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Chapter 4

Alphavirus Nonstructural Proteases and Their Inhibitors

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1 INTRODUCTION

Alphaviruses belong to the Togaviridae (Group IV) family of viruses, and are categorized as C priority pathogens according to the US National Institute of Allergy and Infectious Diseases of United States of America. These viruses are known to be the cause of major fatality due to their high mortality and morbidity rates in underdeveloped regions of Africa, Asia, and some parts of Latin America (Strauss and Strauss, 1994). Alphaviruses are able to infect various vertebrates, such as human, horses, rodent, fish, and birds, as well as invertebrates and include numerous categories of viruses, such as Chikungunya virus (CHIKV), Barmah Forest virus, O'Nyong–Nyong virus, Mayaro virus, Rose River virus, Semiliki Forest virus (SFV), Sindbis virus (SINV), Una virus, Tonate virus, Eastern equine encephalitis, Western equine encephalitis, and Venezuelan equine viruses (Atkins, 2013). The infections caused by these viruses are of potential risk, and many of them have so far no treatment. The treatment, management, and vaccination procedure to combat diseases caused by these viruses have not been rewarding (Rayner et al., 2002). Alphaviruses are generally RNA viruses with a positive-sense, and single-stranded RNA genome (Koonin et al., 1993). These RNA viruses encode nonstructural (NS) proteins (NSPs) that can be used for replication to achieve a maturation state. These protein moieties can be targeted for the development and testing of potential antiviral drugs. The goal of computational study is to provide an alternative tool that is highly reliable and that may be complementary to the current experimental techniques. The two important theoretical approaches, molecular dynamics simulations (Pattanayak and Chowdhuri, 2011, 2012) and docking (Agarwal et al., 2015; Bissoyi et al., 2013), have been found to be quite reliable in this respect. Other computational procedures have been widely utilized

Viral Proteases and Their Inhibitors. http://dx.doi.org/10.1016/B978-0-12-809712-0.00004-6

to investigate the drug–receptor interactions in various types of biochemical problems (Garg et al., 1999; Gupta et al., 1983).

Recently, several protease inhibitors have been developed using computer-aided drug design methodologies (Gupta, 2013; Gupta et al., 1983; Wlodawer and Vondrasek, 1998), synthetic approaches, high-throughput screening method (Mayr and Bojanic, 2009), and drug reposition–based approaches (Sundberg, 2000), which could possibly target the NSPs responsible for virus replication. In addition, protease inhibitors targeting viral NSPs have also been isolated from various sources, such as plant extracts (Bhakat et al., 2014). These protease inhibitors have shown different degrees of inhibition of viral NSPs, which necessitates the development of selective and potent therapeutic agents against the devastating alphaviruses (De Francesco and Carfi, 2007). In this chapter, attempts have been made to discuss the development and design of antiviral drugs against various alphaviruses. In addition, the development of protease inhibitors targeting NSPs of alphaviruses has been also presented.

2 ALPHAVIRUSES

Alphaviruses have been established as a model system to examine the budding and assembly of enveloped viruses. The RNA genome of these viruses encodes multiple copies of capsid proteins (CPs) that assemble along with replicated RNA to form the nucleocapsid core (NC). The icosahedral budding and assembly pattern of these simple enveloped viruses are described in detail here.

2.1 Life Cycle of Virus

Alphaviruses invade the host cells through the clathrin-coated endocytic pathway. Acidic pH at the endosomal membrane enables fusion and results in the release of NC into the cytoplasm. Upon the shifting of CPs to ribosomes, NC becomes uncoated and discharges the 49S RNAs genome into the cytoplasm. These genomic RNAs are translated into the NSPs. The information of negative-sense replica of genomic RNA is transcribed by the NSPs. This RNA works as an archetype for the production of genomic and subgenomic RNA. The subgenomic RNA has greater capability to synthesize proteins, in comparison to the genomic RNA that also encodes the basic proteins of the virus. In the structural polyprotein, CP being at the N-terminus followed by PE2 (E3þE2), 6K, and E1. *Alphavirus* virions are formed by the assembly of 240 copies of CP, E2, and E1 (Fig. 4.1A). The transmembrane E1 glycoprotein mediates the coalescence between viral and endosomal membranes. On the other hand, the transmembrane segment E2 is responsible for the binding of the cell receptor. Endoplasmic reticulum (ER) and Golgi apparatus process the E1 and PE2, a precursor of E2 and E3 as a heterodimer, and transport them in the form of spikes to the cell surface. These spikes are constituted of three heterodimers of E1/E2 that promotes the correct folding of E2 and E3 which work as a chaperone and prevent the

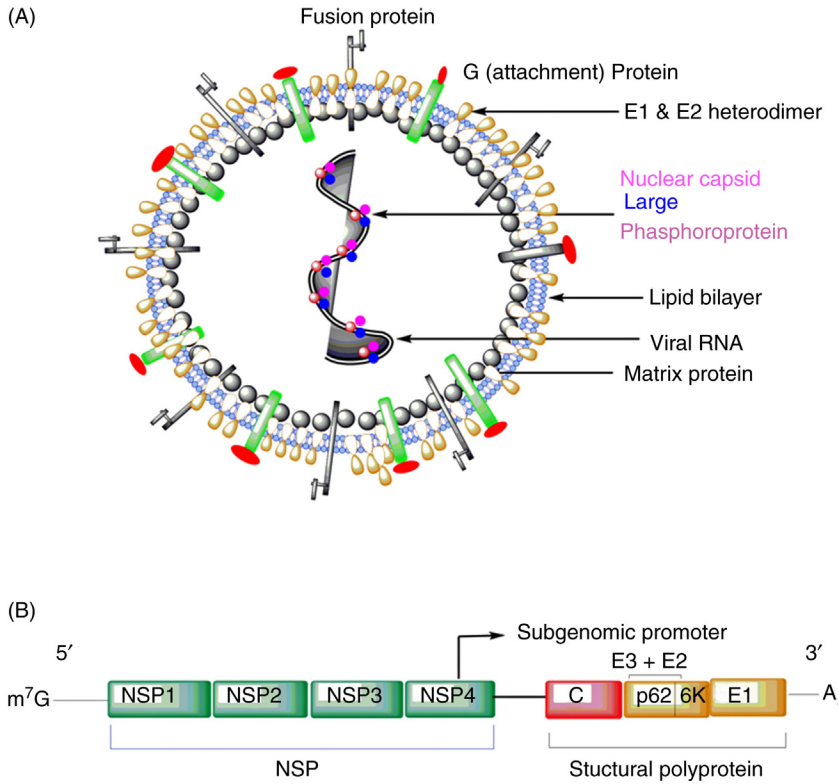


FIGURE 4.1 (A) Structure of *Alphavirus*. The hypothetical alpha capsid arrangement, genome, particle, and proteins, (B) Schematic representation of *Alphavirus* genome showing the RNA sequence. *C*, Capsid; *E*, envelope; *NSP*, nonstructural protein.

premature fusion of E1 with Golgi in an acidic pH environment. During virus entry, fusion of the glycoprotein spike complex produces E2 and E3 by cleavage of PE2 in the Golgi, with the help of a protease similar to furin. The function of 6K is not clear, but it promotes the pathogenic effect of the particle. In the infected cell, an icosahedral NC is formed in the cytoplasm by the encasing of the only copy of genome RNA with 240 copies of CP. Matured virus budded from the host cell membrane by the action of NC and E1/E2 trimeric spikes present in its plasma membrane.

2.2 *Alphavirus* and its Virion Structure

The conformation of alphaviruses (Fig. 4.1A) is clearly defined by the two popular techniques: (1) image reconstruction technique and (2) cryoelectron microscopy (cryo-EM). The outer protein layer of these viruses consists of glycoproteins E1 and E2 (Fig. 4.1). Membrane extension of these

glycoproteins can pass through the lipid bilayer of the host that encloses the NC of the virus. After interaction of the CP and glycoprotein layers, they are proportionally organized in icosahedral configuration of T ¼ 4. A pseudoatomic model of the virus is generated when the amino acid sequence of CP from 106 to 264 and the crystal arrangements of a part of E1 protein extend into the extracellular space and fit in to the cryo-EM density of the SINV. It has been observed that on the exterior surface of the viral membrane, E1 takes the icosahedral shell shape after binding with cryo-EM density. Practically, the position of E1 and E2 is lateral and radial to the lipid bilayer simultaneously. The top of E1 is populated with the E2, which cloaks the fusion of the peptide to prevent early synthesis with cell membranes. These envelope glycoproteins, E1 and E2, are required for cell entry, during which the exterior of E2 is unveiled, which approaches the cellular receptor and protects E1 until the fusion is completed. The E1 and E2 are separated in the endosome at low pH and the fusion of peptide is exposed. The binding of amino acids (106–264) of CP in cryo-EM density of SINV is exhibited in every single subunit of the prominent pentamers, as well as in hexamers (known as capsomeres), and detected in the NC layer made from the CP protease domain comprising of amino acids. Indeed a minor contact has been observed for CP amino acids 114–264 in capsomere(s). Accordingly, NC is stabilized in the absence of glycoproteins by the RNA–RNA and CP–RNA interactions. The connection between CP–RNA and RNA–RNA appears in the RNA–protein layer below the capsomeres.

3 GLOBAL VISION OF NONSTRUCTURAL PROTEINS

The NSPs are essential in the replication process of *Alphavirus*. In addition, these NSPs also play a vital role in the infection and interaction of the virus with host cells. Out of the four NSPs, NSP1 (537 aa), whose structure is shown in Fig. 4.2, binds with membrane proteins and activates guanylyl transferase and methyl transferase, which are involved in the covering of 26S and 42S RNAs (Ahola and Kääriäinen, 1995; Kääriäinen and Ahola, 2002; Koonin et al., 1993; Laakkonen et al., 1994). In the response of *Alphavirus* capping, the methyl group is transferred by NSP1 from S-adenosyl methionine (AdoMet) to GTP. Subsequently, before transferring 7-methyl-GMP (m7GMP) to virus RNA, a covalent complex is formed between m7GMP and NSP1. The response of capping of *Alphavirus* is somewhat different from the capping of the cellular mRNAs, where guanylyl transferase is first transmitted from GMP to RNA, followed by methylation of GMP-RNA (Ahola and Kääriäinen, 1995). The enzymatic actions of NSP1 are necessary for virus replication. A virus becomes noninfectious by single-point mutation because this transformation abolishes the enzymatic activities (Wang et al., 1996). NSP1 is strongly bound with the plasma membrane of infected cells, and when NSP1 is expressed alone then it functions as a single membrane anchor in virus replication complexes (Peränen et al., 1995; Salonen et al., 2003;

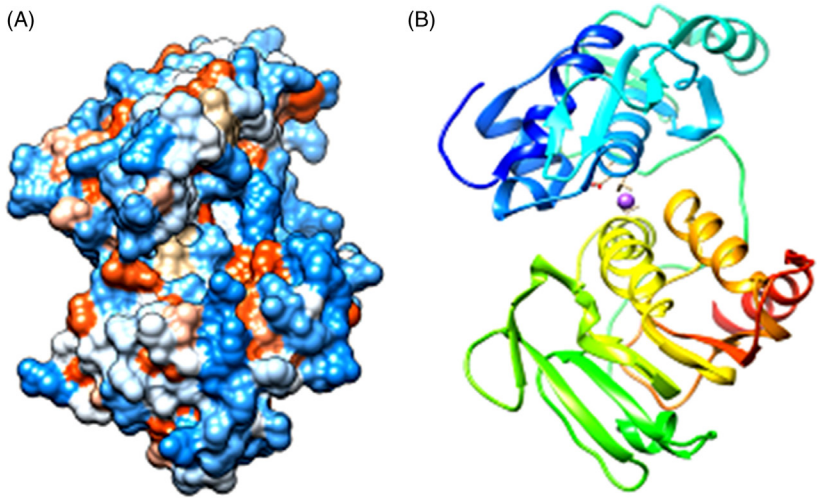


FIGURE 4.2 **3D Protein stucture of NSP1 domain of Chikungunya virus (CHIKV).** (A) 3D representations of surface charge distribution of respective proteins, *blue* color represents the positively charged regions and *orange*, the negatively charged regions, and (B) cartoon representation of the proteins. 3D protein structures of CHIKV NSP1 were designed using TASSER server (Zhang, 2008). The structure is drawn using Chimera visualization tool (Pettersen et al., 2004).

Žusinaite et al., 2007). Amphipathic alpha-helix is formed when amino acid 245–264 of NSP1 mediates the binding of plasma membrane (Ahola and Kääriäinen, 1995; Žusinaite et al., 2007). The binding activities of NSP1 with the membrane can be affected by a single-point mutation and it is lethal for the virus (Žusinaite et al., 2007). In SFV, the Cys 418–420 residues of NSP1 and in SINV, Cys420 of NSP1 tighten the membrane binding by posttranslational palmitoylation (Laakkonen et al., 1998; Wang et al., 1996; Žusinaite et al., 2007). Palmitoylation is required only for the formation of the replication complexes or sustainability of the virus, and not for enzymatic activities of NSP1 (Ahola and Kääriäinen, 1995; Žusinaite et al., 2007). However, removal or exchange of cysteine residues 418–420 in NSP1 of SFV significantly obstructs virus duplication and causes growth of expletive alterations (Žusinaite et al., 2007). It has been observed from the virus expression that palmitoylation-deficient NSP1 is not capable of causing the disease in mice; these viruses induce low levels of viruses in the blood stream, but no infection has been observed in the brain tissues (Ahola and Kääriäinen, 1995). NSP1 is responsible for the initiation of structure on the cell surface, similar to filopodia that is representative of *Alphavirus*-septic cells (Hardy and Strauss, 1989; Laakkonen et al., 1998; Rikkomen, 1996; Žusinaite et al., 2007). The functional activities and significance of filopodia-like structures and palmitoylation remain unidentified.

Of the four NSPs, NSP2 (799 aa in SFV) is known for different types of enzymatic actions and significant roles in virus infection. N-terminal domain of NSP2 (RNA helicase) comprises the activities of nucleoside triphosphatase

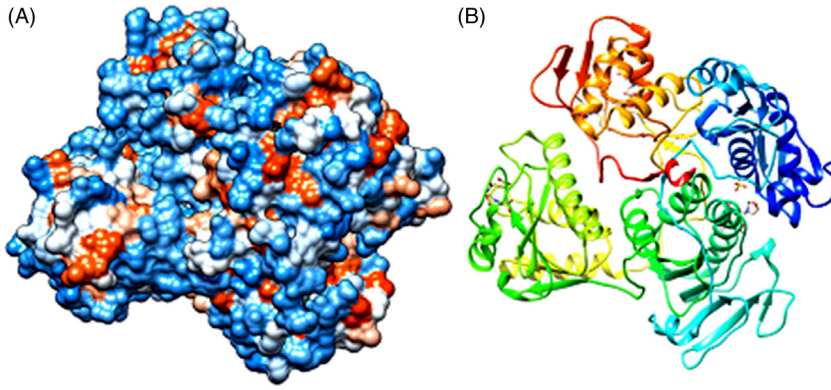


FIGURE 4.3 Representation of NSP2 domain of CHIKV (PDB: 3TRK). (A) The surface view with *blue color* represents the positively charged regions and *orange*, the negatively charged regions. (B) Ribbon-type representation of NSP2 domain of CHIKV. Structures are drawn using Chimera visualization tool (Pettersen et al., 2004).

and RNA triphosphatase as shown in Fig. 4.3 (Bouraï et al., 2012; Lastarza et al., 1994; Merits et al., 2001; Peränen et al., 1990; Rikkomen, 1996). The activities of RNA helicase and RNA triphosphatase are very important for the unwinding of RNA duplexes during replication and removal of 5′-phosphate, respectively, from viral RNA during capping reaction. The removal of 5′-phosphate before the guanylyl transferase activity of NSP1 is a very crucial step that facilitates the attachment of m7GMP.

The papain-like protease activity present at C-terminal domain of NSP2 is responsible for processing of NS polyprotein (Cruz et al., 2010; Fazakerley et al., 2002). Moreover, the protease participates in the formation of the replication complex and subsequently regulates the replication process. This proteolytic activity has also been shown to be hampered due to mutations that abolish its efficient activity and leads to the fatality of the virus (Cruz et al., 2010). The C-terminus of NSP2 contains latent methyl transference activity, which plays an important role in the regulation of minus-strand synthesis and the development of cytopathic effects (Cruz et al., 2010). It has been documented that approximately 50% of NSP2 is present in the nuclear fraction of infected cells, as evidenced by the cell fractionation studies (Peränen et al., 1990). The nuclear transportation of NSP2 starts at the earliest during the infection, and the nuclear localization is mediated through the pentapeptide PRRRV (aa 647–651) (Rikkomen, 1996). The nuclear translocation of NSP2 has been shown to be affected with mutations, which results in attenuated phenotypes with reduced cytotoxicity and transcriptional and translational shutdown of the host cells, yet renders the virus pathogenic in adult mice (Fazakerley et al., 2002; Rikkomen, 1996). In addition, apart from helicase- and methyltransferase-like domains, other sequences/domains within NSP2 are essential for virus-induced

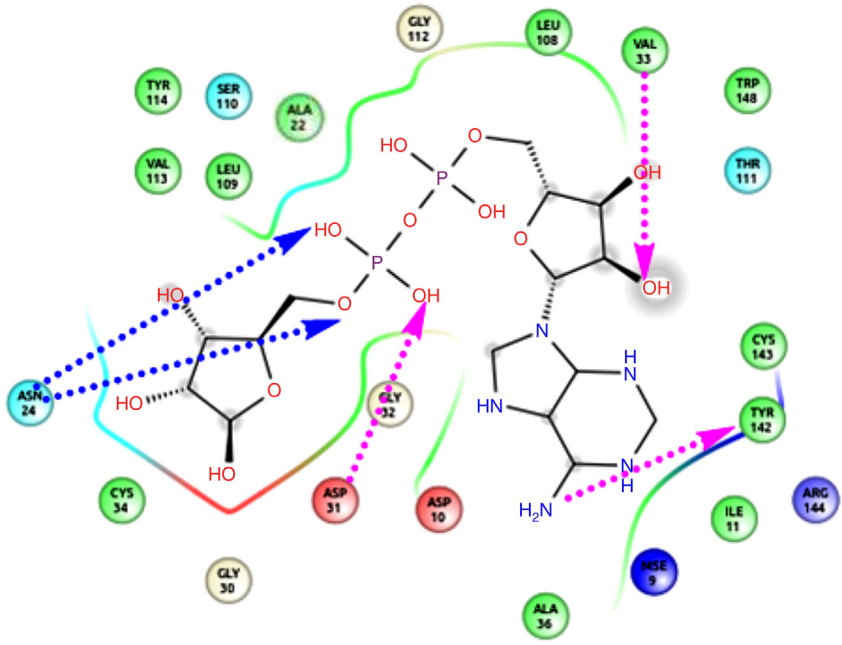


FIGURE 4.4 **A 2D representation of structure of NSP3 macro domain of CHIKV (PDB ID: 3GPO) in complex with ADP ribose.** *Arrows* represent the hydrogen bondings between NSP3 and ADP ribose. Structure drawn using Schrödinger (2011) software.

shutdown of cellular transcription (Akhrymuk et al., 2012; Firth et al., 2011; Gorchakov et al., 2005; Strauss and Strauss, 1994; Varadhachary et al., 2008) and suppression of antiviral responses (Akhrymuk et al., 2012; Gorchakov et al., 2008).

The primary structure of NSP3 (482 aa in SFV) is composed of three discrete domains that contain a small macro domain of 160 amino acids at the N-terminus, which is conserved among alphaviruses, rubella virus, hepatitis E virus, and coronaviruses (Fig. 4.4). This conserved macro domain also shares sequence similarity with the macro domains of proteins of eukaryotic organisms, archaea, and eubacteria (Koonin et al., 1993; Pehrson and Fuji, 1998). The middle domain of *Alphavirus* NSP3 is a conserved region and the third C-terminal domain (starting from Tyr324 in SFV) is hypervariable in length and sequence (Strauss and Strauss, 1994). However, the function of NSP3 is not clearly understood.

The study of replication complexes suggested that NSP3 is found in the cytoplasm in the form of aggregates with irregular boundaries of variable sizes. In addition, a small fraction of NSP3 is localized to the nuclear envelope (Gorchakov et al., 2008; Wang et al., 2006), suggesting that NSP3 might participate in RNA replication. It has also been demonstrated that the enzymatic

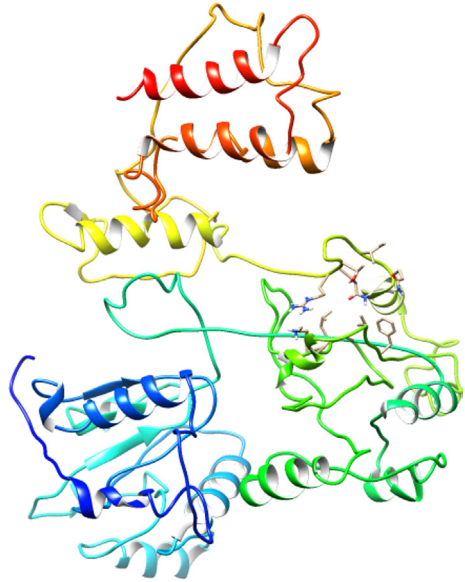


FIGURE 4.5 **Ribbon-type representation of NSP4 domain of CHIKV.**

activity only resides within the macro domain of NSP3 protein, which was identified as ADP-ribose-1-phosphate activity (Malet et al., 2009; Neuvonen and Ahola, 2009). *Alphavirus* macro domain has been found to have the capability of binding to poly-(ADP-ribose), RNA, and ADP-ribose as shown in Fig. 4.5 (Malet et al., 2009; Neuvonen and Ahola, 2009). Interestingly, poly-(ADP-ribose) polymerase-1 (PARP-1) interacts with SINV replication complexes through the C-terminal domain of NSP3 in neuronal cells (Park and Griffin, 2009).

Recent studies have suggested that only NSP3 of *Alphavirus* has the capability to get phosphorylated at serine/threonine residues at the C-terminus (Peränen et al., 1988; Vihinen and Saarinen, 2000; Vihinen et al., 2001). In SFV, 16 phosphorylation sites have been identified that are accumulated in the area of 50–amino acid residues. The complete removal of these sites led to reduced levels of RNA synthesis in cultured cells and subsequently reduced pathogenicity in mice (Vihinen et al., 2001). The phosphorylation of NSP3 is specifically important for minus-strand RNA synthesis, as evidenced by SINV-mutant studies (Vihinen et al., 2001). Thus the SFV and SINV studies have concluded that NSP3 plays a significant role in the modulation of neuropathogenesis in mice (Tuittila and Hinkkanen, 2003). Several studies reported that NSP3 also mediates the movement of *Alphavirus* replication complexes from the plasma membrane to intracellular vesicles. It has been confirmed by the

analysis of subcellular localization of different forms of NSP intermediates (Hahn et al., 1989; Park and Griffin, 2009; Tanno et al., 2007).

Among NSPs of *Alphavirus*, NSP4 (614 aa in SFV) Fig. 4.5 represents the last catalytic subunit of *Alphavirus* that acts as RNA-dependent RNA polymerase (RdRP) (Hahn et al., 1989; Tanno et al., 2007). The C-terminal end of *Alphavirus* NSP4 contains the GDD motif (Gly315, Asp316, and Asp317) and shares sequence homology with RdRPs of other viruses (Argos et al., 1984). CHIKV NSP4 (EC 2.7.7.48) localizes to 2014–2462 sequence position in the polyprotein NSP 1234, which is constituted of 449 amino acids with the activity of polymerase. The potential catalytic site bearing a triad and two allosteric binding sites were identified to be similar to that in hepatitis C virus (HCV) NS5B polymerase. NSP4 of CHIKV included a highly conserved catalytic triad (GDD motif) responsible for polymerase activity.

The voluminous catalytic site is 2769.41 $Å^3$, whereas two allosteric binding sites with volume of 73.216 and 55.904 $Å^3$, were identified on both palm and thumb sites.

However, the N-terminal end of NSP4 is conserved in the alphaviruses. Although NSP4 is the major catalytic core of replication complex in *Alphavirus*, it still needs other NSPs for activity (Rubach et al., 2009). Mutation studies have suggested that the N-terminus of NSP4 interacts with host proteins and other NSPs, particularly NSP1, for its activity (Fata et al., 2002; Shirako et al., 2000). In addition, in vitro experiments have demonstrated that NSP4 also has terminal adenylyl transferase activity, suggesting its role in repair and maintenance of the poly(A)-tail of the viral genome (Shirako and Strauss, 1998; Tomar et al., 2006), which is an essential step for the survival of the virus. The level of NSP4 has been reported to be lower than the levels of other NSPs in the infected cells that are attained by several processes. First, the normal translation of different NS polyproteins is in the form of P123 due to the fact that most of the alpha viruses hold the opal termination codon in their genome. Moreover, the translation of P1234 and NSP4 is found with an efficiency of 15%–25% only. Second, the N-terminal amino acid in NSP4, which is a conserved tyrosine, directs the NSP4 to rapid proteasomal degradation. This destabilizing role of tyrosine has been confirmed by replacing it with a nonaromatic residue that leads to poor RNA replication (Shirako and Strauss, 1998).

3.1 NSP1 Protease Inhibitors

3.1.1 Bone Marrow Stromal Antigen-2–Based Inhibitors

Targeting N-terminal of NS1, which is responsible for methyl and guanyl transferase activities, would have an inhibitory effect on viral replication. Recently, NSP1 protein showed a crucial role in the downregulation of bone marrow stromal antigen-2 (BST-2) and thus paved the way for the development of BST-2–mediated chemicals acting against NSP1 (Tokarev et al., 2009). BST-2 is one

of the important defense mechanisms of host cells, which occurs by the expression of interferon alpha (INFα). Its expression results in retaining the virus at the surface of the infected cells (Jones et al., 2013).

3.1.2 Small Hairpin RNA Molecules

Targeting gene silencers for specific viral proteins have been studied by several groups of researchers (Waterhouse et al., 2001). The small hairpin RNA (shRNA) of E1 and NSP1 show noncell-type specific antiviral effect and in broad-spectrum silencing against different types of strains of alphaviruses (Lam et al., 2012). The cell clones expressing shRNA against CHIKV E1 and NSP1 genes display inhibition activity of CHIKV production by degrading the viral single-stranded RNA.

3.2 NSP2 Inhibitors

With the advancement in the determination of crystal structures of viruses, NSP 2–3 macro domains were determined, which provided a platform for the development of new inhibitors for targeting alphaviruses. Recently, NSP2 is considered to be an important target for the development of antiviral drugs because of its role in viral replication and pathogenesis. Both C- and N-terminals have active sites.

3.2.1 Natural Inhibitors

In the past, the chemical constituents acting as drugs were extracted from natural products. Based on cellular high-throughput screening, 3040 natural compounds led to the discovery of natural NSP2 inhibitors (Lucas-Hourani et al., 2013). The compound ID1452-2 and its four analogs (Fig. 4.6) displayed potent and selective antiviral activity toward CHIKV. This result might initiate future efforts for the discovery of novel natural compounds as NSP2 selective inhibitors.

3.2.2 Druggable Targets Peptides

Recently, six peptide sequences were obtained and a global NSP2 sequence was constructed from various NSP2 strains of *Alphavirus* based on phylogenetic and global conservation (Singh et al., 2012). In silico analysis showed peptides sequences and the position of binding (Table 4.1).

3.2.3 NSP2-Based High-Throughput Screening

The molecular dynamics simulation and molecular docking are methods of high throughput screening. Singh et al. (2012) studied Chikungunya virus NSP2 protease protein to investigate the residues that are involved in the cleavage mechanism. Compounds with a finesse score more than 1.0 were considered for docking studies. The study identified four different compounds (ID27943,

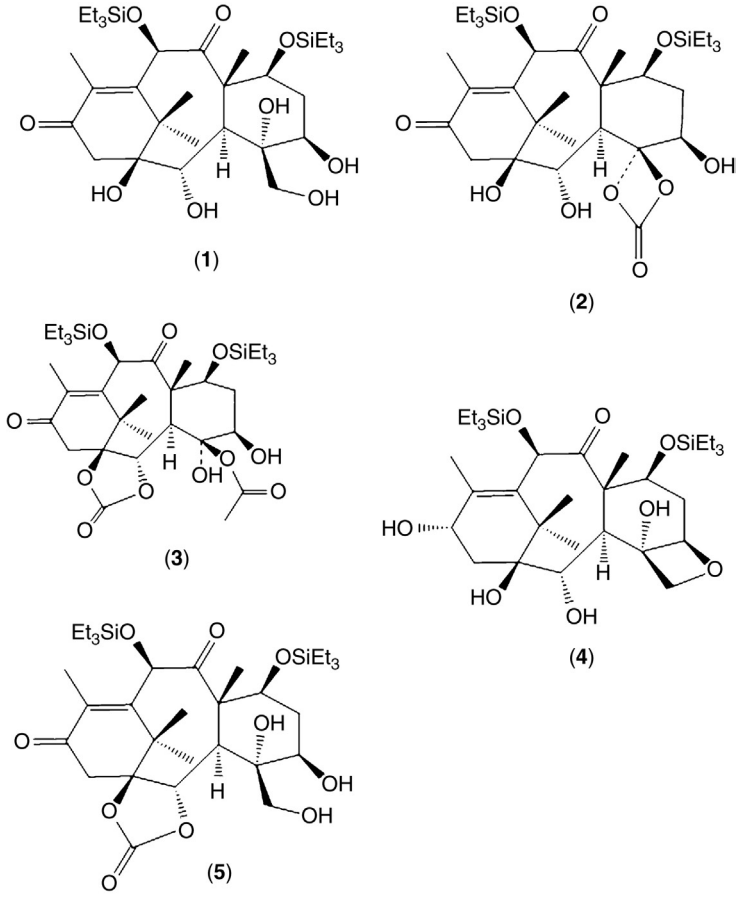


FIGURE 4.6 **Chemical structures of compound ID1452-2 and its four analogs.**

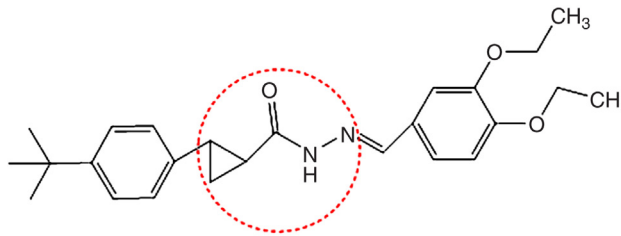
TABLE 4.1 Druggable Target Peptides With Tentative Binding Site at NSP2 Macro Domain

Amino acid positions	Peptide sequences	Position of binding region
22–28	FPADRTT	23, 28
142–159	GTGGRLDSALPRIRIRAKN	142–145, 154
235–249	VRRRGVCVPLWNVTC	236
445–459	PKQSQRLLGELGPYP	443, 458
488–505	PENMHAHVWGGSRQRAIF	504
639–655	WVPSGHKHPHTFSHTPL	642

ID21362, ASN01107557, and ASN01541696) as potential inhibitors toward C terminal domain of NSP2 protein. As per their docking analysis, the residues Gln1039, Lys1045, Glu1157, Gly1176, His1222, Lys1239, Ser1293, Glu1296, and Met1297 were found to have crucial interactions with the NSP complex to be cleaved, and were considered as an individual functional unit. The propagation and replication of CHIKV depends on the NSP2 protein, which inhibits the protein by targeting the main residue. Using molecular modeling and viral screening, Bassetto et al. (2013) explored NSP2 as a target for the discovery and development of different selective inhibitors of CHIKV replication. They discovered a series of inhibitors against CHIKV NSP2. In silico analysis showed that the compounds bind to the central portion of NSP2 protease. The hyrazone and cyclopropyl groups have been shown to play vital roles in antiviral activity of compounds. Furthermore, it was found that antiviral activity increased when the cyclopropyl group was replaced with *trans*-ethenylic moiety shown in Fig. 4.7.

3.2.4 Thienopyrrole Derivatives

The neurotropic alphaviruses, which represent emerging pathogens, have the potential for widespread dissemination and the ability to cause substantial mortality and morbidity. The thieno[3,2-b]pyrroles (Fig. 4.8), heterocyclic compounds possessing physiological activity, have clinical applications and act as antiinflammatory agents. Ilyin et al. (2007) described a solution-phase strategy



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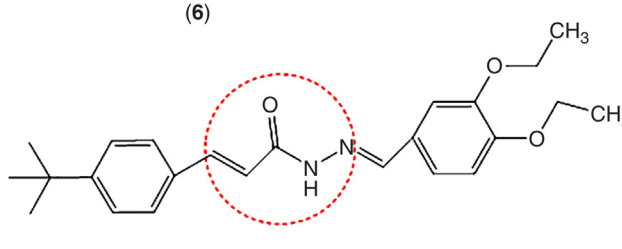


(7)

FIGURE 4.7 Chemical structures of some CHIKV NSP2 protease inhibitors identified based on high-throughput screening. *Red circle* indicates the active functional group involved in binding.

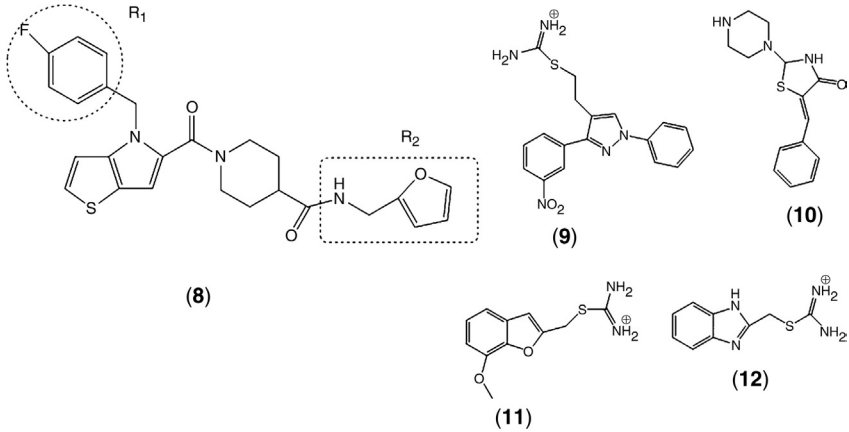


FIGURE 4.8 **The general structures of thienopyrroles and some of their analogs found active against Venezuelan equine encephalitis virus NSP2.**

for the synthesis of novel combinatorial libraries containing a thieno[3,2-b] pyrrole core. Peng et al. (2009) identified thieno[3,2-6]pyrrole compounds with IC_{50} values <10 μM/L and selectivity index >20. Based on structure–activity relationship analysis, 20 potential novel antiviral drugs out of 51,028 compounds were identified. Some of these compounds, shown in Fig. 4.8, were found to possess activity against neutrophilic *Alphavirus*, such as Venezuelan equine encephalitis virus. The thienopyrrole derivatives designed by homology modeling of protein sequences were shown to be effective inhibitors of NSP.

The NSP2 protein, which is responsible for CHIKV replication, causes a general host shutoff. By preventing IFN-induced gene expression, CHIKV suppresses the antiviral IFN response. Bora (2012) used homology modeling to predict the three-dimensional (3D) structure of NSP2 protease. With this model, a ligand [*N*-butyl-9-[3,4-dipropoxy-5-(propoxymethyl)oxolan-2-yl]purin-6-amine] was found to bind more efficiently than the ligand [3-fluoro-5-(6-hexylsulfanylpurin-9-yl)oxolan-2-yl]methanol. Both the ligands are shown in Fig. 4.9. One hydrogen bond was found to be formed between ligand **13** and NSP2. Carbonyl nitrogen atom in the pyrimidine ring of ligand **13** forms a hydrogen bond with the carbonyl oxygen (N–H…O) of Lys103 with a bond distance of 3.00 Å. The ligand **14** has higher gold score than ligand **13**. The former ligand is therefore supposed to be a promising inhibitor of NSP2 protease of CHIKV virus, and has been emerged as one of the promising antiviral drug candidates with potential symptomatic and disease-modifying effects.

Through molecular modeling by autodock and molecular dynamics simulation, Agarwal et al. (2015) elucidated the different structural aspects of NSP2 protein and different ligands, which are shown in the following Fig. 4.10.

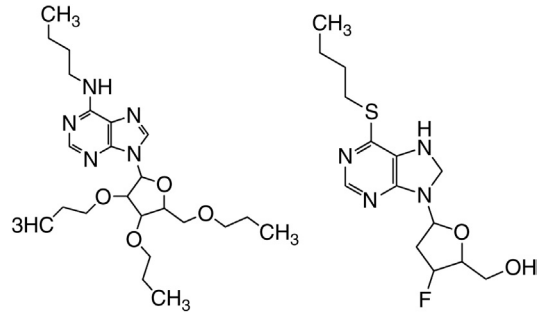


FIGURE 4.9 **Structure of ligand 13 [N-butyl-9-[3,4-dipropoxy-5-(propoxymethyl)oxolan-2-yl] purin-6-amine] and ligand 14 [3-fluoro-5-(6-hexylsulfanylpurin-9-yl)oxolan-2-yl]methanol.**

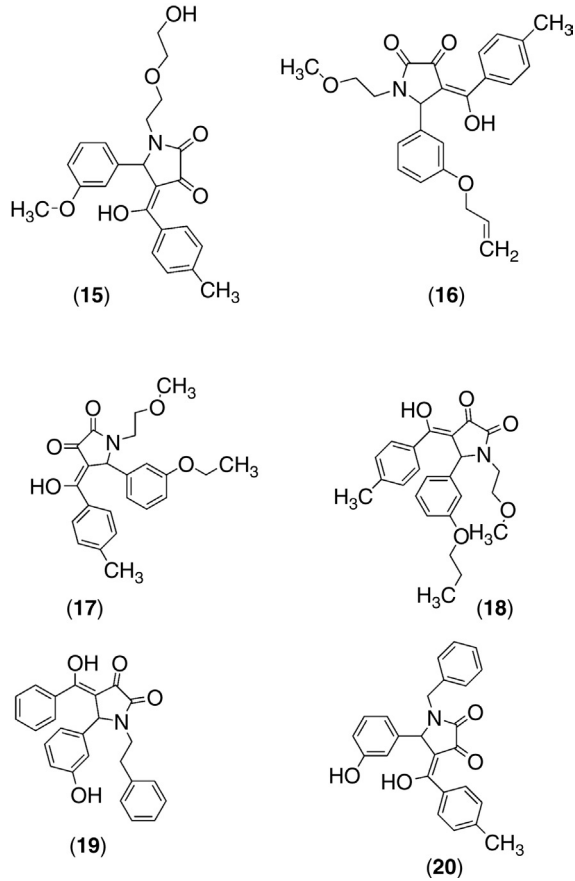


FIGURE 4.10 **Chemical structures of some in silico–predicted S-adenosyl methionine derivatives acting against NSP2 CHIKV protease.**

The ligand **20** (shown in Fig. 4.10) interacted with the protein with three hydrogen bonds having bond length of 2.86, 3.11, and 3.07 Å with Trp1084 and Tyr1047 amino acid residues. Ligand number **15** showed a better affinity toward CHIKV NSP2 protease having binding energy of 8.06 kcal/mol. It interacted with Trp1084 and Asp1246 amino acid residues of CNSP2 protease via two hydrogen bonds having bond length of 3.03 and 2.95 Å. The binding energies of other ligands, such as ligands **16**–**18**, were found in the range of −7.55 to −6.75 kcal/mol. Both ligand **15** and **20** have a common binding site at Trp1084. Ligand **15** interacted with catalytic amino acid residue Cys1013 hydrophobically. Catalytic amino acid residue His1083 was found to be in close proximity to the ligands with distance less than 5 Å, which is insufficient to form hydrophobic interactions. This indicates that the ligands might inhibit protease activity by blocking the binding of substrate to its active site. However, Trp1084 is a conserved residue of the NSP2 protease active site pocket and is present in close proximity to His1083. The catalytic activity of CNSP2 protease was reduced by interaction of the ligands with Trp1084. Interestingly, none of the ligands were found to be involved in π–π interaction with the CNSP2 protease. All the six ligands bonded with Ser1048, Tyr1079, and Gln1241 either through hydrogen bonding or hydrophobic interactions. These three amino acid residues create a microenvironment for the CNSP2 protease activity.

3.2.5 Thiazolidone Derivatives

The derivatives of pyrazole-like 1,3-thiazolidin-4-ones (Fig. 4.11) were synthesized by Jadav et al. (2015) and examined for the antiviral activity against CHIV (LR2006_OPY1) in Vero cell culture through CPE reduction assay. Three aralkylidene derivatives (compounds **6**, **7**, **8**) and *ortho*-methyl–substituted ligands

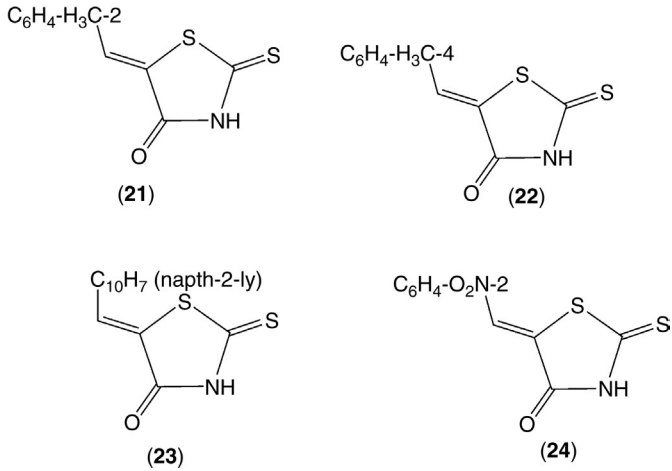


FIGURE 4.11 **Chemical structures of some thiazolidone derivatives.**

were found to be the most active among the rest. Furthermore, to understand the mechanism of protein and ligand interaction, molecular docking was carried out with NSP2 CHIKV protease (PDB: 3TRK). The results showed a strong interaction between aralkylidene with three amino acids (Tyr1047, Tyr1049, and Trp1084), while thiazolidinone showed a strong hydrophobic interaction with Cys1013, Tyr1047, Tyr1049, and Trp1084.

Compounds (**21**–**24**) were found to have antiviral activity at low concentration, particularly 0.42, 4.2, 3.6, 40.1, and 6.8 μM. Compound **21** was the most potent among the aforementioned compounds. Methyl substitution of *ortho*-position of compound **21** was favorable and was found to be 10 times more potent than its *para*-counterpart of compound **22**. The aralkylidene portion gets accommodated and exhibits two H-bonding interactions for compound **21**. Similarly, compound **22** showed interaction with residues in S2 and S1, but not in S3, which does not exhibit any H-bonding interaction with Tyr1047. Thiazolidone derivatives exhibited hydrogen-binding interactions with NSP2 protease. These interactions were between carbonyl oxygen of thiazolidone and the backbone amide hydrogen of Cys1013 and hydrogen of thiazolidone 2-amino nitrogen of the aforementioned compound.

3.3 NSP3 Protease Inhibitors

Like NSP2, the crystalline structure of N-terminal domain of NSP3 also became of great use, positioning NSP3 as a potential drug target for the development of antiviral drugs. To identify potential NSP3 inhibitors, Nguyen et al. (2014) screened 1541 compounds from NCI diverse sets against NSP3 of CHIKV and identified potential inhibitors that targeted CHIKV NSP3 complexed with ADP-ribose. They identified all possible binding pockets. The virtual screening was carried out using three steps. In the first step, docking was centered on the ADP-ribose–binding site, in the second step a blind docking was centered at the middle of the ADP-ribose–binding site, and in the third step docking was centered on the biding sites predicted by meatpacker. This led to the identification of the top 5 compounds as shown in Fig. 4.12, with binding energy of −8 kcal/mol. Furthermore, results also indicated that NSP3 protease residues interact with the ligand through hydrophobic contacts. The residues Val35, Val113, Tyr114, and Trp148 play a crucial role in stabilizing the complex. The binding energy of ADP-ribose having less than −10 kcal/mol, showed that negatively charged ligands can bind very strongly with NSP3. The binding affinities at the three different binding pockets studied by docking analysis revealed that all of the hits for pocket 1 can bind to the protein well, with binding energies less than −10 kcal/mol. Both hydrogen bonding and hydrophobic interactions play important roles in the binding between the ligand and NSP3. Different conformations of ligands bind to different pockets. Pocket 2 might not be a good location for binding, as compared to pockets 1 and 3, which are associated with higher binding affinities. Hydrogen-bonding interactions play an important role in the

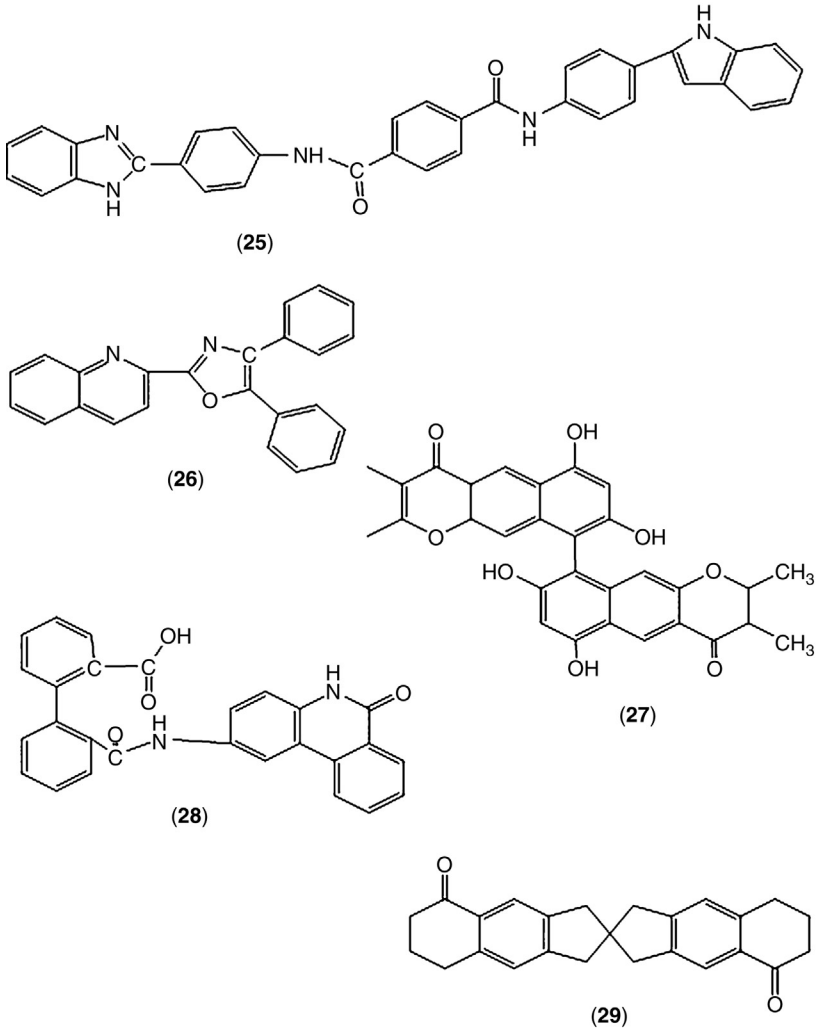
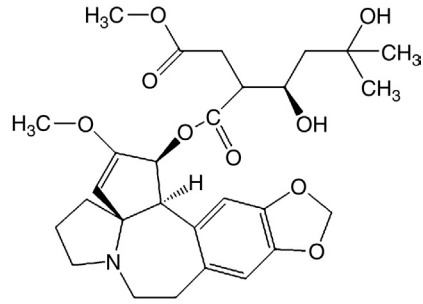


FIGURE 4.12 **Chemical structure of in silico–screened ligands acting against NSP3 domain.**

binding to pocket 1, whereas hydrophobic contacts are responsible for the interactions associated with binding to pocket 2.

3.3.1 Natural Inhibitors for NSP3 Protease

In an effort to identify novel inhibitors against NSP3 protease, Kaur et al. (2013) extensively studied the natural compound library. This study led to the identification of 44 compounds that exhibited more than 70% inhibition of CHIKV virus pathogenesis. Harringtonine, a cephalotaxine alkaloid (Fig. 4.13), which displayed potent inhibition activity against CHIKV infection with minimal



(30)

FIGURE 4.13 **Chemical structure of a cephalotaxine alkaloid, harringtonine.**

cytotoxicity, was studied by Kaur et al. (2013) for elucidation of its antiviral mechanism. Harringtonine inhibited the CHIKV replication cycle, which occurred after viral entry into cells during addition studies, cotreatment assays, and direct transfection of viral genomic RNA. It also affects CHIKV RNA production, as well as viral protein expression. At 5- and 10-μM concentrations, it showed a higher magnitude of inhibition of virus titer for CHIKV-122508 than for CHIKV-0708. The cepholotoxin plant alkaloid (harringtonine) displayed a strong EC_{50} of 0.020 μM with less cytotoxicity. The inhibitory activity of theses compounds was tested on virus cell–based immunofluorescence assay. Recently, an immunofluorescence-based screening was performed for the potential inhibitors of NSP3 CHIKV using the natural compound library. Harringtonine (acephalotoxin alkaloid) displayed an EC_{50} of 0.020 μM with less cytotoxicity (Fig. 4.13).

Silymarin (extracted from milk thistle) has been shown to inhibit hepatitis C virus. It is a complex of more than seven flavonolignans, such as silybin A, silybin B (Fig. 4.14), isosilybin A, isosilybin B, silychristin, isosilychristin, silydianin, and flavonoid. Silymarin is able to suppress the activity of the RLuc marker, which is fused with NSP3 protein of the virus (Lani et al., 2015). Plant-derived flavonoids are polyphenolic compounds endowed with a wide range of biological benefits to human health that not only includes antiinflammatory, antioxidant, antibacterial, and antifungal activities, but also antiviral activity. The increase in the number of drug-resistant microorganisms has brought natural compounds, such as flavonoids, to the forefront as an important natural resource to overcome this problem. A number of studies have successfully shown various types of flavonoids, such as rutin, naringin, baicalein, quercetin, and kaempferol, to be potential antiviral agents against a wide range of important viruses, including Dengue virus, HIV-1, H5N1 influenza A virus, coxsackievirus, and Japanese encephalitis virus (Seyedi et al., 2016). Baicalin is a metabolite of baicalein, which can be extracted from the root of Chinese

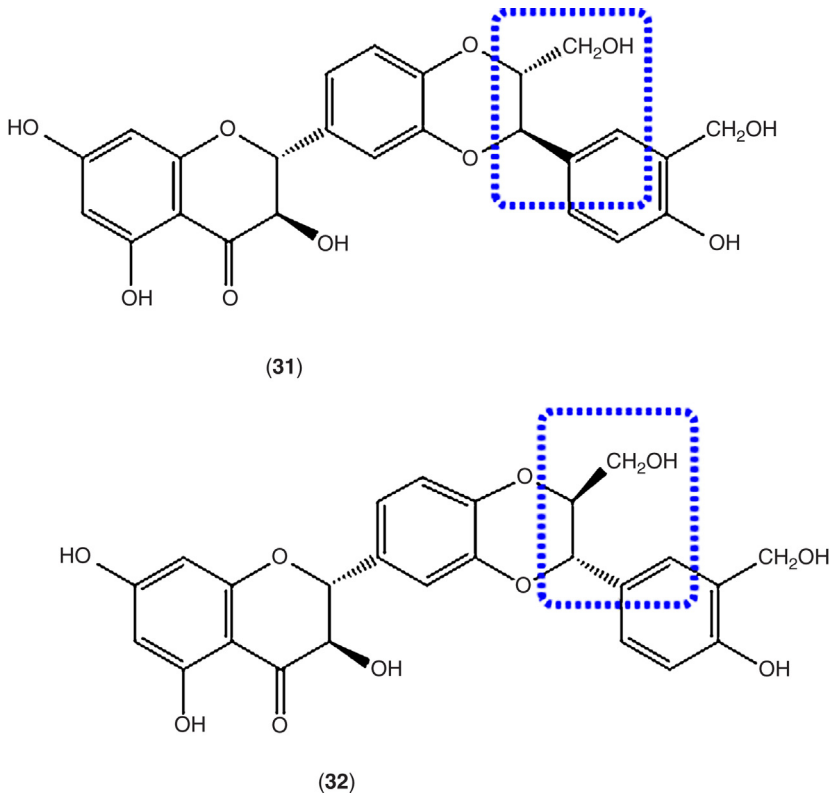


FIGURE 4.14 **Chemical structures of silybin A and silybin B.**

medicinal herbal plant *Scutellaria baicalensis*. Baicalin also serves as a potential antiviral agent against influenza viruses, where it acts as a neuraminidase inhibitor.

Seyedi et al. (2016) screened the potency of three flavonoids, baicalin, naringenin, and quercetagetin (Fig. 4.15), targeting NSP3 protein of CHIKV virus using computational approach. Docking results showed that of the three tested ligands, baicalin showed the highest binding affinity with −9.8 kcal/mol binding energy and low K_i value of 0.064 μM for NSP3, followed by quercetagetin and naringenin, having binding energies −8.6 and −8.4 kcal/mol, respectively. The hydrogen-bond donors came from the protein residues, and the corresponding acceptors were derived from the ligands. The ligands interacted with the residues present in the active site of NSP3 (baicalin: Leu108, Tyr142, Ser110, and Thr111; naringenin: Ser110 and Thr111; quercetagetin: Cys34, Leu108, Arg144, and Asp145). One π–π interaction was found between baicalin and Tyr114 residue of NSP3.

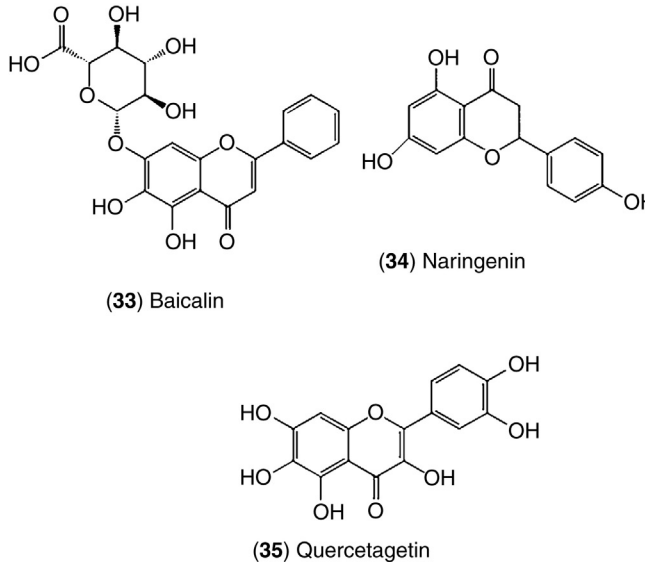


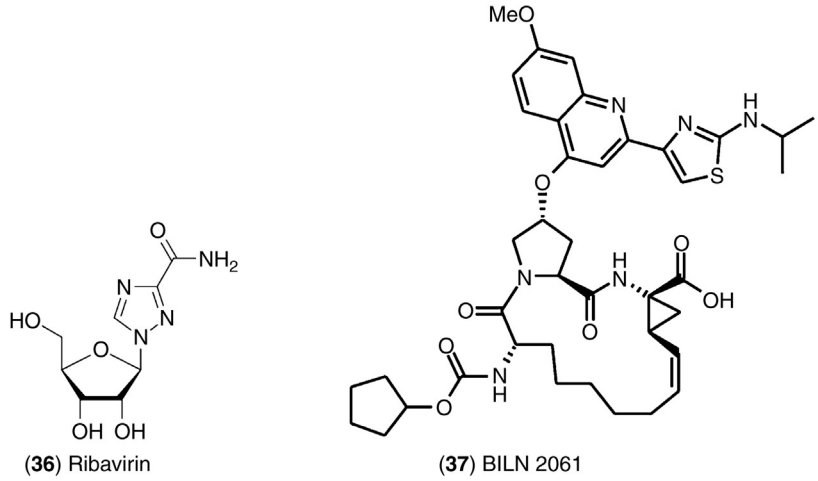
FIGURE 4.15 **Chemical structures of three flavonoids: baicalin, naringenin, and quercetagetin.**

3.4 NSP4 Protease Inhibitors

3.4.1 Nucleoside and Nonnucleoside Inhibitors

In CHIKV NSP4 protease, amino acids Gly315, Asp316, and Asp317 form a catalytic triad and a nearby residue (proximity of 5 Å) acts as the active site. Kumar et al. (2012) identified one core binding site along with two allosteric sites situated in the palm and thumb domains. A docking result showed that nucleoside analogs had the best docking scores in this protease with binding energies in the range of −85.4173 to −78.049 kcal/mol (Kumar et al., 2012). A nucleoside NSP4 inhibitor, ribavirin (**36**, Fig. 4.16), as well as a nonnucleoside inhibitor, BILN 2061 (**37**, Fig. 4.16), could be docked in catalytic, as well as both the allosteric sites, localized in palm, as well as thumb domains, of NSP4, as shown in Fig. 4.17 (Patel et al., 2012). BILN 2061 showed the highest binding affinity with energy of −94.294 kcal/mol for the palm allosteric site.

Some indoles and a few benzimidazole derivatives were also found to better interact with the CHIKV NSP4 palm allosteric site, where the van der Waals interaction was dominant over the hydrogen-bonding interaction. The minimum core for catalytic specific (nucleoside) inhibitors was found to be four hydrogen-bond donors (HBDs), seven hydrogen-bond acceptors (HBAs), and one aromatic ring (R). The minimum core for nonnucleoside inhibitors related to palm allosteric site was found to be one HBD, four HBAs, two Rs, and three



(**36**) Ribavirin (**37**) BILN 2061

FIGURE 4.16 **Structures of a nucleoside inhibitor, ribavirin (36), and a nonnucleoside inhibitor, BILN 2061 (37), of NSP4 protease.**

hydrophobic regions. Ribavirin, a nucleoside analog (inhibitor), interacted with the catalytic site with significant binding energy. Nonnucleoside inhibitors, such as BILN 2061, and diketo acid derivatives, showed better interactions with the palm and thumb allosteric sites, respectively. The selective antiviral activity of favipiravir on the replication of CHIKV, alphaviruses, and favipiravir-resistant CHIKV variants, which all carry a K291R mutation in the RdRp NSP4, were studied by Delang et al. (2014). These authors also studied the effect of T-705 (favipiravir), a nucleotide prodrug that was developed for the treatment of influenza, on NSP4. The characterization of these virus variants in cell culture suggested that a highly conserved part of the viral polymerase of positive-strand RNA viruses is the target of favipiravir. The NSP2 (K49R and E622G) and NSP3 (Opal524W) mutations, which were also found in favipiravir-resistant CHIKV variants, did not result in phenotypic resistance to favipiravir, shown by reverse genetics. The lysine at position 291, which is located in a region of NSP4, possesses a high degree of conservation among alphaviruses. The structure of favipiravir (**38**, Fig. 4.18) bound in the active site of the Norwalk virus (NWV) polymerase structure was found to be superimposed over the cytosine base of CTP. Compared to original Lys291, the charged arginine side chain is much closer to the favipiravir-RTP inhibitor. No such specific interaction or any steric repulsion is observed between K291R and favipiravir-RTP or between Lys291 and favipiravir-RTP. T-705 and its active metabolite T-705 ribofuranosyl triphosphate (**39**, Fig. 4.18) have shown binding affinity for NSP4 protease domain. Metabolic experiment confirmed a direct effect of T-705 on CHIKV RNA synthesis.

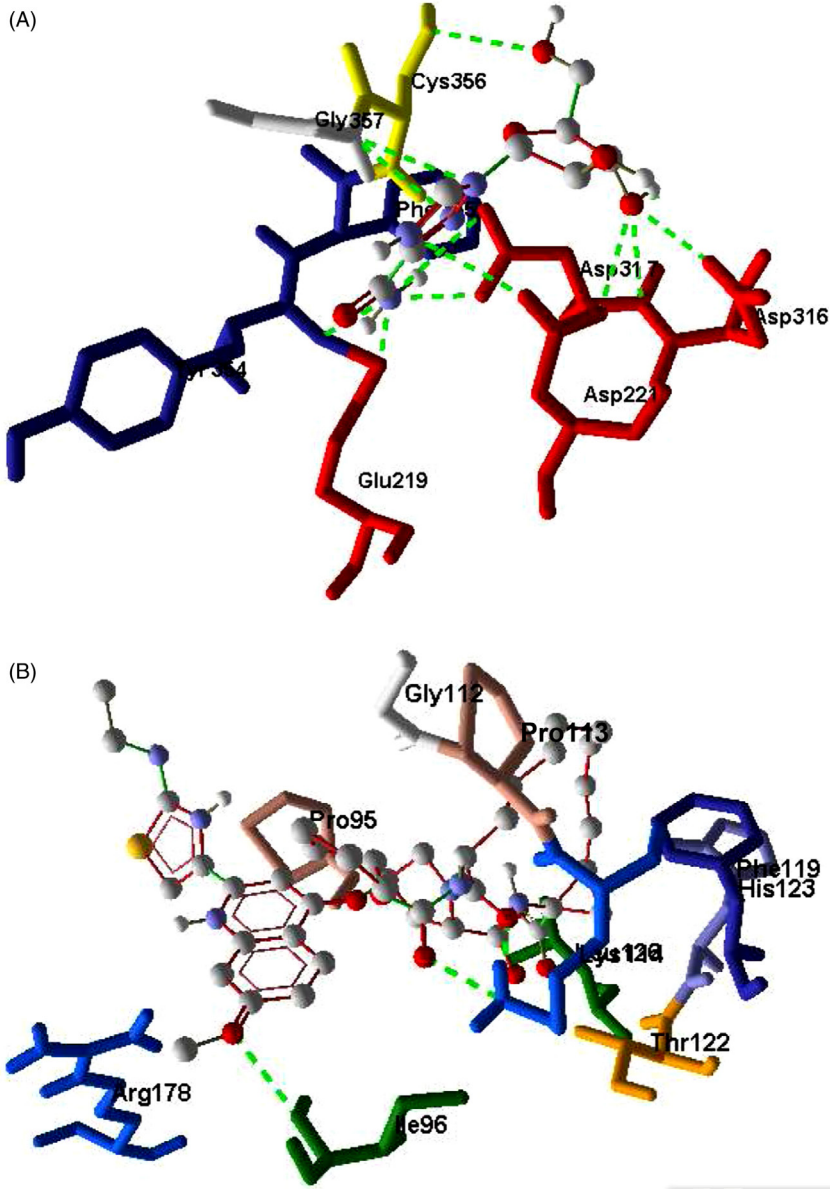
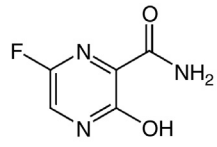
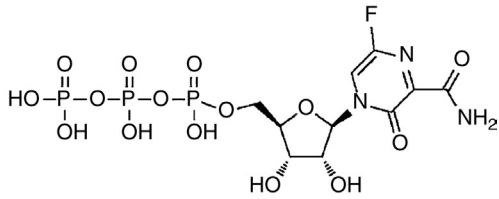


FIGURE 4.17 **Docked poses of ribavirin (A) and BILN2061 (B) interacting with the amino acids present in the catalytic and allosteric sites on the palm and thumb domains.** *(Reprinted from Patel, H., Jasarai, Y.T., Kapoora, R.G., 2012. Exploring the polymerase activity of Chikungunya viral non structural protein 4 (nsP4) using molecular modeling, epharmacophore and docking studies. Int. J. Pharm. Life Sci. 3, 1752–1765.)*



(**38**)



(**39**)

FIGURE 4.18 **Chemical structures of favipiravir or T-705 (38) and its metabolite, T-705 ribofuranosyl triphosphate (39)** .

4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Vaccines and therapeutic strategies are required to combat viruses and manage viral infections in all epidemic situations. The available vaccines and therapeutic strategies to combat viruses are very limited, which include viral polymerase inhibitors, recombinant interferons, and boosting host antiviral system. Recently, protease inhibitors were successfully developed to inhibit and manage hepatitis C and HIV viruses. In the present chapter, it has been shown that NSP2 from CHIKV can be effectively targeted by chemicals, that interact with numerous cellular proteins to control interferon signaling and gene transcription. Various forms of scaffold and chemical entities have already been considered for enhancing their capacity as drug molecules targeting NSPs. The crystallographic structures and functions of viral key proteins provide necessary information to develop potential targeted inhibitors against these viral proteins. These developed protease inhibitors ultimately combat diseases arising from different viruses, such as Mayaro virus, Ross River virus, CHIV, SINV, O'Nyong–Nyong virus, Eastern equine encephalitis virus, Semliki Forest virus, Una virus, Venezuelan equine encephalitis virus, Tonate virus, Western equine encephalitis virus, etc. Although significant improvement has been made in the direction of discovering novel protease inhibitors targeting viral NSP, the lack of clinically approved drug molecules is a limitation for protection against sudden disease occurrence. This chapter is equipped with useful information for developing chemical libraries to design *Alphavirus*-targeting inhibitors, and to construct pharmacophores for ligand-based drug design. This will increase the chances of discovery for small-molecule inhibitors. In conclusion, in near future

NSP inhibitor–based discovery of antiviral drugs need to be considered by both private and public organizations. Hopefully, with the grooming of business-friendly sectors, discovery of effective drugs based on NSP inhibitors would be a reality.

ACKNOWLEDGMENTS

A. Bissoyi expresses his gratefulness to the Department of Science and Technology (DST), Government of India, for the financial support to this work through Grant No. YSS/2015/000618 and to the National Institute of Technology, Raipur, for providing necessary facilities to execute the project. One of the authors (S.K. Pattanayak) would like to thank Prof. S. Chowdhuri and Prof. B.S. Mallik for their kind support and helpful communications.

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