inhibitors (NNIs). NIs (also known as type 1 inhibitors) are nucleoside/nucleotide analogs that target the active sites of the polymerase and generally compete with natural NTP substrates of RdRp to block their incorporation into viral genome during replication and lead to incomplete replication or mutations of viral genome. The success of NIs against several viruses including HIV-1, herpesviruses, HBV, and HCV has already proved the therapeutic potential of this class of compounds [270–272]. In addition, NI generally displays broad-spectrum antiviral activities across related RNA viruses suggesting its potential as pan-flaviviral therapeutics. Various cell-based and cell-free assays have been developed for high-throughput screening of flaviviral RdRp inhibitors [273–276].

So far, several NIs that inhibit WNV, DENV, and other RNA viruses have been identified [34]. For example, favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) and related compounds selectively inhibit viral RNA-dependent RNA polymerase and have potent anti-influenza activity [277, 278]. This antiviral drug is currently being evaluated in clinical trials against influenza virus. In addition, favipiravir also blocks replication of many other RNA viruses, including WNV and are promising drug candidate against a broad range of RNA viral diseases [279]. Two other nucleoside analogs called 7-deaza-2’-C-methyl-adenosine and 5-aza-7-deazaguanosine (ZX-2401), which are the derivatives of triphosphates of 2’-C-methyl-adenosine and 2’-C-methyl-guanosine, respectively, are also broad-spectrum antiviral compounds targeting viral RdRp that inhibit DENV, HCV and WNV [280–282]. Similarly, two other NI inhibitors, NITD-008 (beta-D-2’-ethynyl-7-deazaadenosine triphosphate) and NITD203 (3’5’-O-diisobutyryl-2’-C-acetylene-7-deaza-7-carbamoyladenosine) inhibit all four of DENV serotypes and WNV.

In contrast to NI inhibitors, antiviral NNI inhibitors (also known as type 2 inhibitors) interfere with the function of viral polymerase by occupying its allosteric sites, thus preventing viral
RNA synthesis. Analysis of RdRp crystal structure of WNV and DENV3 revealed a cavity that plays a critical role in viral replication, suggesting a potential target for screening of structure-based allosteric inhibitors [55, 283]. N-sulfonylanthranilic acids derivatives identified by high-throughput screening are examples of allosteric inhibitors of RdRp activity of DENV [284]. However, these compound were specific to DENV and did not show any activity against WNV RdRp. A recent study demonstrated that a conformational change occurred in DENV-3 polymerase after binding with an inhibitor [285]. However, a similar antiviral activity of NNI inhibitors targeting polymerase of WNV has not been reported yet.

**Methyltransferase Inhibitor**

Messenger RNA (mRNA) of WNV possesses a 5′ cap that plays important roles in stability of mRNA and its translation. The methyltransferase (MTase) activity of the N-terminal domain of NS5 is responsible for N-7 and 2′-O-methylation of the viral RNA cap [64, 286] [287]. In addition, MTase activity is also responsible for evading host’s antiviral interferon response and plays an important role in WNV pathogenesis [288]. Several structural and functional studies along with identification of several potential inhibitors suggest that targeting MTase represents a novel approach for the development of novel therapeutics against WNV and other flaviviruses [289–294]. Flaviviruses MTase catalyzes sequential methylations of the viral RNA cap using S-adenosyl-L-methionine (SAM) as the methyl donor and contains a single binding site for SAM in its crystal structure [289, 294]. In addition to MTase activity, binding of GTP has been shown in MTase domain of several members of flavivirus [295]. Several assays have been developed for high-throughput screening of methyltransferase inhibitors by structural-based and ligand-based methods [296, 297]. Rational design of SAM analogs has identified several inhibitors targeting MTase activity of DENV and WNV [34, 298].

Nonspecific inhibition of host MTase is one of the potential drawbacks of SAM analogs. A specific inhibition of flaviviral, but not host, MTase can be achieved by targeting a pocket near the SAM-binding site [290, 298]. Two nucleoside analogs were identified that potently inhibited the MTase of WNV without inhibiting human MTase. One of these compounds (GRL-003) showed antiviral activity against WNV in cell culture [299]. In addition, several screening studies against YFV and DENV NS5 have identified hits targeting MTase activity, some of which showed antiviral activity against WNV in cell culture [204, 300, 301]. However, an extensive multistage molecular docking approach to screen a library of about 5 millions of commercial compounds against two active sites of DENV MTase/GTase failed to identify any specific hits [302]. Recently, 5′-silylated nucleoside scaffold derived from 3′-azidothymidine (AZT) demonstrated antiviral activity against WNV and DENV, which binds MTase [303].
Several potential tools, including rational design and phase display library, have been developed for high-throughput screening of specific antiviral peptides [304, 305]. Enfuvirtide, a 36-amino-acid peptide based on the stem region of the HIV gp41, exemplifies an efficient antiviral peptide currently in clinical use [306]. Thus, antiviral peptides may serve as a novel therapeutic measure against WNV. Several antiviral peptides targeting both structural and non-structural protein of WNV and other flavivirus have been identified.

Targeting WNV E protein by antiviral peptides is a potential strategy that blocks virus attachment and entry into the host cells. Several short antiviral peptides (13–16 amino acid residuals) that bind to WNV E protein have been identified by screening of a murine brain cDNA phage display library [307]. One of those peptides (P9) reduces viremia and fatality after WNV infection in mice. P9 can efficiently penetrate the murine blood–brain barrier, implying that it may have antiviral activity in the CNS [307]. Similarly, a peptide inhibitor (WN83) targeting domain II of WNV E protein designed by using a physicochemical algorithm approach potently inhibits WNV infectivity [308]. Another peptide designed to target domain II of DENV E shows antiviral activity against both DENV and WNV [308]. In addition, a rational drug design approach has been used to identify a peptidomimetic that mimic NS2B/NS3 protease substrate and inhibits its activity. The mechanism proposed for the peptidomimetic is that NS2B/NS3 cleaves between P₁ and P₁’ in a peptide substrate consisting of P₂P₁P₁’, where P₁ and P₂ are basic amino acids (Arg or Lys) and P₁’ is a side-chain amino acid (Gly, Ser, or Ala) [56, 231]. Thus, a preferred peptide substrate contains several positively charged amino acids. A common method for screening peptide inhibitors of NS2B/NS3 protease employs a fluorophore conjugated peptide substrate containing basic amino acids at the P₁ and P₂ positions. Cleavage of peptide substrate by NS2B/NS3 protease results in a release of fluorophore and increase of fluorescence [242, 243]. Several peptide inhibitors of NS2B/NS3 protease have been identified against WNV [62, 309–311]. A novel agmatine dipeptide inhibitor with improved inhibitory activity against WNV NS2B/NS3 has been recently identified [309]. In addition, a recombinant peptide called retrocyclin-1 (RC-1) has been shown to inhibit NS2B/NS3 protease [312]. However, most of these peptides showed poor activity in the cell-based assay and has not been tested for their in vivo efficacy. Thus, all of the peptide inhibitor of NS2B/NS3 protease that has been identified so far failed at the early development stages. Potential limitations of this approach include poor pharmacokinetic properties due to charged nature of peptide, lack of specificity, requirement of intravenous delivery, rapid degradation in plasma, and costly production.
RNA interference (RNAi) is a cellular process first described in the nematode *Caenorhabditis elegans* [313, 314]. This process specifically degrades RNA in a sequence-specific manner and is conserved in mammalian cells [315, 316]. RNAi is a natural defense of eukaryotic cells against viral infections, and may be a promising strategy for developing a potential antiviral therapeutic. Numerous siRNA targets were identified in the genomic region of WNV encoding both structural and non-structural proteins, and siRNA targeting these proteins effectively inhibits WNV replication [191, 317–321]. Besides the siRNA targeting coding regions, siRNA that targets noncoding regions have also been identified to inhibit WNV replication in a sequence-specific manner [191]. Although anti-WNV siRNAs efficiently block viral replication in cell cultures, similar successes are difficult to achieve in animal models [191]. Quick degradation by serum nucleases, failure to reach target cells, and rapid renal excretion due to their small size and anionic character are hindering the clinical application of antiviral siRNAs. Several delivery systems, including cell-penetrating peptide [322, 323], nanoparticles [324, 325], and viral vectors [326], may improve siRNA stability and enhance delivery efficiency. Despite many challenges, use of antiviral siRNA as anti-WNV therapeutics remains promising.

Cytokines signaling controls diverse immune functions during infection, autoimmune disease, and cancer. Various immunomodulatory or immunostimulatory cytokines and chemokines have been identified to play a protective or pathological role in WNV infection. For examples, type-I interferons (IFNs) [327, 328], interleukin (IL)-23 [192], interferon-γ (IFN-γ) [83], IL-1β [329], macrophage migration inhibitory factor (MIF) [330], CXCL10 [331], and CCL5 [332, 333] protect against WNV infection, whereas IL-10 [190] and IL-22 [334] favor WNV pathogenicity. Pharmacological blockade of IL-10 by neutralizing antibody has been shown to protect mice against WNV challenge [190]. Type I IFNs (IFNα/β) inhibit many flaviviruses including WNV and have been used as therapeutics against hepatitis C virus [335]. Although the therapeutic effect of type I IFNs in WNV has yet to be evaluated, its application may be limited due to the antagonistic role of WNV NS5 protein in IFN signaling [336, 337]. Interestingly, treatment with pegylated IFN-λ, also known as a type III interferon, has been recently shown to protect mice against lethal WNV infection by decreasing blood–brain barrier permeability [338]. Thus, strategies targeting the expression of cytokine and chemokine, blocking their signaling, or direct use of recombinant cytokines may be novel approaches for treating WNV infection or controlling its pathology.
Viruses utilize host cellular system for entry, genome replication, transcription, synthesis of viral proteins, and production of viral progenies. In addition, interactions of viral proteins with cellular proteins may evade host immune defense and favor viral replication and pathogenesis. Several host pathways and enzymes including clathrin-mediated endocytosis cyclophilins [339], ubiquitin-proteasome system [340], unfolded protein response [341], nucleotide biosynthesis [342, 343], post-translational protein modification [344–346], and lipid metabolism [347–349] have been suggested in flavivirus replication and pathogenesis. Targeting host factors may be used as a strategy for developing antiviral therapeutic against flaviviruses, including WNV infection [350–352].

So far, many inhibitors targeting host proteins have been developed and tested against WNV and other flaviviruses, such as HCV. Host cyclophilin, a family of cellular peptidyl-prolyl isomerases, may serve as a component of flavivirus replication complex and play a role in flaviviral replication. Targeting this enzyme by cyclosporine inhibits replication of WNV [339]. Targeting lipid signaling and metabolism by a bioactive lipid signaling modulator 4-hydroxyphenyl retinamide (4-HPR, fenretinide) also inhibits replication of WNV and other flaviviruses. Similarly, ribavirin and mycophenolic acid target inosine monophosphate dehydrogenase (IMPDH), an enzyme in purine biosynthesis, and thereby inhibit replication of flaviviruses [342, 343]. In addition, NITD-982 and brequinar that block pyrimidine biosynthesis also inhibit replication of broad range of RNA viruses, including WNV and other flaviviruses [353, 354]. Besides blocking viral genome replication, antiviral targeting of other steps, such as virus maturation, assembly, and viral dissemination into brain has also been suggested and tested against WNV. For instance, inhibitors of alpha-glucosidase I and II, enzymes that play a role in processing of N-linked oligosaccharides of the viral glycoproteins, also inhibit WNV and other flaviviruses [205, 355]. Although the mechanism by which WNV enters the brain is still poorly understood, the two potential routes include axonal retrograde transport (ART) from the peripheral nervous system and direct hematogenous diffusion via a breakdown in the blood–choroid plexus barrier [356]. Nocodazole, a microtubule inhibitor that blocks ART, delays WNV entry into brain [357]. The 3’ or the 5’ terminal stem-loop in flaviviral RNA contains essential cis-acting elements and plays important roles in viral replication [358, 359]. Interestingly, a range of cellular proteins have been identified to interact with 3’ stem-loop of flaviviral RNAs [352, 359–362], suggesting a potential strategy to design inhibitors targeting this virus–host protein interaction.

Targeting host factors may raise a higher barrier to viral resistance emergence and provide broad-spectrum antiviral effects.
However, current understanding of virus–host cell interaction and research on targeting of host factors to block viral infections are still limited. In addition, there are some potential drawbacks of this approach, including undesirable drug-induced side-effects and difficulties for drug delivery into brain to control WNV encephalitis. Further understanding of virus–host interaction will facilitate identification of novel antiviral agents.

6 Conclusions and Perspectives

Development and testing of various methods for treatment and prevention of WNV infection, such as protective vaccines, therapeutic antibodies, antiviral compounds, peptides, and siRNA have been proposed and intensively studied. Although a number of WNV veterinary vaccines have already been licensed and are in use for years, human vaccine candidates are still in various stages of development and testing. Some therapeutic antibodies that show excellent efficacy in small animal models and are currently being tested in clinical trials represent a promising class of WNV therapeutic. Recent technological advancement and increased understanding of the biology of WNV and other flaviviruses along with structural/functional characterization of viral proteins have provided a solid foundation for the development of small molecule inhibitors as future WNV therapeutics. However, efforts for development of an effective drug for prevention or control of WNV infection in human still remain unsuccessful. Some of the reasons include a low incidence of diseases, low commercial interest by pharmaceutical companies, high cost of mass vaccination, and difficulties with running clinical trials due to unpredictable and sporadic nature of WNV outbreaks [363, 364]. Another challenge for developing successful WNV therapeutics is to ensure safety and efficacy in target populations that mostly include children, elderly, and immune-compromised individuals. Despite all these difficulties, the quest for development of effective treatment and prevention methods against WNV infection are likely to be facilitated by recent technological advancement and should continue to meet the public health needs.

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