Small Molecule Inhibitors of West Nile Virus.

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Abstract: West Nile virus is a human pathogen which is rapidly expanding worldwide. It is a member of the Flavivirus genus and it is transmitted by mosquitos between its avian hosts and occasionally in vertebrate hosts. In humans, the infection is often asymptomatic, but the most severe cases result in encephalitis or meningitis. Around 10% of cases of neuroinvasive disease are fatal. To date there is no effective human vaccine or effective antiviral therapy available to treat WNV infections For this reason, research in this field is rapidly growing. In this article we will review the latest efforts in the design and development of novel WNV inhibitors from a medicinal chemistry point of view, highlighting challenges and opportunities for the researchers working in this field.

Keywords: drug design, Flavivirus, West Nile Virus, X-ray structure.

Running head: West Nile Virus Inhibitors

INTRODUCTION

West Nile Virus (WNV) is a member of the genus *Flavivirus*, a group of enveloped viruses with a positive-sense RNA genome. This genus contains many important human pathogens (e.g.: Dengue, Japanese Encephalitis and Yellow Fever viruses), which together infect millions of people worldwide and are the cause of tens of thousands of fatalities annually [1]. The West Nile virus has been reported in dead or dying birds of at least 326 species [2]. In humans, it was first isolated in the West Nile province of Uganda in 1937 from the blood of a woman suffering from a mild febrile illness [3].

WNV is an emerging human pathogen with an expanding geographical distribution, spreading rapidly in recent years throughout North America [4, 5]. It has been the cause of an increasing number of human infections with associated sever disease and fatalities. WNV is transmitted by mosquitoes within its avian host populations and to incidental vertebrate hosts, including humans and horses. Infection in humans is generally asymptomatic or causes a mild febrile disease in about 20-30% of cases. The most severe cases of WNV infection result in encephalitis or meningitis. Around 10% of cases of neuroinvasive disease are fatal while the survivors may suffer from long-term cognitive and neurological impairment [6]. The most important risk factors for these more serious complications are aging and a compromised immune system [7]. Although vaccines are available against some flaviviruses, unfortunately there is no effective human vaccine or effective antiviral therapy available for WNV, indeed their development remains an high priority for the World Health Organization [8, 9].

The present review will concentrate primarily on the medicinal chemistry effort in the development of potential inhibitors of WNV. Other aspects of flavivirus infections, including WNV, are covered in excellent elsewhere [10-12].

WEST NILE VIRUS PROTEIN TARGETS

WNV contains a single-stranded positive sense RNA genome, which encodes three structural proteins, capsid, pre-membrane and envelope (C, prM, E), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Non-structural protein 1 (NS1) has been implicated in host immune response evasion, likewise the function NS2A is poorly defined. NS2B functions as cofactor protein in the protease function of NS3, which is a multifunctional protein, consisting of the N-terminal protease domain and C-terminal helicase, nucleoside triphosphatase and RNA triphosphatase activities [13]. The function of NS4A is a matter of debate while NS4B membrane protein is thought to anchor and target the replication complex to the endoplasmic reticulum (ER) membrane. NS5 is the largest of the non-structural proteins and it contains a classic RNA-dependent RNA polymerase domain as well as methyltransferase and guanylyltransferase domains for mRNA capping necessary for virus that replicates its mRNA in the cytoplasm [14].

E protein inhibitors

Wang *et al.* from Novartis performed a virtual screening using a 586,000 compounds library (a subset of the corporate compound collection) revealing a hydrophobic pocket in the DENV-2 strain S1 envelope protein. The top-ranked compounds were also docked in the crystal structure of the WNV E protein [15, 16], which contain a hydrophobic pocket that is presumably important for low-pH mediated membrane fusion. After performing a high-throughput docking within this

hydrophobic pocket, compound **1** was evaluated in cell-based assays showing an EC_{50} of 0.564 ± 0.17 µM [17].

An *in silico* screening of the Maybridge chemical database was performed on the dengue E protein structure. The biological evaluation of the most promising compounds obtained from the computer-based simulation revealed two hit structures with low micromolar antiviral activity against dengue virus. Interestingly, one of these compounds (**2**) also has antiviral activity against both WNV and YFV [18].

NS2B/NS3 protease inhibitors

Historically, WNV NS3 protease and the NS2B cofactor have always been indicated as attractive targets for drug development, due to their essential catalytic activity [19, 20]. The WNV NS2B-NS3 is a serine-like protease, important for viral replication. Viral proteases in general are proving to be successful antiviral targets (e.g. for HIV) and inhibitors of serine protease in particular look promising for treating flaviviruses (e.g. hepatitis C [21]). Several homology models for WNV NS2B/NS3 protease were reported [22-24], unfortunately all these structures were significantly different from the subsequent crystal structures of the active protease (PDB id: 2FP7 [25], PDB id: 2IJO [26]) due to the unusual binding mode of the NS2B cofactor which encircles and stabilizes the protease structure [27].

Peptide-like inhibitors. A common strategy used for the inhibition of the NS2B/NS3 protease involved covalent inhibitors that compete with the substrate for the catalytic site. Such peptide-based inhibitors have their C-terminal carboxyl group chemically modified into reactive electrophilic "warheads". A popular warhead is the aldehyde functional group and peptide-aldehydes have been shown to inhibit the WNV NS2B/NS3 protease at submicromolar concentrations [28]. In this study peptidomimetics containing a P1 decarboxylated arginine (agmatine) compound (4-phenyl-phenacetyl-Lys-Lys-agmatine) showed potent inhibition of the WNV protease (IC_{50} 4.7 ± 1.2 µM). This inhibitor does not bind covalently to the catalytic serine in the active site but instead competes with the natural substrate for the active site with a binding affinity of *Ki* 2.05 ± 0.13 µM. Selectivity for WNV NS2B/NS3 protease was demonstrated using thrombin, a mammalian serine protease involved in blood clotting that is selective for peptide substrates with a P1 Arg. The compound did not inhibit thrombin at concentration of 100 µM, showing a good selectivity towards the WNV NS2B/NS3 protease [29]. In 2013 the same research group has reported thirty-seven novel agmatine dipeptides [30]. The most potent inhibitor displayed an IC_{50} of 2.6 ± 0.3 µM against the West Nile virus NS2B/NS3 protease, a two-fold improvement over the inhibitor described previously.

Recently a series of new substrate analogues of NS2B/NS3 protease based on *trans*-(4-guanidino)cyclohexylmethylamide (GCMA) were identified. These GCMA inhibitors are stable, readily accessible, and have a better selectivity profile than the previously described agmatine analogues. Furthermore, they possess negligible affinity for the trypsin-like serine proteases thrombin, factor Xa, and matriptase. A crystal structure in complex with the WNV protease was determined for one of the most potent inhibitors, 3,4-dichlorophenylacetyl-Lys-Lys-GCMA (K_i=0.13 µM) [31].

It should be noted that, despite their potency, warhead peptidomimetics have several undesirable characteristics, including lack of selectivity over other trypsin-like proteases due to their high reactivity and low chemical stability, limiting their potential for drug development [32].

Non-peptide inhibitors. Recently, different non-peptidomimetic inhibitors have been reported in literature. Compound **3** has been identified by automatic fragment-based docking of about 12,000 compounds and is able to inhibit WNV protease NS3 with an IC_{50} of 183 µM [33].

A more potent compound, the sultam thiourea TYT-1 (**4**), was identified by screening a 3,500 molecules library in a in a cell based assay that measured the compound ability to protect the cells from WNV-induced cytopathic effects (CPE). Compound **4** proved to one on the most potent WNV NS3 inhibitor reported to date ($IC_{50 =} 0.7$ µM) [34].

Exploratory studies, using a combinatorial approach based on the 1-Oxo-1,2,3,4-tetrahydroisoquinolinee scaffold, have led to the identification of an hit (**5**), which inhibited WNV protease at ($IC_{50} = 30$ µM) [35]. The design rationale for this compound is based on the observation that molecules containing the aforementioned scaffold are substrate of chymotrypsin, hence a problem of selectivity might arise for compound **5** and related analogues.

Compounds **6** and **7**, two 8-hydroxyquinoline (8-HQ) derivatives, exhibited inhibition of WNV protease in vitro, with a *Ki* of 3.2±0.3 µM and 3.4±0.3 µM respectively [36]. In further studies, 15 compounds having 8-HQ scaffold were biologically tested as WNV protease inhibitor and compound **7** proved to be the most potent of the series with an IC_{50} of 2.01±0.08 µM. It is noteworthy that the compound containing the naphthalene-1-ol moiety instead of the 8-HQ group showed a significantly reduced activity, underlying the importance of this heterocyclic ring for the inhibition of WNV NS2B/NS3 protease by this class of compounds [37].

The aminobenzamide scaffold was also utilized in the synthesis of a series of structurally diverse *meta* and *para*-substituted derivatives [38]. The four analogues that exhibited activity against WNV protease, all have a (pehnoxy)phenyl group present in the *meta* or *para* position. The most potent aminobenzamide derivative is compound **8**, which showed a low micromolar NS3/NS2B protease inhibition ($IC_{50} = 5.5±0.08$ µM). These compounds showed also activity against the DENV protease.

Recently, Tiew *et al.* have identified a series of triazolic compounds active on both Dengue virus and WNV NS2B/NS3 protease [39]. The compounds were obtained from the benz[*d*]isothiazol-3(2*H*)-one scaffold using a click-chemistry derived library. The most interesting molecules (**9**-**10**) display weak inhibitory activity toward the NS2B-NS3 protease.

3-Aryl-2-cyanoacrylamides were identified as a new class of nitrile-containing inhibitors of the NS3/NS2B protease by Nitsche *et al.* [40]. The most relevant structural features required for activity are: a *para*-substituted aromatic system with high electron density; an amide or acid residue; a planar geometry. Consequently, the most active molecule was the hydroxyl derivative (**11**), with a K_i of 44.6 µM.

The 5-amino-1-(phenyl)sulfonyl-pyrazol-3-yl based inhibitors seems to interfere with the binding of the NS2B cofactor with the NS3 protease. The hit compounds (**12**-**13**), which showed a sub-micromolar activity, were obtained by high throughput screening (HTS), using the National Institutes of Health's 65,000 compound library [41]. However, these derivatives were rapidly hydrolyzed in an aqueous buffer (pH 8) to the corresponding pyrazol-3-ol and, for this reason, the authors designed and synthesized a new series of analogues with improved chemical stability. The

two ester isosteres derivative, compound **14**, which contain alkene group, and compound **15**, an amide analogue, while less potent than the original hits (IC_{50} of 13.8 μM and 16.0 μM respectively), are highly stable inhibitors of WNV NS2B/NS3 proteinase with an degradation time of 13 and 96 h. [42].

NS5 – RNA-dependent RNA polymerase inhibitors

Puig-Basotti *et al.* reported the results of an HTS study, which was performed by screening nearly 100,000 compounds in WNV replicon assay [43]. From this exercise, five novel inhibitors have been identified with EC_{50} (50% effective concentration) values of <10μM and TI (therapeutic index) values of >10. Viral titer reduction assays, using various flaviviruses and nonflaviviruses, showed that the compounds have distinct antiviral spectra. One compound (**16**) suppresses both viral translation and RNA synthesis ($EC_{50} = 12$ μM), while the other four compound suppress only RNA synthesis. Examination of the antiviral spectrum revealed unspecific profile for compounds **17**-**19** with an EC_{50} of 0.2, 8 and 0.7 μM respectively. Compound **20**, appear to be the only one able to block RNA synthesis by selective inhibition of WNV NS5 ($EC_{50} = 14$ μM).

NTPase/Helicase inhibitors

Compound **21** exhibited helicase inhibitory activity against WNV NTPase/ Helicase with an IC_{50} value of 3-10 μg/ml when a RNA substrate was employed. However no inhibition could be detected when the same experiment was repeated using a DNA substrate [44]. Interestingly, compound **22,** the ribose analogue of compound **21**, showed activity against NTPase/helicase of WNV when DNA substrate was employed ($IC_{50}=23$μM), but no inhibition could be detected when the same experiment was repeated using an RNA substrate[45]. As flaviviridae are RNA viruses, the observed results are surprising, especially considering that the 2-deoxyribose analogue **21** has shown activity against the NTPase/helicase of WNV only when using a RNA substrate. The significance and implications of these results are not clear at the moment. There are several reports that demonstrate non-covalent, tight-binding interactions of analogues of nucleobases, nucleosides, and nucleotides, to major or minor grooves of DNA or RNA double helices [46, 47]. However, preliminary studies show that compound **22** does not form a tight complex with either an RNA or a DNA substrate, suggesting that its mechanism of action may involve direct interaction with the enzyme. Related ribose analogues **23** and **24** were also found to inhibit the WNV NTPase/helicase with an IC_{50} of approximately 50 and 3 μM respectively [48, 49].

Other Inhibitors

Many investigators have noted the broad antiviral properties of ribavirin (**25**) [50], currently a mainstay of therapy in combination with IFN-a for the treatment of HCV. Although ribavirin shows some activity against WNV *in vitro* ($EC_{50} \sim 200$ μM), *in vivo* studies have been less promising, with reports of ribavirin treatment increasing mortality in Syrian golden hamsters infected with WNV New York strain CDC996625 [51].

A recent paper has reported weak anti-WNV activity of the tetracycline antibiotic minocycline (**26**) in a cytopathic effect assay in Vero cells, with an IC_{50} of approximately 20 μM [52]. Olivo *et al.* have also disclosed a series of aminoquinoline compounds that possess antiviral activity against a range of viruses, including WNV, although no details on the mechanism of action have been

provided. Compound **27** has activity against a DENV-2 sub-genomic replicon cell line with EC_{50} 5 μM and against a WNV sub-genomic replicon cell line with an EC_{50} of 1.9 μM [53].

CONCLUSION

WNV virus is rapidly spreading worldwide and, despite viral infection could lead to severe encephalitis, neither a vaccine nor an antiviral therapy is currently available. In this paper we have provided a summary of the current medicinal chemistry efforts in identifying small molecule inhibitors of WNV. Indeed, several interesting lead are now appearing in literature. However, we believe there are still areas that require a more intense investigation. In particular, it is easy to notice that researchers have been focused their attention mainly on the NS3 protease, while, in comparison, little has been reported on the inhibition of the NS5 polymerase and methyltransferase. Interestingly, several crystal structures of the latter have become available in the last few years (Table 1) and they could be used in virtual screening simulations or other structure-based drug design methodologies. For this reason, it is possible to foresee an increase interest around this target in the near future. It should be mentioned that the research around the development of antiviral compounds active against Dengue virus is at a more advanced stage compared to WNV [54, 55]. Given the close similarity between these two viruses, it is likely that some of the anti-Dengue molecules reported in literature could be also useful against WNV. Indeed, several of the compounds reported in this review show activity against both viruses. Furthermore, the knowledge and the insight gained with the dramatic development of novel anti-HCV agents could also be used as foundation for the identification of suitable WNV inhibitors. Finally, it should not be overlooked that the most severe cases affect the central nervous system, thus the future antiviral therapy agents should be able to cross the blood-brain barrier. This clearly represents one of the biggest challenges for the researchers in the field.

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Disclosure Statement
The authors declare no competing interests.

ABBREVIATIONS

8-HQ	=	8-hydroxyquinoline
DNA	=	Deoxyribonucleic acid
EC_{50}	=	50% effective concentration
HTS	=	High Throughput Screening
IC_{50}	=	50% inhibitory concentration
RNA	=	Ribonucleic acid
WNV	=	West Nile Virus

REFERENCES

1. Gould LH & Fikrig E. West Nile virus: a growing concern? *J Clin Invest* 2004; **113:**1102-1107.

2. The CDC database http://www.cdc.gov/ncidod/dvbid/westnile/birds&mammals.htm 2012.

3. Smithburn KC, Hughes TP, Burke AW & Paul JH. A Neurotropic Virus Isolated from the Blood of a Native of Uganda. *The American Journal of Tropical Medicine and Hygiene* 1940; **s1-20:**471-492.

4. Mackenzie JS, Gubler DJ & Petersen LR. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. *Nat Med* 2004; **10:**S98-109.

5. Komar N & Clark GG. West Nile virus activity in Latin America and the Caribbean. *Rev Panam Salud Publica* 2006; **19:**112-117.

6. Davis LE, DeBiasi R, Goade DE, Haaland KY, Harrington JA, Harnar JB, Pergam SA, King MK, DeMasters BK & Tyler KL. West Nile virus neuroinvasive disease. *Ann Neurol* 2006; **60:**286-300.

7. Petersen LR & Marfin AA. West Nile virus: a primer for the clinician. *Ann Intern Med* 2002; **137:**173-179.

8. Pugachev KV, Guirakhoo F & Monath TP. New developments in flavivirus vaccines with special attention to yellow fever. *Curr Opin Infect Dis* 2005; **18:**387-394.

9. De Filette M, Ulbert S, Diamond M & Sanders NN. Recent progress in West Nile virus diagnosis and vaccination. *Vet Res* 2012; **43:**16.

10. Bollati M, Alvarez K, Assenberg R, Baronti C, Canard B, Cook S, Coutard B, Decroly E, de Lamballerie X, Gould EA, Grard G, Grimes JM, Hilgenfeld R, Jansson AM, Malet H, Mancini EJ, Mastrangelo E, Mattevi A, Milani M, Moureau G, Neyts J, Owens RJ, Ren J, Selisko B, Speroni S, Steuber H, Stuart DI, Unge T & Bolognesi M. Structure and functionality in flavivirus NS-proteins: perspectives for drug design. *Antiviral Res* 2010; **87:**125-148.

11. Parkinson T & Pryde DC. Small molecule drug discovery for Dengue and West Nile viruses: applying experience from hepatitis C virus. *Future Med Chem* 2010; **2:**1181-1203.

12. Botting C & Kuhn RJ. Novel approaches to flavivirus drug discovery. *Expert Opin Drug Discov* 2012; **7:**417-428.

13. Gorbalenya AE, Donchenko AP, Koonin EV & Blinov VM. N-terminal domains of putative helicases of flavi- and pestiviruses may be serine proteases. *Nucleic Acids Res* 1989; **17:**3889-3897.

14. Brinton MA. The molecular biology of West Nile Virus: a new invader of the western hemisphere. *Annu Rev Microbiol* 2002; **56:**371-402.

15. Kanai R, Kar K, Anthony K, Gould LH, Ledizet M, Fikrig E, Marasco WA, Koski RA & Modis Y. Crystal structure of west nile virus envelope glycoprotein reveals viral surface epitopes. *J Virol* 2006; **80:**11000-11008.

16. Nybakken GE, Nelson CA, Chen BR, Diamond MS & Fremont DH. Crystal structure of the West Nile virus envelope glycoprotein. *J Virol* 2006; **80:**11467-11474.

17. Wang QY, Patel SJ, Vangrevelinghe E, Xu HY, Rao R, Jaber D, Schul W, Gu F, Heudi O, Ma NL, Poh MK, Phong WY, Keller TH, Jacoby E & Vasudevan SG. A small-molecule dengue virus entry inhibitor. *Antimicrob Agents Chemother* 2009; **53:**1823-1831.

18. Kampmann T, Yennamalli R, Campbell P, Stoermer MJ, Fairlie DP, Kobe B & Young PR. In silico screening of small molecule libraries using the dengue virus envelope E protein has identified compounds with antiviral activity against multiple flaviviruses. *Antiviral Res* 2009; **84:**234-241.

19. Chambers TJ, Grakoui A & Rice CM. Processing of the yellow fever virus nonstructural polyprotein: a catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites. *J Virol* 1991; **65:**6042-6050.

20. Falgout B, Pethel M, Zhang YM & Lai CJ. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. *J Virol* 1991; **65:**2467-2475.

21. Abbenante G & Fairlie DP. Protease inhibitors in the clinic. *Med Chem* 2005; **1:**71-104.

22. Nall TA, Chappell KJ, Stoermer MJ, Fang NX, Tyndall JD, Young PR & Fairlie DP. Enzymatic characterization and homology model of a catalytically active recombinant West Nile virus NS3 protease. *J Biol Chem* 2004; **279:**48535-48542.

23. Ganesh VK, Muller N, Judge K, Luan CH, Padmanabhan R & Murthy KH. Identification and characterization of nonsubstrate based inhibitors of the essential dengue and West Nile virus proteases. *Bioorg Med Chem* 2005; **13:**257-264.

24. Zhou H, Singh NJ & Kim KS. Homology modeling and molecular dynamics study of West Nile virus NS3 protease: a molecular basis for the catalytic activity increased by the NS2B cofactor. *Proteins* 2006; **65:**692-701.

25. Erbel P, Schiering N, D'Arcy A, Renatus M, Kroemer M, Lim SP, Yin Z, Keller TH, Vasudevan SG & Hommel U. Structural basis for the activation of flaviviral NS3 proteases from dengue and West Nile virus. *Nat Struct Mol Biol* 2006; **13:**372-373.

26. Aleshin AE, Shiryaev SA, Strongin AY & Liddington RC. Structural evidence for regulation and specificity of flaviviral proteases and evolution of the Flaviviridae fold. *Protein Sci* 2007; **16:**795-806.

27. D'Arcy A, Chaillet M, Schiering N, Villard F, Lim SP, Lefeuvre P & Erbel P. Purification and crystallization of dengue and West Nile virus NS2B-NS3 complexes. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 2006; **62:**157-162.

28. Stoermer MJ, Chappell KJ, Liebscher S, Jensen CM, Gan CH, Gupta PK, Xu WJ, Young PR & Fairlie DP. Potent cationic inhibitors of West Nile virus NS2B/NS3 protease with serum stability, cell permeability and antiviral activity. *J Med Chem* 2008; **51:**5714-5721.

29. Lim HA, Joy J, Hill J & San Brian Chia C. Novel agmatine and agmatine-like peptidomimetic inhibitors of the West Nile virus NS2B/NS3 serine protease. *Eur J Med Chem* 2011; **46:**3130-3134.

30. Lim HA, Ang MJ, Joy J, Poulsen A, Wu W, Ching SC, Hill J & Chia CS. Novel agmatine dipeptide inhibitors against the West Nile virus NS2B/NS3 protease: A P3 and N-cap optimization study. *Eur J Med Chem* 2013; **62C:**199-205.

31. Hammamy MZ, Haase C, Hammami M, Hilgenfeld R & Steinmetzer T. Development and Characterization of New Peptidomimetic Inhibitors of the West Nile Virus NS2B-NS3 Protease. *ChemMedChem* 2013.

32. Becker GL, Sielaff F, Than ME, Lindberg I, Routhier S, Day R, Lu Y, Garten W & Steinmetzer T. Potent inhibitors of furin and furin-like proprotein convertases containing decarboxylated P1 arginine mimetics. *J Med Chem* 2010; **53:**1067-1075.

33. Ekonomiuk D, Su XC, Ozawa K, Bodenreider C, Lim SP, Yin Z, Keller TH, Beer D, Patel V, Otting G, Caflisch A & Huang D. Discovery of a non-peptidic inhibitor of west nile virus NS3 protease by high-throughput docking. *PLoS Negl Trop Dis* 2009; **3:**e356.

34. Barklis E, Still A, Sabri MI, Hirsch AJ, Nikolich-Zugich J, Brien J, Dhenub TC, Scholz I & Alfadhli A. Sultam thiourea inhibition of West Nile virus. *Antimicrob Agents Chemother* 2007; **51:**2642-2645.

35. Dou D, Viwanathan P, Li Y, He G, Alliston KR, Lushington GH, Brown-Clay JD, Padmanabhan R & Groutas WC. Design, synthesis, and in vitro evaluation of potential West Nile virus protease inhibitors based on the 1-oxo-1,2,3,4-tetrahydroisoquinoline and 1-oxo-1,2-dihydroisoquinoline scaffolds. *J Comb Chem* 2010; **12:**836-843.

36. Mueller NH, Pattabiraman N, Ansarah-Sobrinho C, Viswanathan P, Pierson TC & Padmanabhan R. Identification and biochemical characterization of small-molecule inhibitors of west nile virus serine protease by a high-throughput screen. *Antimicrob Agents Chemother* 2008; **52:**3385-3393.

37. Ezgimen M, Lai H, Mueller NH, Lee K, Cuny G, Ostrov DA & Padmanabhan R. Characterization of the 8-hydroxyquinoline scaffold for inhibitors of West Nile virus serine protease. *Antiviral Res* 2012; **94:**18-24.

38. Aravapalli S, Lai H, Teramoto T, Alliston KR, Lushington GH, Ferguson EL, Padmanabhan R & Groutas WC. Inhibitors of Dengue virus and West Nile virus proteases based on the aminobenzamide scaffold. *Bioorg Med Chem* 2012; **20:**4140-4148.

39. Tiew KC, Dou D, Teramoto T, Lai H, Alliston KR, Lushington GH, Padmanabhan R & Groutas WC. Inhibition of Dengue virus and West Nile virus proteases by click chemistry-derived benz[d]isothiazol-3(2H)-one derivatives. *Bioorg Med Chem* 2012; **20:**1213-1221.

40. Nitsche C, Steuer C & Klein CD. Arylcyanoacrylamides as inhibitors of the Dengue and West Nile virus proteases. *Bioorg Med Chem* 2011; **19:**7318-7337.

41. Johnston PA, Phillips J, Shun TY, Shinde S, Lazo JS, Huryn DM, Myers MC, Ratnikov BI, Smith JW, Su Y, Dahl R, Cosford ND, Shiryaev SA & Strongin AY. HTS identifies novel and

specific uncompetitive inhibitors of the two-component NS2B-NS3 proteinase of West Nile virus. *Assay Drug Dev Technol* 2007; **5:**737-750.

42. Sidique S, Shiryaev SA, Ratnikov BI, Herath A, Su Y, Strongin AY & Cosford ND. Structure-activity relationship and improved hydrolytic stability of pyrazole derivatives that are allosteric inhibitors of West Nile Virus NS2B-NS3 proteinase. *Bioorg Med Chem Lett* 2009; **19:**5773-5777.

43. Puig-Basagoiti F, Qing M, Dong H, Zhang B, Zou G, Yuan Z & Shi PY. Identification and characterization of inhibitors of West Nile virus. *Antiviral Res* 2009; **83:**71-79.

44. Ujjinamatada RK, Agasimundin YS, Zhang P, Hosmane RS, Schuessler R, Borowski P, Kalicharran K & Fattom A. A novel imidazole nucleoside containing a diaminodihydro-S-triazine as a substituent: inhibitory activity against the West Nile virus NTPase/helicase. *Nucleosides Nucleotides Nucleic Acids* 2005; **24:**1775-1788.

45. Ujjinamatada RK, Baier A, Borowski P & Hosmane RS. An analogue of AICAR with dual inhibitory activity against WNV and HCV NTPase/helicase: synthesis and in vitro screening of 4-carbamoyl-5-(4,6-diamino-2,5-dihydro-1,3,5-triazin-2-yl)imidazole-1-beta-D-ribo furanoside. *Bioorg Med Chem Lett* 2007; **17:**2285-2288.

46. Marsch GA, Ward RL, Colvin M & Turteltaub KW. Non-covalent DNA groove-binding by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Nucleic Acids Res* 1994; **22:**5408-5415.

47. Morales JC & Kool ET. Functional hydrogen-bonding map of the minor groove binding tracks of six DNA polymerases. *Biochemistry* 2000; **39:**12979-12988.

48. Borowski P, Lang M, Haag A, Schmitz H, Choe J, Chen HM & Hosmane RS. Characterization of imidazo[4,5-d]pyridazine nucleosides as modulators of unwinding reaction mediated by West Nile virus nucleoside triphosphatase/helicase: evidence for activity on the level of substrate and/or enzyme. *Antimicrob Agents Chemother* 2002; **46:**1231-1239.

49. Borowski P, Deinert J, Schalinski S, Bretner M, Ginalski K, Kulikowski T & Shugar D. Halogenated benzimidazoles and benzotriazoles as inhibitors of the NTPase/helicase activities of hepatitis C and related viruses. *Eur J Biochem* 2003; **270:**1645-1653.

50. Sidwell RW, Huffman JH, Khare GP, Allen LB, Witkowski JT & Robins RK. Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. *Science* 1972; **177:**705-706.

51. Morrey JD, Day CW, Julander JG, Blatt LM, Smee DF & Sidwell RW. Effect of interferon-alpha and interferon-inducers on West Nile virus in mouse and hamster animal models. *Antivir Chem Chemother* 2004; **15:**101-109.

52. Michaelis M, Kleinschmidt MC, Doerr HW & Cinatl J, Jr. Minocycline inhibits West Nile virus replication and apoptosis in human neuronal cells. *J Antimicrob Chemother* 2007; **60:**981-986.

53. Olivo PD, Buscher BA, Dyall J, Jocket-Balsarotti JI, O'Guin AK, Roth RM, Zhou Y, Franklin GW & Starkey GW. 4-Aminoquinoline compounds for treating virus-related conditions. WO2006121767A2, 2006.

54. Noble CG, Chen YL, Dong H, Gu F, Lim SP, Schul W, Wang QY & Shi PY. Strategies for development of Dengue virus inhibitors. *Antiviral Res* 2010; **85:**450-462.

55. Noble CG & Shi PY. Structural biology of dengue virus enzymes: towards rational design of therapeutics. *Antiviral Res* 2012; **96:**115-126.

56. Kaufmann B, Vogt MR, Goudsmit J, Holdaway HA, Aksyuk AA, Chipman PR, Kuhn RJ, Diamond MS & Rossmann MG. Neutralization of West Nile virus by cross-linking of its surface proteins with Fab fragments of the human monoclonal antibody CR4354. *Proc Natl Acad Sci U S A* 2010; **107:**18950-18955.

57. Dong H, Liu L, Zou G, Zhao Y, Li Z, Lim SP, Shi PY & Li H. Structural and functional analyses of a conserved hydrophobic pocket of flavivirus methyltransferase. *J Biol Chem* 2010; **285:**32586-32595.

58. Cherrier MV, Kaufmann B, Nybakken GE, Lok SM, Warren JT, Chen BR, Nelson CA, Kostyuchenko VA, Holdaway HA, Chipman PR, Kuhn RJ, Diamond MS, Rossmann MG & Fremont DH. Structural basis for the preferential recognition of immature flaviviruses by a fusion-loop antibody. *EMBO J* 2009; **28:**3269-3276.

59. Robin G, Chappell K, Stoermer MJ, Hu SH, Young PR, Fairlie DP & Martin JL. Structure of West Nile virus NS3 protease: ligand stabilization of the catalytic conformation. *J Mol Biol* 2009; **385:**1568-1577.

60. Mastrangelo E, Milani M, Bollati M, Selisko B, Peyrane F, Pandini V, Sorrentino G, Canard B, Konarev PV, Svergun DI, de Lamballerie X, Coutard B, Khromykh AA & Bolognesi M. Crystal structure and activity of Kunjin virus NS3 helicase; protease and helicase domain assembly in the full length NS3 protein. *J Mol Biol* 2007; **372:**444-455.

61. Zhang Y, Kaufmann B, Chipman PR, Kuhn RJ & Rossmann MG. Structure of immature West Nile virus. *J Virol* 2007; **81:**6141-6145.

62. Zhou Y, Ray D, Zhao Y, Dong H, Ren S, Li Z, Guo Y, Bernard KA, Shi PY & Li H. Structure and function of flavivirus NS5 methyltransferase. *J Virol* 2007; **81:**3891-3903.

63. Malet H, Egloff MP, Selisko B, Butcher RE, Wright PJ, Roberts M, Gruez A, Sulzenbacher G, Vonrhein C, Bricogne G, Mackenzie JM, Khromykh AA, Davidson AD & Canard B. Crystal structure of the RNA polymerase domain of the West Nile virus non-structural protein 5. *J Biol Chem* 2007; **282:**10678-10689.

64. Nybakken GE, Oliphant T, Johnson S, Burke S, Diamond MS & Fremont DH. Structural basis of West Nile virus neutralization by a therapeutic antibody. *Nature* 2005; **437:**764-769.

65. Dokland T, Walsh M, Mackenzie JM, Khromykh AA, Ee KH & Wang S. West Nile virus core protein; tetramer structure and ribbon formation. *Structure* 2004; **12:**1157-1163.

66. Volk DE, Beasley DW, Kallick DA, Holbrook MR, Barrett AD & Gorenstein DG. Solution structure and antibody binding studies of the envelope protein domain III from the New York strain of West Nile virus. *J Biol Chem* 2004; **279:**38755-38761.

Table 1. Crystallographic structures of WNV proteins available in the Protein Data Bank.

Code	Ligands	Technique	Resolution (Å)	Protein	Reference	
3J0B	-	Electron Microscopy	10.3	E	2012	-
3IYW	CR4354 Fab	Electron Microscopy	13.7	E	2010	[56]
3LKZ	AdoMet	X-ray	2.00	NS5	2010	[57]
3I50	-	X-ray	3.00	E	2009	[58]
3IXX	E53 Fab	Electron Microscopy	15.0	E		
3E90	Naph-KKR-H	X-ray	2.45	NS3	2009	[59]
2P5P	-	X-ray	2.80	E	2008	-
2QEQ	-	X-ray	3.10	NS3	2007	[60]
2IJO	-	X-ray	2.30	NS2B/NS3	2007	[26]
2OF6	-	Electron Microscopy	24.0	E	2007	[61]
2OY0	SAH	X-ray	2.80	NS5	2007	[62]
2GGV	-	X-ray	1.80	NS2B/NS3	2007	[26]
2HCN	-	X-ray	2.35	NS5		
2HCS	-	X-ray	2.50	NS5	2007	[63]
2HFZ	-	X-ray	3.00	NS5		
2I69	-	X-ray	3.10	E	2006	[15]
2HG0	-	X-ray	3.00	E	2006	[16]
2FP7	Peptide	X-ray	1.68	NS2B/NS3	2006	[25]
1ZTX	E16 Fab	X-ray	2.50	E	2005	[64]
1SFK	-	X-ray	3.20	C	2004	[65]
1S6N	-	NMR	-	E	2004	[66]

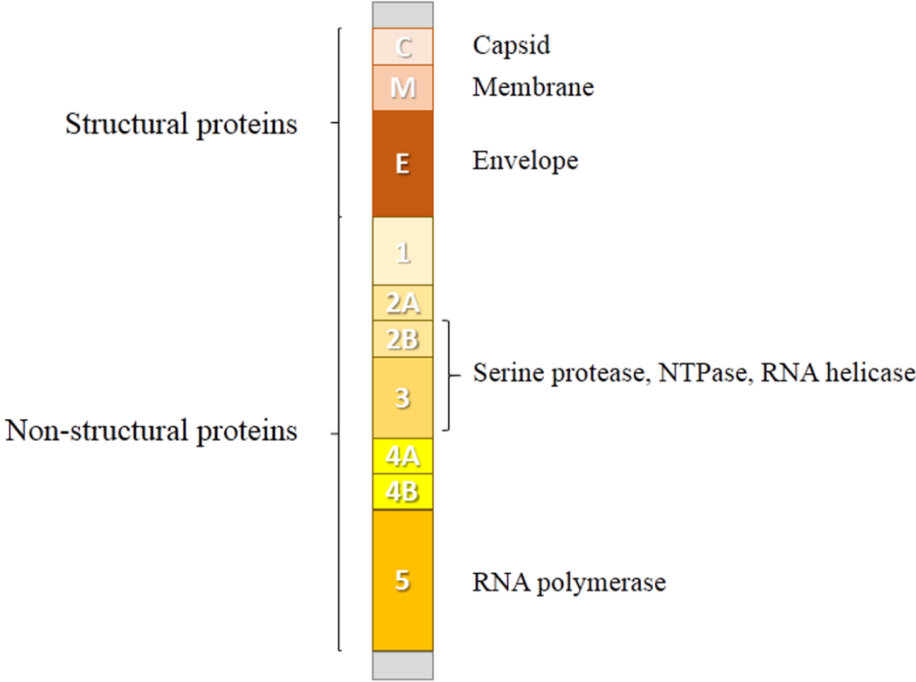**Fig. (1).** The RNA genome of West Nile virus.

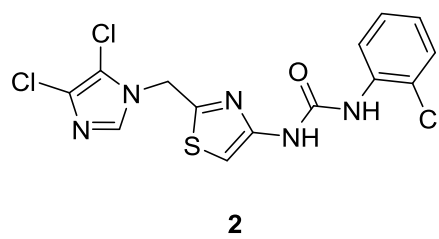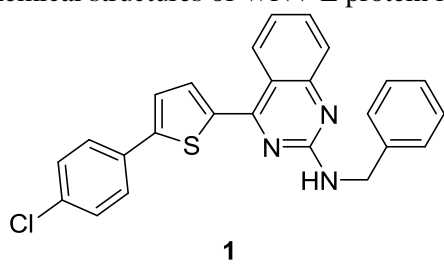**Fig. (2).** Chemical structures of WNV E protein inhibitors.

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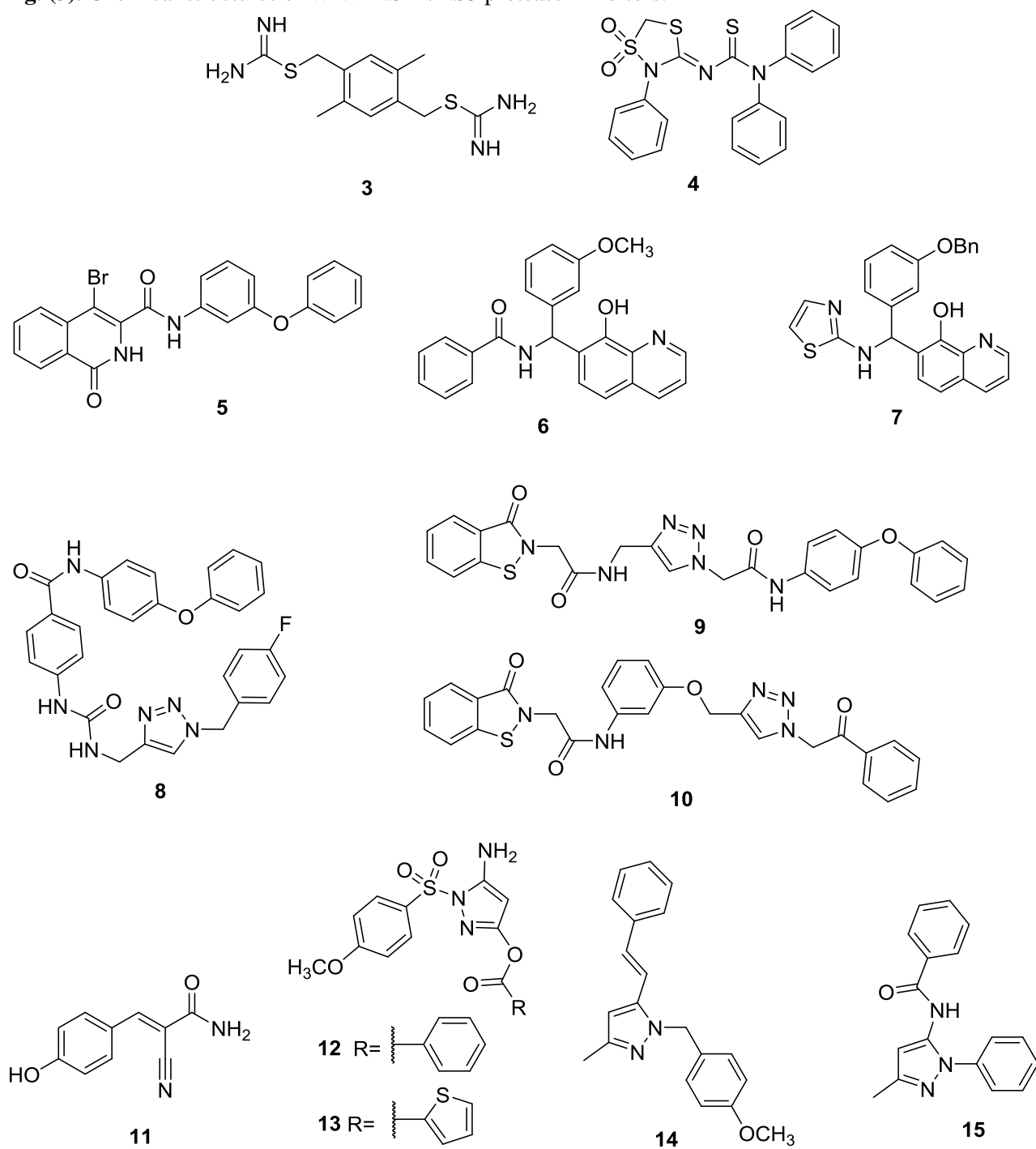**Fig. (3).** Chemical structures of WNV NS2B/NS3 protease inhibitors.

Fig. (4). Chemical structures of WNV NS5 inhibitors.

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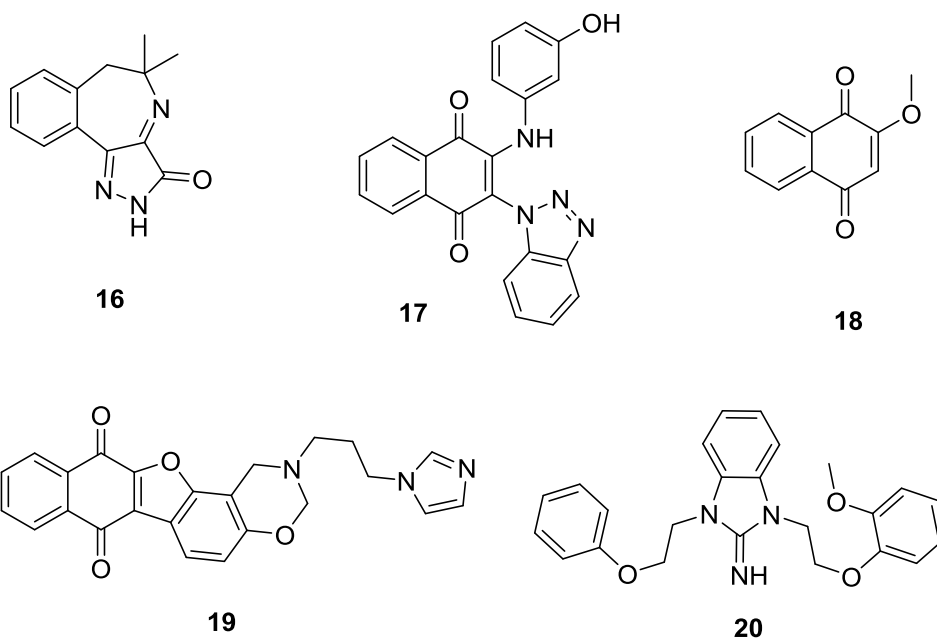
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Fig. (5). Chemical structures of WNV NTPase/Helicase inhibitors.

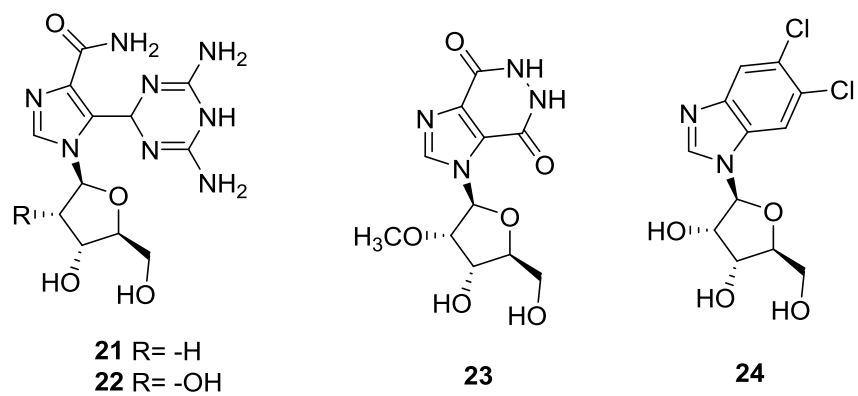
21 R= -H
22 R= -OH

23

24

Fig. (6). Chemical structures of inhibitors with a non-specific or unknown mode of action.