

# 21. Novel Therapeutics Against West Nile Virus

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## Abstract

No effective therapy is currently available for clinical treatment of flavivirus infections. Recent advances in the structural and molecular biology of flaviviruses have provided new opportunities for the development of antiviral therapies. This chapter summarizes the current status of West Nile virus (WNV) therapeutics. First, strategies for identifying and characterizing small molecular inhibitors are reviewed. These strategies include structure-based rational design, biochemical enzyme-based screening, and reverse genetic system-based screening. Second, known WNV inhibitors are summarized. Both small and macromolecular inhibitors have been identified to inhibit WNV. The macromolecular inhibitors include WNV antibodies, interferon, and nucleic acid-based agents (i.e., antisense oligomer and siRNA). Since the antibody-based therapy is reviewed elsewhere in this book, this chapter emphasizes the nonantibody macromolecular and small molecular inhibitors. Finally, new potential antiviral targets and issues related to WNV therapeutics are discussed.

## Keywords

antiviral, siRNA, small molecule inhibitor, replicon, flavivirus

## 1 Introduction

Flavivirus infection is a major public health threat. Human vaccines for flavivirus infections are currently available only for yellow fever virus (YFV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV). For West Nile virus (WNV), vaccines are only approved for equine use (Davis et al., 2001; Minke et al., 2004; Ng et al., 2003).

Development of a vaccine for dengue virus (DENV) has been challenging, principally because of the need to simultaneously immunize and induce long-lasting protection against all four DENV serotypes; an incompletely immunized individual may be sensitized to dengue hemorrhagic fever or dengue shock syndrome. Because of these complications, it is critical to develop effective therapies for the treatment of flavivirus infections. This chapter focuses on the current development of WNV therapeutics, including (1) strategies for the identification of novel small molecular inhibitors, (2) known WNV inhibitors, and (3) potential new antiviral targets and issues related to the antiviral development. Since members from the *Flavivirus* genus share a common replication strategy, antiviral studies of flaviviruses other than WNV are also selectively discussed.

## **2 Strategies for the Identification of Novel Small Molecular Inhibitors**

Each step of the WNV life cycle is a potential target for antiviral intervention. Three approaches are commonly taken to identify inhibitors, including structure-based rational design, biochemical enzyme-based screening, and reverse genetic system-based screening. Each approach has its own advantages and potential problems. Once combined, these approaches can provide complementary information about individual inhibitors.

### **2.1 Rational Design**

The single open reading frame of the flavivirus RNA genome encodes a long polyprotein that is co- and posttranslationally processed into ten mature proteins by a combination of host proteases and the viral protease, resulting in three structural proteins [capsid (C), membrane (M), and envelope (E)] and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (Lindenbach et al., 2007). The atomic structures of many flavivirus proteins and domains have been solved. This structural information makes the rational design of inhibitors possible. Among the three structural proteins, the structure of C has been solved by NMR (Ma et al., 2004) and crystallography (Dokland et al., 2004). The structures of the E ectodomain are available in both pre- and postfusion forms (Bressanelli et al., 2004; Modis et al., 2003, 2004; Rey et al., 1995). Moreover, the virion structures of WNV and DENV have been determined by using a combination of cryoelectron microscopy and fitting of the known structure of E into the electron density map (Kuhn

et al., 2002; Mukhopadhyay et al., 2003; Zhang et al., 2003c). Among the seven nonstructural proteins, two have enzymatic activities. The N-terminal region of NS3 exhibits a protease activity (with cofactor NS2B), which is responsible for all cleavages of the polyprotein on the cytoplasmic side of the endoplasmic reticulum (ER). The C-terminal portion of NS3 has 5'-RNA triphosphatase, NTPase, and helicase activities. The 5'-RNA triphosphatase activity is required for formation of the cap structure of the viral genome. The NTPase and helicase activities are needed to unwind RNA structure during viral replication. The N-terminal region of NS5 encodes a methyltransferase, which is required to methylate the cap guanine N7 and the ribose 2'-OH of the first nucleotide during cap formation (m7GpppAm). The C-terminal portion of NS5 contains an RNA-dependent RNA polymerase (RdRp). Crystal structures of individual domains of the protease complexed with a Bowman-Birk inhibitor (Murthy et al., 2000) or with the NS2b peptide (Aleshin et al., 2007; Erbel et al., 2006), helicase (Mancini et al., 2007; Mastrangelo et al., 2007b; Wu et al., 2005; Xu et al., 2005), methyltransferase (Assenberg et al., 2007; Egloff et al., 2002, 2007; Mastrangelo et al., 2007a; Zhou et al., 2007), and RdRp (Malet et al., 2007; Yap et al., 2007) have been solved for a number of flaviviruses. Furthermore, the crystal structure of full-length NS3 of DENV-4 was recently resolved (Luo et al., 2008). The structure of the full-length NS5 remains to be determined.

Despite the breakthrough in structure solving of flavivirus proteins, few inhibitors have been developed through rational design. A virtual screening, using DENV-2 NS5 methyltransferase structure, recently revealed a compound that inhibited DENV 2'-O cap methylation with an  $IC_{50}$  value of 60  $\mu$ M (Luzhkov et al., 2007). The study used *S*-adenosylmethionine (SAM), the methyl donor, as a starting molecule to search for analogs that could specifically dock into the SAM-binding pocket of the DENV-2 methyltransferase. Since the SAM molecule bound in the same pocket donates methyl groups to both N7 and 2'-O positions during cap methylations (Dong et al., 2008), the identified compound is expected to inhibit N7 methylation of the viral RNA cap. Given that the flavivirus methyltransferases are highly conserved in structure and sequence, it is likely that this compound would inhibit methyltransferases from flaviviruses other than DENV. However, experiments are needed to verify these speculations and to demonstrate the potency of the compound in a viral infection assay. Along the same line, sinefungin (SIN), a SAM analog in which the transferring methyl group is replaced by an amino group, inhibits both N7 and 2'-O methylations of the WNV RNA cap, with an  $IC_{50}$  value of approximately 14  $\mu$ M (Dong et al., 2008). In BHK cells, SIN inhibits WNV infection with an

$IC_{50}$  of 27  $\mu$ M,  $CC_{50}$  of 4.5 mM, and therapeutic index ( $TI = CC_{50}/IC_{50}$ ) of 167. The cytotoxicity of SIN is expected, because the compound can also bind to host methyltransferase and suppress cap methylations of cellular mRNA. As the residues that form the SAM-binding pocket between the host and viral methyltransferases are poorly conserved (Martin and McMillan, 2002), the specificity of SIN binding to the viral methyltransferase could be improved to reduce its cytotoxicity.

## 2.2 Biochemical Enzyme-Based Screening

NS3 and NS5 are key enzymes of the viral replication complex and are ideal targets for antiviral screening. Of the numerous high-throughput screening (HTS) assay formats, the scintillation proximity assay (SPA), a radioactive homogeneous assay technology has been commonly used to screen large compound libraries. The SPA method relies upon the fact that the energy emitted from a radioisotope will only travel a limited distance in an aqueous environment. When a radioisotope-labeled molecule binds to the microsphere, the radioisotope is brought into close proximity to the scintillant and effective energy transfer from the  $\beta$ -particle occurs, resulting in light emission. When the radioisotope remains free in solution, it is too distant from the scintillant; the  $\beta$ -particle dissipates the energy into the aqueous medium and remains undetected. The SPA method has been used to assess DENV-3 RdRp activity (Yap et al., 2007), and can be readily adapted to flavivirus helicases and methyltransferases:

1. In the RdRp assay, a 5' biotin-labeled oligomer is annealed to an RNA template and extended to incorporate  $^3$ H-labeled GTP in the presence of recombinant RdRp. The newly synthesized RNA is then captured onto the streptavidin-coupled SPA beads, and the amount of  $^3$ H-GTP incorporation (indicating the RdRp activity) is quantified by scintillation counting.
2. An SPA-formatted methyltransferase assay could be similarly developed. As flavivirus methyltransferase requires specific viral RNA for cap methylation (Dong et al., 2007a), the biotin-labeled RNA substrate should contain the 5' terminal sequence of the viral genome. Upon transfer of the  $^3$ H-labeled methyl group from SAM to RNA cap, the biotin-labeled RNA is captured onto the streptavidin SPA beads and measured through a scintillation counter.
3. For the helicase assay, one strand of the double-stranded RNA substrate (derived from annealing of two RNAs with complementary sequences with 3' end overhangs) should be labeled with radioactive

nucleotide, while the other RNA strand is coupled with biotin. After unwinding of the double-stranded RNA, the biotinylated RNA could be captured by the streptavidin SPA beads, and counted in a scintillation counter. The helicase activity will be reflected by a reduction of the scintillation signal.

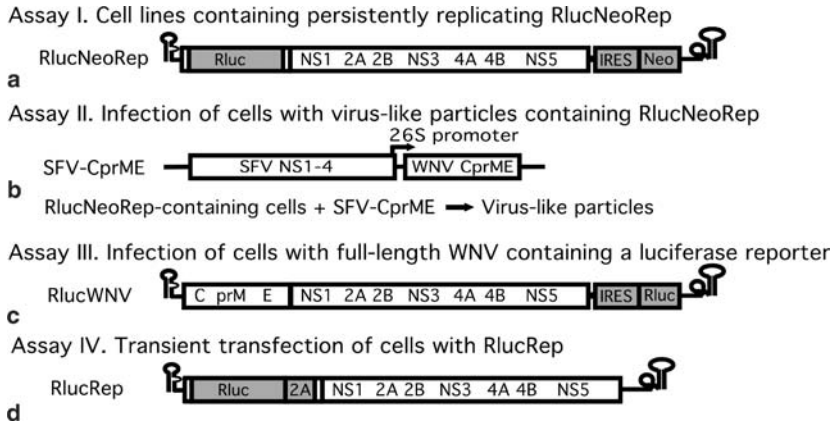
For the NS3 protease assay, a fluorogenic peptide substrate-based assay has been widely used (Mueller et al., 2007; Yusof et al., 2000). This method uses a peptide substrate coupled to a highly fluorescent dye, whose fluorescence is quenched while linked to the uncleaved peptide substrate. Upon protease-mediated cleavage, the fluorescent dye is released from the peptide, thereby producing an increase in fluorescence intensity.

The above assays have made it possible to screen for potential inhibitors of key viral enzymes in an HTS fashion. The major advantage of the enzyme-based assays is that the identified compounds possess known targets. The availability of structures of these proteins will facilitate further improvement of the inhibitors. However, compared to the cell-based screening assays (see next section) the enzyme-based screening does not examine cellular uptake and nonspecific binding of serum proteins to the compounds. The latter factors could dramatically affect the potency of the compounds in cell culture and animals.

### 2.3 Genetic Cell-Based Screening

Genetic cell-based assays have two major advantages: more than one target and step are analyzed during replication and the uptake of compounds into cells is required, which represents a more authentic therapeutic environment. Inhibitors identified through cell-based assays have had a higher success rate in subsequent animal experiments. Since most flavivirus infections cause cytopathic effects (CPE), the traditional cell-based screening assay involves viral infection of cultured cells and monitoring of inhibition through measurement of CPE (Jordan et al., 2000; Morrey et al., 2002) or quantification of viral RNA by RT-PCR (Shi et al., 2001). Unfortunately, these assays are labor-intensive and lack sensitivity. Thus, while informative, they are not ideal for screening large compound libraries.

To overcome the above problems, three cell-based HTS assays have been developed for WNV drug discovery (Fig. 1) (Puig-Basagoiti et al., 2005). These antiviral assays were optimized in a 96- or 384-well format, validated with known WNV inhibitors, and proven useful in identifying new inhibitor(s) through screening compound libraries:



**Figure 1.** Cell-based assays for development of WNV therapeutics. A *Renilla* luciferase (Rluc) gene was engineered into a replicon or into a full-length viral genome to monitor viral replication. Potential inhibitors could be indicated through suppression of luciferase signals upon compound incubation. (a) A reporting cell line assay. The cell line contains a persistently replicating replicon that harbors dual reporter genes, Rluc and neomycin phosphotransferase (Neo, driven by an EMCV IRES), resulting in RlucNeoRep. The assay allows screening inhibitors of all targets involved in viral replication. (b) Virus-like particle (VLP) infection assay. WNV structural proteins are provided *in trans* to package luciferase-reporting replicon. The example here uses Semliki forest virus vector (containing viral nonstructural proteins, SFV NS1–4) to express WNV structural proteins C-prm-E under the control of the SFV 26S subgenomic promoter (SFV-CprME). Transfection of RlucNeoRep-containing cells with SFV-CprME packages RlucNeoRep RNA into VLPs. Infections of naïve cells with such VLPs result in the replication of RlucNeoRep, yielding Rluc signals. The VLP-based assay allows screening inhibitors of viral entry and replication. (c) A full-length luciferase-reporting WNV infection assay. An EMCV IRES-Rluc was inserted at the 3' UTR of the genome, resulting in RlucWNV. Infection of cells with RlucWNV generates Rluc signals. This assay allows screening inhibitors of all steps of viral infection cycle, including entry, replication, and assembly. (d) A transient luciferase-reporting replicon. A fragment containing Rluc fused with a foot-and-mouth disease virus (FMDV) 2A protease was engineered to replace the viral structural genes, resulting in RlucRep (Lo et al., 2003a). Transfection of cells with RlucRep results in two distinct Rluc peaks: the first peak (at 1–6 h posttransfection) represents input RNA translation, and the second peak (at >10 h posttransfection) represents viral RNA synthesis. The transient RlucRep system is useful to dissect whether a compound inhibits viral translation or RNA synthesis.

1. *Cell lines expressing WNV replicons.* Cell lines have been established that persistently replicate the WNV subgenomic replicon in the absence of structural genes (Lo et al., 2003b). Many of these cell lines harbor replicons that contain a *Renilla* luciferase (Rluc; substituting for viral structural genes) and a neomycin phosphotransferase gene [Neo; driven by an encephalomyocarditis virus internal ribosomal

entry site (EMCV IRES) in the 3' UTR] (RlucNeoRep, Fig. 1a). Incubation of the RlucNeoRep-containing cells with candidate inhibitors results in a decrease in Rluc activity. The reporting cell lines allow for screening inhibitors at all steps involved in viral translation and RNA replication, but do not interrogate steps of viral assembly and entry. One major advantage of the replicon cell line is that the assay can be performed in a biosafety level 2 (BSL-2) laboratory, as no infectious particles are involved, whereas BSL-3 containment is required for working with the full-length WNV.

2. *Packaged virus-like particles (VLPs) containing replicon RNA.* The luciferase replicon can be packaged into VLPs by supplying structural proteins *in trans* (Khromykh et al., 1998). The structural proteins of WNV can be expressed from a 26S subgenomic promoter from the Semliki forest virus (SFV) expression vector (SFV-CprME, Fig. 1b). Alternatively, the structural proteins could be provided in an inducible expression cell line (Harvey et al., 2004), or in a constitutive expression cell line harboring a noncytopathic Venezuelan equine encephalitis virus (VEEV) replicon (Fayzulín et al., 2006). Compared with the replicon cell line assay, the VLP-infection system allows for screening inhibitors of viral entry as well as translation and RNA synthesis.
3. *A full-length WNV with reporter gene activity.* An IRES-Rluc fragment was inserted into the 3' UTR of the WNV genome (RlucWNV, Fig. 1c). Transfection of the RlucWNV RNA into BHK cells produced full-length reporting WNV (Deas et al., 2005). Unfortunately, the reporting virus was not stable; after multiple rounds of infection in cell culture, the engineered IRES-Rluc was deleted from the full-length virus. Nevertheless, an early passage of this reporting virus can be used to screen for inhibitors against all steps of the viral life cycle (Puig-Basagoiti et al., 2005). Recent studies showed that stable reporting YFV could be generated when a heterologous gene was inserted between E and NS1 (Bredenbeek et al., 2006), or when a foreign gene was engineered upstream of the C of the open reading frame (Shustov et al., 2007). These strategies may help to generate WNV that express reporter genes in a more stable fashion.

Besides the above cell-based assays, a transient Rluc-reporting replicon of WNV was developed to analyze mode of action of inhibitors (Lo et al., 2003a). In this replicon, the Rluc was fused in-frame with a foot-and-mouth disease virus (FMDV) 2A protease. The resulting Rluc-2A was engineered to replace the viral structural genes, resulting in

the replicon RlucRep (Fig. 1d). Transfection of cells with RlucRep generates two distinct Rluc peaks: one at 1–6 h posttransfection and another at >10 posttransfection. The two Rluc peaks can be used to differentiate between translation of the input RNA and translation of the replicating RNA, respectively. A similar approach was used to develop reporting replicons for other flaviviruses (Alvarez et al., 2005; Jones et al., 2005; Puig-Basagoiti et al., 2006). The transient RlucRep is useful to examine whether a compound inhibits viral translation and/or RNA synthesis.

### 3 Current WNV Inhibitors

Although a number of compounds have been reported to inhibit WNV enzymes or viral replication in cell culture, few have shown *in vivo* efficacy. Based on the molecular weights, these antiviral agents can be divided into two categories: macromolecular inhibitors and small molecular inhibitors.

#### 3.1 Macromolecular Inhibitors

##### 3.1.1 Antibody

Antibody-based therapy has yielded the most promising results for treatment of WNV infection (Ben-Nathan et al., 2003; Engle and Diamond, 2003; Julander et al., 2005). The results agreed with previous studies showing that passive administration of monoclonal antibodies prevented and alleviated encephalitis caused by St. Louis encephalitis virus (SLEV) (Mathews and Roehrig, 1984), JEV (Kimura-Kuroda and Yasui, 1988), and YFV (Schlesinger et al., 1985). Humanized monoclonal antibodies against WNV E protein have shown efficacy in mice, even administered as a single dose at day 5 postinfection when WNV has already infected the CNS. These antibodies showed high neutralizing activity in cell culture and provide equivalent protection in mice and hamsters in comparison to gamma-globulin (Gould et al., 2005; Morrey et al., 2006, 2007; Oliphant et al., 2005; Throsby et al., 2006). Intravenous immunoglobulin containing high titers of anti-WNV antibodies appeared effective in patients with WN encephalitis in an open-label study (Shimoni et al., 2001). However, the latter results require further confirmation with appropriate controls. The current status of the antibody-based therapy is detailed elsewhere in this book.



### 3.1.2 Interferon

Interferon- $\alpha$ -2b is currently under clinical trial for treatment of patients with WNV-mediated meningoencephalitis. Although interferon- $\alpha$ -2b inhibits WNV replication *in vitro* (Anderson and Rahal, 2002), the effect is dramatically reduced once viral replication has initiated, probably due to the blockage of interferon signaling by several of the nonstructural proteins (Best et al., 2005; Lin et al., 2004; Liu et al., 2005; Munoz-Jordan et al., 2003, 2005). Mice that lack expression of the receptor for interferon- $\alpha/\beta$  (and are therefore defective in interferon response) exhibited higher mortality, shorter survival time, and altered cellular tropism of infection in comparison with wild-type mice upon WNV inoculation (Samuel and Diamond, 2005). Treatment of WNV-infected primary neurons with interferon- $\alpha/\beta$ , however, appeared to independently increase the survival of neurons in culture. Significant recovery of neurologic function in five patients with WNV CNS disease was reported when treated with interferon- $\alpha$ -2b soon after symptom onset (Sayao et al., 2004). However, one interferon- $\alpha$ -2b failure case was reported, possibly due to delayed diagnosis and treatment, and other complications (Chan-Tack and Forrest, 2005). More controlled studies are needed to demonstrate the efficacy of the interferon treatment.

### 3.1.3 Small Peptides

Antiviral peptides were identified from a murine brain cDNA phage display library when screened for binding to the WNV E protein (Bai et al., 2007). The most potent peptide, with a sequence of CDVIALLACHLNT, had an  $IC_{50}$  of 2.6  $\mu$ M in cell culture. The peptides were able to penetrate the blood-brain barrier in mice. Mice challenged with WNV that had been preincubated with the peptide exhibited reduced viremia and mortality compared with the mice challenged with untreated virus. However, the peptide did not elicit any protection when administered intraperitoneally (i.p.) in mice at 3 h post-WNV challenge.

Short peptides representing fragments from the viral E protein have also been screened for inhibition of WNV and DENV (Hrobowski et al., 2005). The underlying rationale was that some peptides may interfere with the intramolecular interactions required for virus-host membrane fusion. The effort resulted in several peptides (representing junction regions between domains I and II) that had  $IC_{50}$  values in the 10  $\mu$ M range. Since flavivirus membrane fusion occurs in endosomes, efficient delivery of the E peptides into cells is required to further improve the efficacy.

### 3.1.4 Antisense Phosphorodiamidate Morpholino Oligomers

Two phosphorodiamidate morpholino oligomers (PMOs) were reported to potently inhibit WNV infection in cell culture (Deas et al., 2005). One PMO targets the first twenty nucleotides of the WNV genome, another PMO targets the 3' conserved sequence 1 (CS1) RNA that is required for genome cyclization during viral replication (Khromykh et al., 2001; Lo et al., 2003a). Conjugation of an arginine-rich peptide with the PMO results in more efficient cellular delivery and greater potency. Mode-of-action analyses showed that the PMOs targeting the 5' end and the 3' CS1 distinctly suppressed RNA translation and synthesis, respectively. Since the CS1 RNA sequence is conserved among mosquito-borne flaviviruses, the CS1 PMO inhibited a broad spectrum of flaviviruses in cell culture. PMO-resistant viruses can be selected in cell culture (Deas et al., 2007). Viruses resistant to the 5' end PMO accumulated mutations within the PMO-targeted region, whereas viruses resistant to the 3' CS1 PMO accumulated mutations downstream of the PMO-targeted region. In vivo analysis showed that the PMOs could partially protect mice from WNV disease, even when administered at day 5 postinfection (Deas et al., 2007). In these studies, viral inoculation and PMO treatment were administered subcutaneously (s.c.) and i.p., respectively. Since the arginine-rich peptide (conjugated to the PMO) is essential for cellular uptake, but is also responsible for toxicity, modification of the peptide composition to reduce its toxicity while maintaining its function is needed to improve the PMO-mediated therapy.

### 3.1.5 siRNA

Synthetic short interfering RNA (siRNA) inhibits WNV both in cell culture and in mice. The potency of siRNA depends on the routes of viral inoculation and siRNA delivery. In one study, both viral inoculation and siRNA [complexed with transfection reagents cationic lipid formulation (JetSI) and the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE)] were administered intracranially (i.c.) (Kumar et al., 2006). Mice receiving siRNA treatment at 6 h postviral inoculation exhibited 100% survival, whereas the mock-treated WNV-infected mice showed 100% mortality. In another study, pretreatment of mice with siRNA through a hydrodynamic injection (into tail vein) 24 h before i.p. inoculation of WNV yielded partial protection (Bai et al., 2005). In a third cell-based study, WNV replication was greatly reduced when siRNA was introduced by cytoplasmic-targeted transfection prior

to, but not after the establishment of viral replication (Geiss et al., 2005). However, when siRNA was transfected through electroporation, suppression of viral replication could be achieved after the establishment of viral replication. The latter results indicate that the transfection reagent is critical for delivering the siRNA into the viral replication compartment.

## 3.2 Small Molecular Inhibitors

### 3.2.1 Inhibitors of Nucleoside Triphosphate Synthesis

Inhibitors of nucleotide triphosphate synthesis have been reported to have anti-WNV activities (Anderson and Rahal, 2002; Jordan et al., 2000; Morrey et al., 2002). Among those, ribavirin and mycophenolic acid (MPA) are inhibitors of cellular IMP dehydrogenase (IMPDH), an enzyme that is essential for de novo biosynthesis of guanine nucleotides. Ribavirin is a guanosine analog that competitively inhibits the IMPDH, whereas MPA is a nonnucleoside, noncompetitive inhibitor of IMPDH. Both ribavirin and MPA exhibit a broad spectrum of antiviral activity, primarily through depletion of intracellular GTP pools (Leysen et al., 2005). Besides inhibition of GTP synthesis, two other antiviral mechanisms have been demonstrated for ribavirin: (1) ribavirin can be incorporated into viral RNA through base-pairing with cytidine or uridine, leading to lethal levels of mutagenesis within the virus population (Crotty et al., 2000; Day et al., 2005) and (2) ribavirin blocks viral cap methylation through binding to a GTP-binding pocket of the DENV-2 NS5 methyltransferase (Benarroch et al., 2004). Although both ribavirin and MPA inhibit WNV in cell culture, treatment of WNV-infected hamsters with ribavirin led to increased mortality (Morrey et al., 2004). Furthermore, an increased mortality was observed among 37 patients who received ribavirin treatment in a WNV outbreak in 2000 (Chowers et al., 2001). In agreement with the WNV results, no clinical benefit was observed when DENV-infected mice and monkeys were treated with ribavirin (Koff et al., 1983; Malinoski et al., 1990). Similar to ribavirin, an increased mortality was observed when WNV-infected mice were treated with MPA (Noueiry et al., 2007). Collectively, the results suggest that ribavirin and MPA are not promising candidates for treatment of WNV infection in vivo.

Several other nucleoside inhibitors were reported to have anti-WNV activity in cell culture, with TIs of >10. The inhibitors are 6-azauridine ( $IC_{50}$  5  $\mu$ M and  $CC_{50}$  600  $\mu$ M), 2-thio-6-azauridine ( $IC_{50}$  3  $\mu$ M and  $CC_{50}$  > 3.5 mM), 6-azauridine triacetate ( $IC_{50}$  3.5  $\mu$ M and  $CC_{50}$  210  $\mu$ M),

pyrazofurin ( $IC_{50}$  6  $\mu$ M and  $CC_{50}$  110  $\mu$ M), and cyclopentenylcytosine ( $IC_{50}$  1  $\mu$ M and  $CC_{50}$  110  $\mu$ M) (Morrey et al., 2002). Additionally, 5-aza-7-deazaguanosine (ZX-2401) was shown to have a broad-spectrum antiviral activity against viruses in the *Flaviviridae* family, including WNV, hepatitis C replicon, and pestivirus (bovine viral diarrhea virus) (Ojwang et al., 2005). No in vivo efficacy has been reported for these compounds against WNV.

### 3.2.2 NTPase/Helicase Inhibitors

A series of nucleoside analogs have been identified that inhibit WNV NS3NTPase/helicase:

1. An imidazo[4,5-d]pyridazine nucleoside was reported to inhibit WNV helicase with an  $IC_{50}$  of about 30  $\mu$ M in both enzyme and viral titer reduction assay (Borowski et al., 2002). The antiviral activity of the compound appears to be specific to the WNV NS3, because it did not inhibit hepatitis C virus helicase.
2. A set of ring-expanded heterocyclic nucleoside analogs, which contain either the imidazo[4,5-e][1,3]diazepine ring (Zhang et al., 2003a) or the 6-aminoimidazo[4,5-e][1,3]diazepine-4,8-dione ring systems (Zhang et al., 2003b), inhibited WNV NS3 NTPase/helicase with  $IC_{50}$  in low micromolar concentrations.
3. Random screening of a compound library containing a broad range of unrelated small molecules revealed 5,6-dichloro-1-( $\beta$ -D-ribofuranosyl)benzotriazole which inhibited the WNV helicase with an  $IC_{50}$  of about 0.3  $\mu$ M (Borowski et al., 2003). However, the potency of the compounds described in 2 and 3 has not been examined in cell culture.

### 3.2.3 Protease Inhibitors

Both small molecular and peptide inhibitors have been reported to inhibit the WNV NS3 protease. Based on the crystal structure of the DENV protease in complex with the mung bean Bowman–Birk inhibitor, five compounds were identified that could mimic the interactions between substrate P1 arginine and protease and therefore inhibit DENV protease activity (Ganesh et al., 2005). Due to the structural conservation among flavivirus proteases, these compounds also inhibited WNV protease, three of which showed  $IC_{50}$  of 13–35  $\mu$ M. With the recent advances in structure solving of flavivirus protease complexes [NS2b-protease-peptide inhibitor (Erbel et al., 2006) and NS2b-protease-aprotinin inhibitor

(Aleshin et al., 2007)], the computational approach could be extended to improve the current compounds or to search for new inhibitors that block the interactions among substrate, NS2b, and protease.

Flavivirus NS3 is a serine protease that belongs to the trypsin superfamily. The cleavage sites of the flavivirus NS3 protease are conserved and contain unique dibasic residues at the P1 and P2 sites (lysine or arginine) followed by a residue with a short side chain at the P1' position (most commonly glycine, serine, or alanine). Peptides mimicking the conserved cleavage substrate have been explored as possible inhibitors of the flavivirus protease. For WNV, tetrapeptide variants, derived from the benzoyl-norleucine-lysine-arginine-arginine (Bz-nKRR) sequence, inhibited the protease with  $IC_{50}$  of 1–200  $\mu$ M (Knox et al., 2006). Similarly, at micromolar concentrations peptides with  $\alpha$ -keto amide backbones inhibited DENV protease (Leung et al., 2001). The potency of the compounds may be improved through structural optimization, based on the recent crystal structure of NS2b-protease-inhibitor (Bz-nKRR) (Erbel et al., 2006). Besides the peptide inhibitors, standard serine protease inhibitors have been tested against flavivirus proteases. Aprotinin inhibits both WNV and DENV proteases with 160 nM of  $IC_{50}$  and 26–65 nM of  $IC_{50}$ , respectively (Leung et al., 2001; Mueller et al., 2007). The mode of action of aprotinin was recently revealed by the structure of a tertiary complex consisting of WNV NS2b-protease-aprotinin (Aleshin et al., 2007). Aprotinin binds in a substrate-mimetic fashion. In comparison with the structure of the NS2b-protease complex without substrate, the tertiary complex suggests an “induced fit” mechanism of the flavivirus protease, providing an opportunity to develop inhibitors that block the conformational change required for catalytic activity of the protease.

#### 3.2.4 Host Glucosidase Inhibitors

The host glycosylation machinery is required for modifications of flavivirus proteins. Inhibitors of ER  $\alpha$ -glucosidase, such as *N*-nonyl-deoxynojirimycin and castanospermine, were reported to inhibit JEV and DENV at low micromolar concentrations in cell culture (Courageot et al., 2000; Wu et al., 2002). The  $\alpha$ -glucosidase inhibitors block the trimming step of N-linked glycosylation of prM and E, leading to delayed formation of preM/E heterodimer and nonproductive virion assembly. The *N*-nonyl-deoxynojirimycin compound could partially protect mice from JEV infection at 200 mg/kg/day (Wu et al., 2002). Similarly, castanospermine significantly reduced the mortality of A/J

mice infected with DENV-2 at doses of 50 and 250 mg/kg/day. However, castanospermine did not substantially suppress WNV infection in cell culture or in mice (Whitby et al., 2005).

### 3.2.5 Other Small Molecular Inhibitors

A number of new classes of WNV inhibitors have been identified through HTS using luciferase-reporting replicon cell lines:

1. Triaryl pyrazoline was reported to inhibit WNV without detectable cytotoxicity ( $IC_{50}$  28  $\mu$ M and  $CC_{50} \geq 300$   $\mu$ M). Besides WNV, this compound also inhibited other flaviviruses (DENV, YFV, and SLEV), an alphavirus (Western equine encephalitis virus), a coronavirus (mouse hepatitis virus), and a rhabdovirus (vesicular stomatitis virus). However, the compound did not suppress an orthomyxovirus (influenza virus) or a retrovirus (HIV-1). Mode-of-action analysis with WNV showed that the compound did not block viral entry or virion assembly, but specifically suppressed viral RNA synthesis (Goodell et al., 2006; Puig-Basagoiti et al., 2006).
2. One pyrozolopyrimidine compound was shown to have antiviral activity ( $IC_{50}$  11  $\mu$ M and  $CC_{50}$  85  $\mu$ M) (Gu et al., 2006).
3. Several compounds within the sulfonamide family were recently reported to inhibit WNV in cell culture at the submicromolar range with TIs of  $>10$ . Besides WNV, these compounds also inhibit other flaviviruses. One of these compounds was shown to specifically block translation of a YFV replicon, but not an alphavirus (Sindbis virus) replicon or an EMCV IRES-containing mRNA (Noueiry et al., 2007). Experiments are needed to determine the exact target and the in vivo efficacy of the above compounds.

Besides the above novel molecules, minocycline (a tetracycline derivative that inhibits bacterial translation through binding to the 30S ribosomal subunit thus preventing the amino-acyl tRNA from binding to the A site of the ribosome) was recently shown to inhibit WNV infection in cell culture ( $IC_{50}$  18–25  $\mu$ M and  $CC_{50}$  140  $\mu$ M). The compound also inhibited WNV-induced apoptosis and suppressed virus-induced activation of c-Jun N-terminal kinase and its target c-jun (Michaelis et al., 2007). Minocycline was previously shown to protect mice from fatal Sindbis virus encephalitis, although it did not inhibit Sindbis replication in cell culture (Irani and Prow, 2007). It will be interesting to examine whether minocycline has anti-WNV activity in animal models.

## 4 New Antiviral Targets and Challenges on Antiviral Development

Significant progress has been made toward the development of therapeutics against WNV. Among the known inhibitors, antibody-based treatment has so far shown the most promising results. Due to the quasispecies nature of WNV and the error-prone mutation of the viral RdRp, one potential problem of the antibody-based therapy is rapid development of resistant viruses. Therefore, novel classes of inhibitors should be developed in parallel to the antibody-mediated therapy. The establishment of HTS assays has made it possible to screen large compound libraries for inhibitors. One challenge for the development of effective therapeutics of WNV infection is that candidate inhibitors must effectively reach the CNS to clear the virus from infected neurons. Patients with the most severe WNV disease often have immune deficits and present to clinical attention relatively late in their disease course. Ultimately, a combinatory strategy that inhibits viral replication, boosts protective immune responses, and minimizes CNS injury may be more effective than any single antiviral agent.

Improvement of our understanding of the basic virology and pathogenesis of flavivirus will continue to open new avenues for antiviral development. For example, crystallographic studies showed that viral E protein (Bressanelli et al., 2004; Modis et al., 2003, 2004) undergoes a sequential structural change during the fusion-activating transition. Small molecular inhibitors could be developed to block the structural transitions essential for viral/host membrane fusion. The recent characterization of flavivirus NS5 methyltransferase has demonstrated that guanine N7 methylation of the viral RNA cap is essential for WNV replication (Zhou et al., 2007). Since flavivirus methyltransferase specifically methylates the cap structure from viral RNA, the NS5 methyltransferase represents an antiviral target (Dong et al., 2007a). The findings that flavivirus proteins antagonize host innate immune responses have also provided opportunities to develop antiviral therapies (Munoz-Jordan et al., 2003). Inhibitors that block the interferon antagonism of viral protein(s) may allow the innate antiviral response to effectively suppress viral infection and, therefore, reduce viral burden. Finally, host proteins essential for viral replication could potentially be targeted for antiviral therapy. For instance, host Src family kinase c-Yes was found to be critical for maturation of WNV particles (Hirsch et al., 2005). Inhibitors of the c-Src protein kinase were recently shown to potently inhibit four serotypes of DENV, with  $IC_{50}$  values in submicromolar range (Chu and Yang, 2007).

In clinical settings, it will be essential to develop a specific and rapid assay for diagnosis of WNV infection prior to initiation of chemotherapy. Upon human infection with WNV after mosquito inoculation, viremia is detected during the first 1–2 weeks postinfection, after which time symptoms develop and antibodies are produced (Prince et al., 2005). Various RT-PCR-based assays have been developed for sensitive detection of the viral RNA (Shi and Kramer, 2003). It is expected that early treatment with antiviral agents will yield better therapeutic outcome than late treatment during the course of infection. Since viremia diminishes when symptoms develop and antibodies are produced, serology is a key diagnostic method for WNV infection (Shi and Wong, 2003). The current serological assays detect WNV-specific antibody against viral structural proteins by enzyme immunoassays (EIA) (Martin et al., 2002; Tardei et al., 2000) or enzyme-linked immunoabsorbent assay for IgG (ELISA) (Davis et al., 2001; Tardei et al., 2000). These assays lack specificity because of cross-reactivity of the structural proteins among flaviviruses. A luminex assay using recombinant NS5 of WNV as an antigen was reported to differentiate WNV infection from infections by other flaviviruses (DENV and SLEV), and also to differentiate between natural WNV infection and flavivirus vaccination (Wong et al., 2003). It remains to be determined whether NS5 from other flaviviruses could be used for serologic diagnosis. Other viral-type specific diagnostic methods are needed not only for ant flavivirus therapy, but also for vaccine development.

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