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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 severe 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 a 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 EC₅₀ 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 (IC₅₀ 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 *K_i* 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 IC₅₀ 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 cell based assay that measured the compound ability to protect the cells from WNV-induced cytopathic effects (CPE). Compound **4** proved to be one of the most potent WNV NS3 inhibitor reported to date ($IC_{50} = 0.7 \mu M$) [34].

Exploratory studies, using a combinatorial approach based on the 1-Oxo-1,2,3,4-tetrahydroisoquinoline scaffold, have led to the identification of a hit (**5**), which inhibited WNV protease at ($IC_{50} = 30 \mu M$) [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 K_i of $3.2 \pm 0.3 \mu M$ and $3.4 \pm 0.3 \mu M$ 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 $2.01 \pm 0.08 \mu M$. 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 ($IC_{50} = 5.5 \pm 0.08 \mu M$). 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 seem 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 μ M and 16.0 μ M 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 $<10\mu$ M 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 ($EC_{50} = 12 \mu$ M), 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 μ M respectively. Compound **20**, appear to be the only one able to block RNA synthesis by selective inhibition of WNV NS5 ($EC_{50} = 14 \mu$ M).

NTPase/Helicase inhibitors

Compound **21** exhibited helicase inhibitory activity against WNV NTPase/ Helicase with an IC_{50} value of 3-10 μ g/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 ($IC_{50}=23\mu$ M), 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 μ M 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- α for the treatment of HCV. Although ribavirin shows some activity against WNV *in vitro* ($EC_{50} \sim 200 \mu$ M), *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 μ M [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 EC₅₀ 5 μM and against a WNV sub-genomic replicon cell line with an EC₅₀ 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

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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.

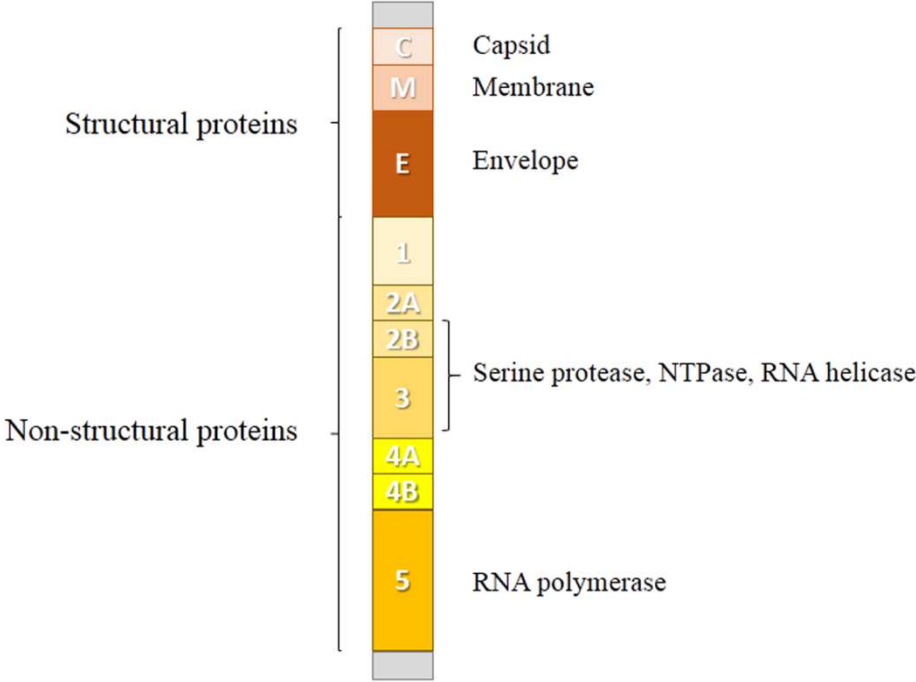


Fig. (2). Chemical structures of WNV E protein inhibitors.

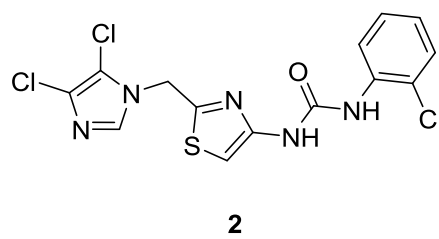
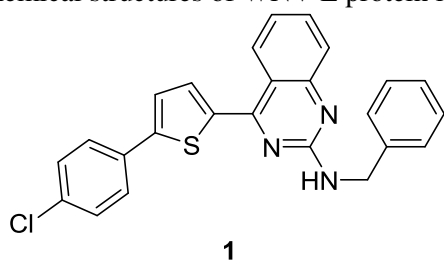


Fig. (3). Chemical structures of WNV NS2B/NS3 protease inhibitors.

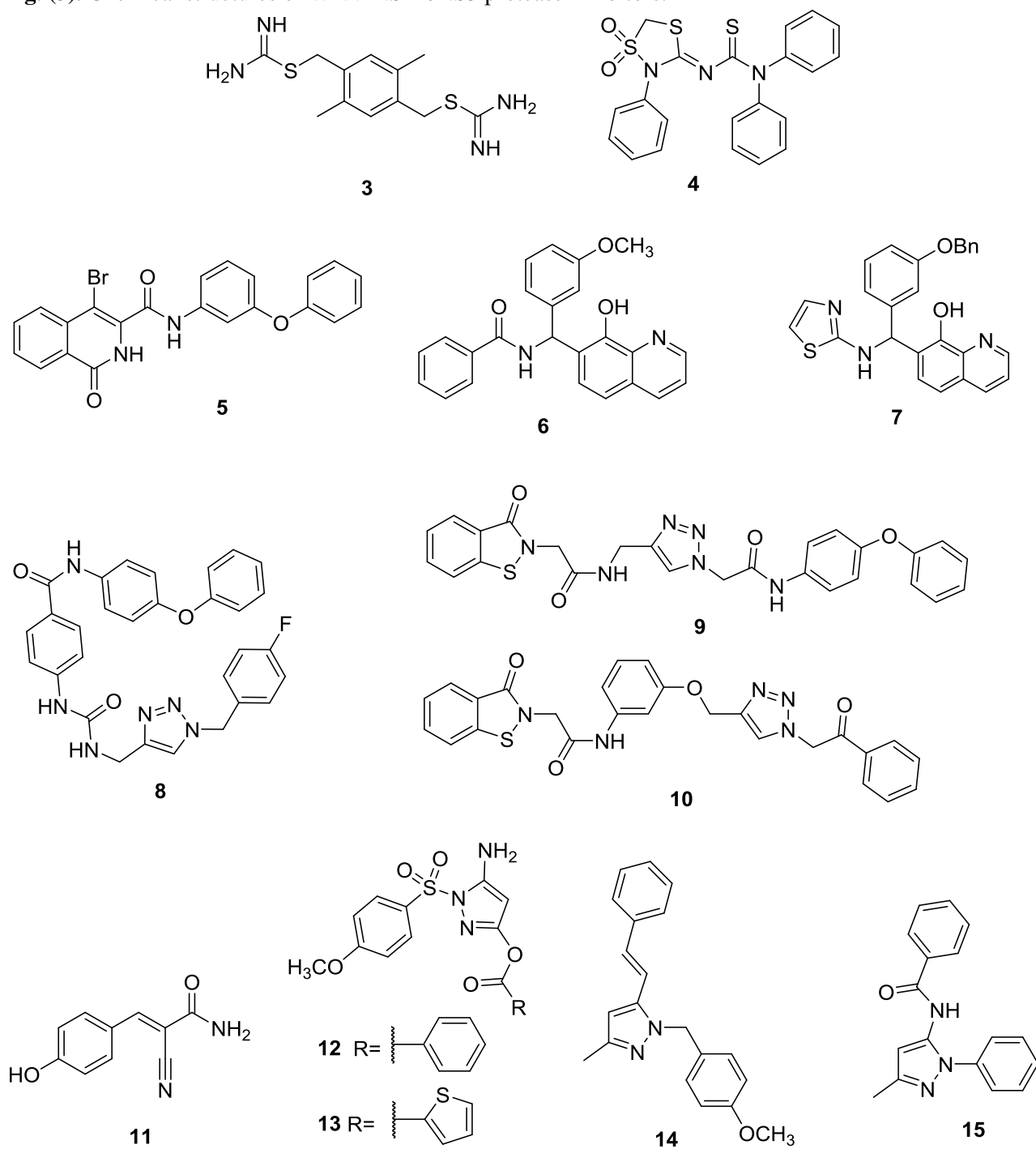


Fig. (4). Chemical structures of WNV NS5 inhibitors.

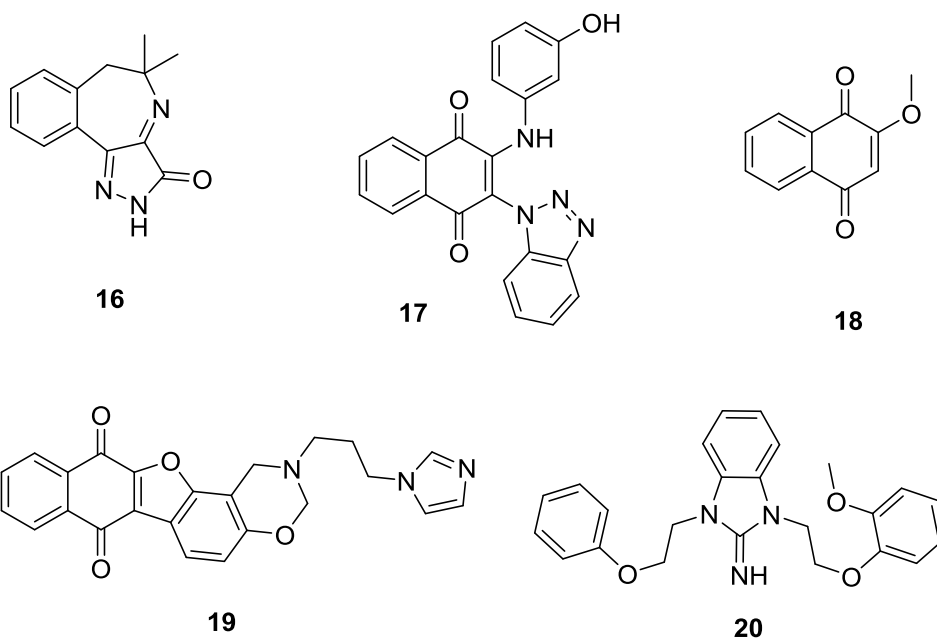


Fig. (5). Chemical structures of WNV NTPase/Helicase inhibitors.

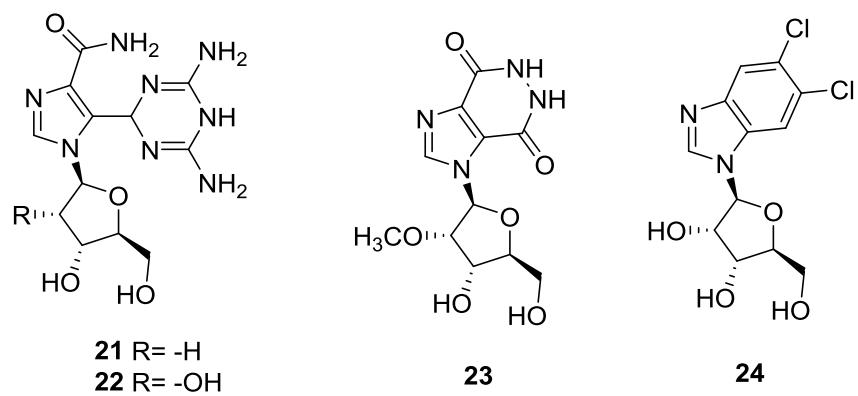


Fig. (6). Chemical structures of inhibitors with a non-specific or unknown mode of action.

