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A Small Molecule Inhibitor of Serine Protease Inhibits the Replication of Flavivirus in Vitro

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Abstract Flavivirus plays a significantly in human disease and mortality. The N-terminal domain of the flaviviral nonstructural NS3 protein codes for the serine, chymotrypsin fold proteinase NS3pro. The cofactor encoded by the upstream gene in the genome nonstructural NS2Bis essential for proteolytic activity of NS3pro. The processing and replication of the flaviviral polyprotein was exhibited by the two-component NS2B-NS3pro, this makes NS2B-NS3pro a promising target for anti-flavivirus drugs because of its essential function in the posttranslational processing of the viral polyprotein precursor. Based on the above hypothesis we identified and synthesized some anti viral compounds. (1'R, 2'S, 6'R)-2-hydroxy 4, 6-dimethoxyisopanduratin A 4 was synthesized in 38 % yield from commercially available products over three steps. Together with its precursor 2, 4, 6-trimethoxyisopanduratin A 3, both of these compounds showed promising competitive inhibitory activities towards dengue 2 virus NS3 protease with the Ki values of 39.68 and 19.84 µM, respectively. The starting materials β-trans-ocimene showed competitive inhibitory activities, but 2', 4', 6'-trimethoxychalcone was observed to be non-competitive.

Keywords: *flavivirus, NS2B/NS3pro, serine protease, small molecule compounds*

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1. Introduction

West Nile virus (WNV) and Dengue virus (DV) belongs to flavivirus were transmitted to humans by the bites of infected female Aedes mosquitoes [1]. WHO, reported that there were more than 890,000 reported cases of Dengue in the Americas in 2007 alone, of which 26,000 cases were Dengue hemorrhagic fever [2]. Anti-flaviviral therapies and vaccines are currently unavailable. Flavivirus a RNA genome virus with 11 Kb positive sense genome after translation has a single C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 polyprotein precursor [3] The nascent polyprotein should be inserted into the endoplasmic reticulum membrane for its expression and processing by the host and viral proteinases [4], this generates into three mature structural proteins (C, prM, and E) and non-mature seven nonstructural (NS) proteins (NS1-NS5) [5]. The flaviviral full length NS3 protein sequence is a multifunctional protein in which the N-terminal *180-residue portion encodes serine proteinase (NS3pro) and the C-terminal *440-residue portion codes for an RNA helicase [6]. The presence of the NS2B cofactor is necessary for NS3pro to exhibit its proteolytic activity [7]. NS3pro is responsible for the cleavage of the capsid protein C, and also at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 boundaries and, in addition, at the junction of NS4A/2K peptide [8,9]. Inactivating mutations of the NS3pro cleavage sites in the poly-protein abolish replication of the virus [10]. Therefore, NS2B-NS3pro is a promising anti-flaviviral drug target [11,12,13]. Dengue hemorrhagic fever (DF) and dengue shock syndrome (DSS) are the deadly diseases caused by the dengue virus, which have no reported vaccine until now [14]. One of the promising approaches to an effective antiviral therapy is the development of small molecule inhibitors directed against the viral NS3 serine protease, which is an essential component for the maturation of the dengue virus polyprotein. Thus the NS3pro is an attractive target for potential chemotherapeutic agents.

2. Materials and Methods

As part of our ongoing effort to search for a lead therapeutic agent for DF/DHF, we synthesized Panduratin A and its regioisomer isopanduratin A are bioactive Diels-Alder adducts isolated from natural products [15]. Despite of its excellent bioactivities, the synthesis and structural-activity relationship of these cyclohexenyl 2', 4', 6'-trimethoxychalcones were never been explored. Here, we report an efficient route to the regioselective synthesis of (1'R, 2'S, 6'R)-2, 4, 6-trimethoxyisopanduratin A. Our strategy employed the Diels-Alder cyclization of 2', 4', 6'-trimethoxychalcone and acyclic monoterpene ocimene. In this communication, we described the inhibitory activities of these compounds towards the DEN-2 virus NS3 protease.

The synthesis of Methoxy isopanduratin A is illustrated in Scheme 1. Aldol condensation of the commercially available 2', 4', 6'-trimethoxyacetophenone and benzaldehyde

in the presence of aqueous KOH in ethanol gave the desired 2', 4', 6'-trimethoxychalcone 1 in 86 % yields. The 2', 4', 6'-trimethoxychalcone was then engaged in Diels–Alder reactions with β-trans-ocimene 2 [16]. Reacting 2', 4', 6'-trimethoxychalcone 1 with five equivalent excess of β-trans-ocimene 2 at 170°C led to the sole regioselective formation of (1'R, 2'S, 6'R)-2, 4, 6-trimethoxyisopanduratin A 3 in 82 % yield. Attempt to improve the reaction conditions by using Lewis acid catalysts such as BF3-Et2O, AlCl3, and ZnCl2 resulted in extensive polymerization of the ocimene with no significant adduct formation. Demethoxylation of the adduct 3 by using boron tribromide gave 2-hydroxy-4, 6-dimethoxyisopanduratin A 4 in 54 % yield. Demethoxylating in longer period, however, resulted in a complete decomposition of the substrates . Reagents and conditions: (i) benzaldehyde, EtOH, 50 % KOH aq, 24 h, 86 % (ii) ocimene, 170°C, pressure tube, 48h, 82 % (iii) BBr3, CH2Cl2, 0˚C, 1 h, 54 %.

The construct DEN-2 NS2B/NS3Pro was then cloned in the pET expression vector (Invitrogen) and verified by DNA sequencing and transformed into Escherichia coli BL21 CodonPlus (DE3) cells (Stratagene). The transformed cells were grown in 2 liters of LB broth at 37°C to reach $A600_{OD}$ 0.5 and the protein expression was induced at 18°C using 0.8 mM isopropyl-D-thiogalactoside for an additional 16 hours. The cells were then collected by centrifugation, resuspended in 40 ml of Buffer A (50 mM Tris-HCl, pH 7.8, 200 mM NaCl) supplemented with the Complete Proteinase Inhibitors mixture (Roche Applied Sciences) and 1 mg/ml lysozyme (Sigma) and snap frozen at □-80 °C. After thawing, the cells were sonicated (6 x 30 s with a 60 s interval). The cell debris was removed by centrifugation (19,000 x g; 30 min). The supernatant fraction was filtered using a 0.45-m filter (Whatman). The NS2B-NS3pro construct was each purified from the supernatant fraction using HiTrap Co2-chelating chromatography (GE Healthcare). The Hisx6-tagged constructs were eluted with a 15–500 mM gradient of imidazole concentrations. After that the, the isolated protein were additionally purified by FPLC on a Mono Q 5/50 column (GE Healthcare) equilibrated in 20 mM Tris-HCl buffer, pH 7.8, containing 100 mM NaCl and 1 mM dithiothreitol. The construct was eluted using a linear gradient of 0.1–1 M NaCl. The fractions was analyzed using SDS-PAGE followed by Coomassie staining.

The standard 200 µL reaction mixtures comprised 100 µM fluorogenic peptide substrate Boc-Gly-Arg-Arg-MCA (S), 1 µM dengue 2 protease complexes and with or without compounds of varying concentrations buffered at pH 8.5 by 200 mM Tris–HCl. The screening was done at a range of inhibitor concentrations between 100 and 500 ppm indicated a significant inhibition of the NS3 protease. Each test was done in triplicate.

The complete cleavage of the refolded precursor protein was verified by SDS-PAGE gel electrophoresis and kinetic parameters for reaction of the consist of the enzyme under the conditions of our assay, the Km value for this substrate was estimated to be 63 µM, kcat to be 0.026×10^{-2} S^{-1} and the catalytic efficiency was expressed as kcat/Km to 167 M^{-1} S^{-1}. All solutions for bioassay were prepared in methanol prior tests showed the absence of methanolic inhibition even at 30 % v/v. Enzyme inhibition by compounds was analyzed by titration experiments that were performed in the presence of increasing amounts of the inhibitor. Recombinant NS2B/NS3pro (1 µM) was pre-incubated for 30 min at 37°C with the test compounds at 100, 200, 300, 400 and 500 ppm concentration and the reaction was started by addition of the (S) substrate.

3. Results and Discussion

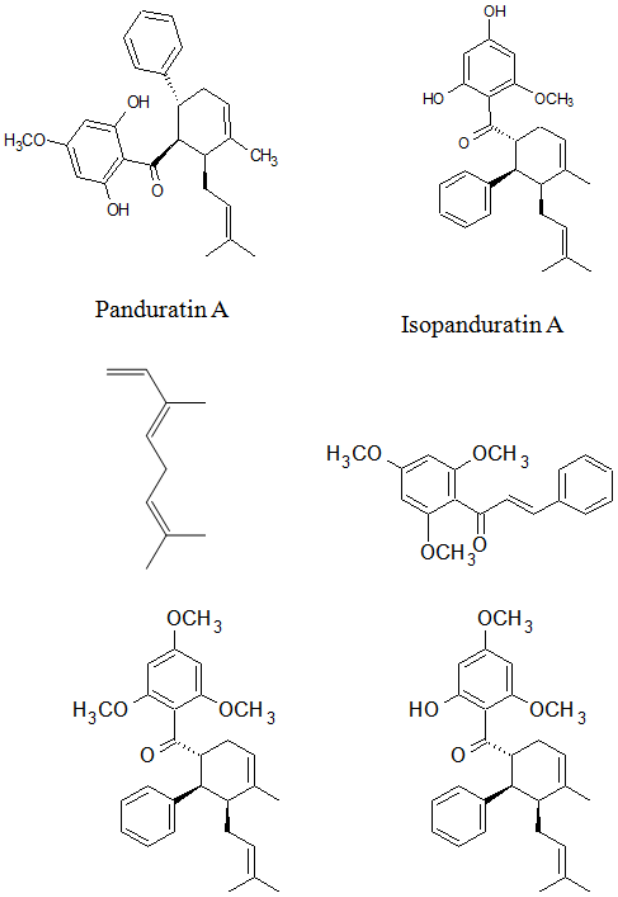

Figure 1. The structures of the Panduratin A derivatives

Both the 2-hydroxy, 4, 6-dimethoxyisopanduratin A 4 and its precursor 2, 4, 6-trimethoxyisopanduratin A 3 had a marked effect on enzyme activity in assays with the synthetic substrate (S) comparatively, whereas 2', 4', 6'-trimethoxychalcone and β-transocimene had less effect on the enzyme. To obtain better estimates for Ki values, the enzyme was incubated in the presence of inhibitor with the above mentioned concentration and substrate concentration was varied between 50 and 150 µM. Experimental data were analyzed by using double reciprocal plots and the observed velocities for substrate conversion in the presence of product peptides were compatible with competitive inhibitor modality. Table 1 shows the percentage of inhibition of the compounds tested at different concentrations. The results indicated an increase in inhibitory activities with increasing concentration of compounds tested. Compounds 3 and 4 showed an average of 95% inhibition at the higher dose of compounds. Compound 3 showed a 27% of inhibition even at the lower dose when compared to compound 4. Figure 2 shows Lineweaver–Burk plots for the inhibition of the NS3 protease by the compound 2-hydroxy, 4, 6-dimethoxyisopanduratin A 4. Similar plots were obtained

for all other compound 2, 4, 6-trimethoxyisopanduratin A 3, β-trans-ocimene 2 and 2', 4', 6'-trimethoxychalcone 1. Consistent with a competitive mode of enzyme inhibition recorded an increase in apparent Km values in the presence of increasing inhibitor concentrations, whereas kcat values remained unaffected, except for 2', 4', 6'-trimethoxychalcone 1 which showed the non-competitive inhibition. The Ki for all compounds tested were calculated from the Lineweaver–Burk plot. A graph of reciprocal of apparent maximum velocity to inhibitor concentration was plotted and the Ki value was calculated from the gradient (1/ Vmax Ki). The Ki value for 4 was determined to be 39.68 µM showed in Figure 4. Ki values for the examined compounds were represented in Table 1. Of all the compounds tested, the 3 had the lowest Ki value 19.84 µM.

Table 1. Ki values for the Compounds

Compounds	Ki Value (µM)	Inhibition Mechanism
2	31.74	Competitive
3	19.84	Competitive
4	39.68	Competitive
1	55.54	Non Competitive

Table 2. Percentage inhibition of dengue 2 NS2B/3 virus protease cleavage of substrate (S) by 3 and 4 ± standard error

Compounds	Percentage inhibition of dengue 2 virus protease NS3 concentration used in ppm				
	100	200	300	400	500
3	27.1 ± 4.8	66.7 ± 0.1	87.7 ± 0.6	93.7 ± 0.5	99.8 ± 1.1
4	22.1 ± 4.9	63.7 ± 0.2	83.7 ± 0.7	91.7 ± 0.6	92.8 ± 1.2

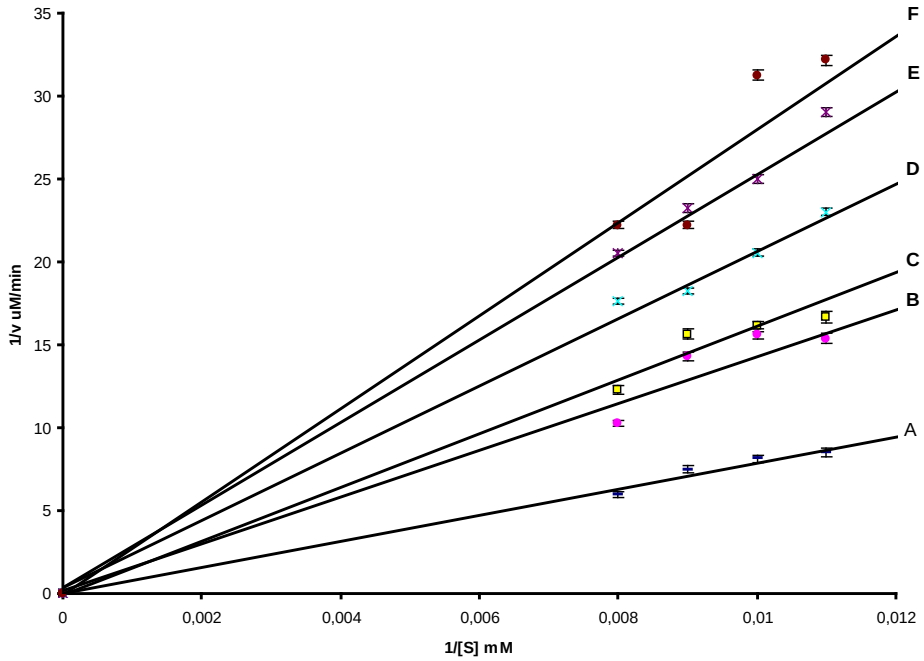

Figure 2. Lineweaver–Burk plot of inhibitor 2, 4, 6-trimethoxyisopanduratin A **3**. Plots: A, absence of inhibitor; B, I = 100 ppm; C, I = 200 ppm; D, I = 300 ppm; E, I = 400 ppm; F, I = 500 ppm

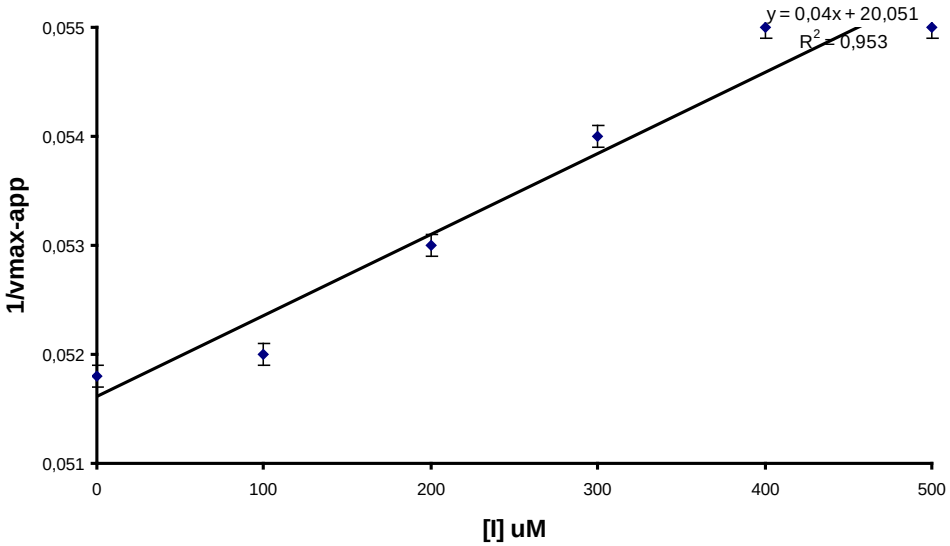

Figure 3. Plot of reciprocal of apparent maximum velocity, 1/Vmax-app, Vmax-app in mM/min, to inhibitor compound 2, 4, 6-trimethoxyisopanduratin A **3** concentration, I µM

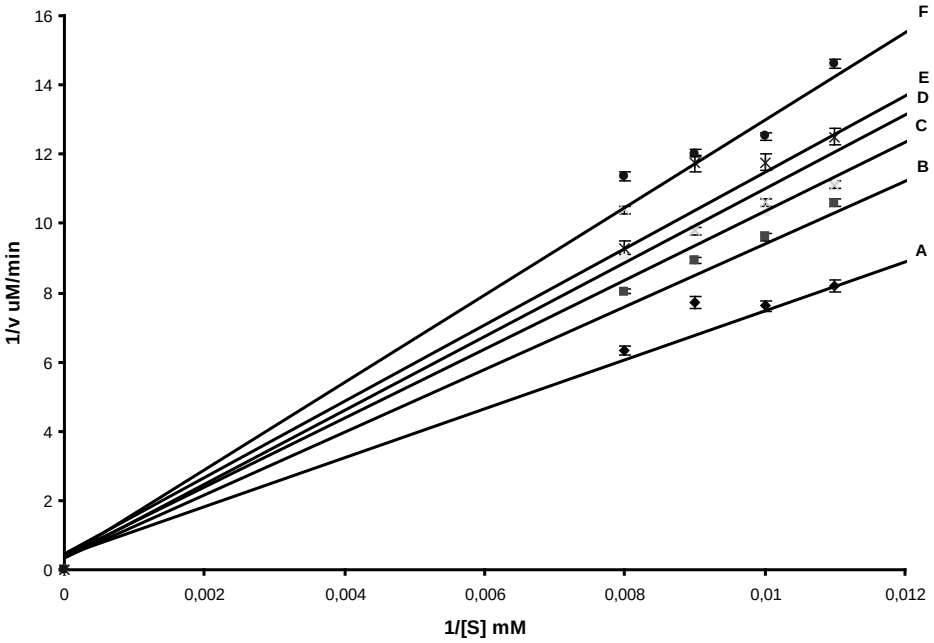

Figure 4. Lineweaver–Burk plot of inhibitor compound (1'*R*, 2'*S*, 6'*R*)-2-hydroxy 4, 6-dimethoxyisopanduratin A **4**. Plots: A, absence of inhibitor; B, I = 100 ppm; C, I = 200 ppm; D, I = 300 ppm; E, I = 400 ppm; F, I = 500 ppm

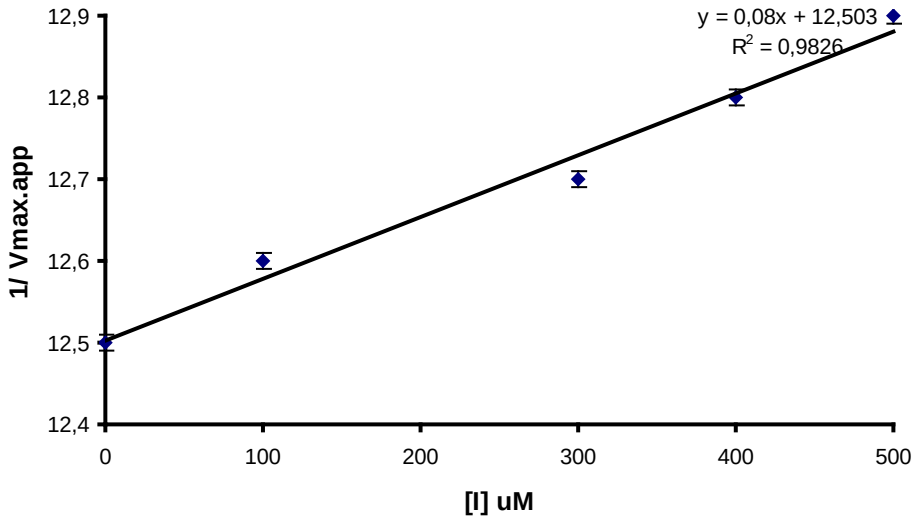

Figure 5. Plot of reciprocal of apparent maximum velocity, 1/vmax-app Vmax-app in mM/min, to inhibitor (1'*R*, 2'*S*, 6'*R*)-2-hydroxy 4, 6-dimethoxyisopanduratin A **4** concentration, I μM

The serine proteases present in the flavivirus are the best targets for the development of antiviral agents because of their pathological properties, which provide the virus for its replication and Infection. Recent studies with regard to human immunodeficiency virus type 1 and the hepatitis C virus showed that the anti viral small-molecules directed against the proteases of these viruses recorded effectively against these virus in the clinical trail report [17,18,19]. Such compounds target protease active sites and effectively inhibit viral replication. Therefore, in the case of DENV infection, the most attractive target on the DENV protease is the protease catalytic site of NS3 [2]. Difficulties in designing the inhibitors against DENV NS2B/NS3 protease complex because of its catalytic site of DENV NS2B/NS3 protease complex is flat [2]. Other sites forming the pocket can be an alternative strategy to designing the small molecule inhibitor because small potent inhibitors can stably bind. Recent studies regarding the action of the NS2B/NS3 protease complex provide a potential alternative strategy for the development of DENV inhibitors. Monoclonal antibody against NS2B blocks the activity of the protease by interfering with the interaction between NS2B and NS3pro [20]. Many studies reported that both the integrity and the productive conformation of the NS2B cofactor are critical for the activity of the NS3pro [1,7,21,22]. It is reported that NS2B-NS3pro normally exists in open and closed conformations. Open, the active conformation, the NS2B cofactor interacts with the active site of the enzyme, but the closed, inactive conformation, the NS2B cofactor remains bound to the NS3pro domain, but it is shifted away from the proteinase active site [23,24]. Taken together, [27,28,29] the work has demonstrated that disrupting the interactions of the NS2B cofactor with the NS3 protease is the promising strategy for rational structure-based inhibitor development. So these hypothesis considered to determine the NS2B-NS3pro is a valid therapeutic target for anti-DENV drugs [10,25,26].

In conclusion, we report a three step entry into a so far little known family of products, the (1'R, 2'S, 6'R)-2-hydroxy 4, 6-dimethoxyisopanduratin A with 38 % overall yield. The small Ki values of the competitive inhibitors for these compounds warrant further investigation as in vitro inhibitors for the DEN-2 virus NS3 protease. So these compounds can be potential compounds against the dengue virus after a animal study.

Conflict of Interests

No potential conflict of interests was disclosed

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References

[1] 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 May;16(5):795-806.

[2] Erbel P, Schiering N, D'Arcy A, Renatus M, Kroemer M, Lim SP, et al. Structural basis for the activation of flaviviral NS3 proteases from dengue and West Nile virus. Nat Struct Mol Biol. 2006 Apr;13(4):372-3.

[3] Chambers TJ, Droll DA, Tang Y, Liang Y, Ganesh VK, Murthy KH, et al. Yellow fever virus NS2B-NS3 protease: characterization of charged-to-alanine mutant and revertant viruses and analysis of polyprotein-cleavage activities. J Gen Virol. 2005 May;86(Pt 5):1403-13.

[4] Wu H, Bock S, Snitko M, Berger T, Weidner T, Holloway S, et al. Novel dengue virus NS2B/NS3 protease inhibitors. Antimicrob Agents Chemother. 2015 Feb;59(2):1100-9.

[5] Zhu X, He Z, Yuan J, Wen W, Huang X, Hu Y, et al. IFITM3-containing exosome as a novel mediator for anti-viral response in dengue virus infection. Cell Microbiol. 2015 Jan;17(1):105-18.

[6] Lescar J, Luo D, Xu T, Sampath A, Lim SP, Canard B, et al. Towards the design of antiviral inhibitors against flaviviruses: the case for the multifunctional NS3 protein from Dengue virus as a target. Antiviral Res. 2008 Nov;80(2):94-101.

[7] Johnston PA, Phillips J, Shun TY, Shinde S, Lazo JS, Huryn DM, et al. HTS identifies novel and specific uncompetitive inhibitors of the two-component NS2B-NS3 proteinase of West Nile virus. Assay Drug Dev Technol. 2007 Dec;5(6):737-50.

[8] Monath TP. Dengue: the risk to developed and developing countries. Proc Natl Acad Sci U S A. 1994 Mar 29;91(7): 2395-400.

[9] Gubler DJ, Clark GG. Dengue/dengue hemorrhagic fever: the emergence of a global health problem. Emerg Infect Dis. 1995 Apr-Jun;1(2):55-7.

[10] Arias CF, Preugschat F, Strauss JH. Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. Virology. 1993 Apr;193(2):888-99.

[11] Lobigs M. Flavivirus premembrane protein cleavage and spike heterodimer secretion require the function of the viral proteinase NS3. Proc Natl Acad Sci U S A. 1993 Jul 1;90(13):6218-22.

[12] Chambers TJ, Hahn CS, Galler R, Rice CM. Flavivirus genome organization, expression, and replication. Annu Rev Microbiol. 1990;44:649-88.

[13] Gorbalenya AE, Koonin EV, Donchenko AP, Blinov VM. Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. Nucleic Acids Res. 1989 Jun 26;17(12):4713-30.

[14] Yusof R, Clum S, Wetzel M, Murthy HM, Padmanabhan R. Purified NS2B/NS3 serine protease of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids in vitro. J Biol Chem. 2000 Apr 7;275(14):9963-9.

[15] Cheenpracha S, Karalai C, Ponglimanont C, Subhadhirasakul S, Tewtrakul S. Anti-HIV-1 protease activity of compounds from Boesenbergia pandurata. Bioorg Med Chem. 2006 Mar 15;14(6):1710-4.

[16] Ficht TA, Chang LJ, Stoltzfus CM. Avian sarcoma virus gag and env gene structural protein precursors contain a common amino-terminal sequence. Proc Natl Acad Sci U S A. 1984 Jan; 81(2): 362-6.

[17] Asselah T, Marcellin P. New direct-acting antivirals' combination for the treatment of chronic hepatitis C. Liver Int. 2011 Jan;31 Suppl 1:68-77.

[18] Naggie S, Patel K, McHutchison J. Hepatitis C virus directly acting antivirals: current developments with NS3/4A HCV serine protease inhibitors. J Antimicrob Chemother. 2010 Oct; 65(10): 2063-9.

[19] Wensing AM, van Maarseveen NM, Nijhuis M. Fifteen years of HIV Protease Inhibitors: raising the barrier to resistance. Antiviral Res. 2010 Jan;85(1):59-74.

[20] 20. Phong WY, Moreland NJ, Lim SP, Wen D, Paradkar PN, Vasudevan SG. Dengue protease activity: the structural integrity and interaction of NS2B with NS3 protease and its potential as a drug target. Biosci Rep. 2011 Oct;31(5):399-409.

[21] Sidique S, Shiryaev SA, Ratnikov BI, Herath A, Su Y, Strongin AY, et al. 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 Oct 1;19(19):5773-7.

[22] Shiryaev SA, Ratnikov BI, Chekanov AV, Sikora S, Rozanov DV, Godzik A, et al. Cleavage targets and the D-arginine-based inhibitors of the West Nile virus NS3 processing proteinase. Biochem J. 2006 Jan 15;393(Pt 2):503-11.

[23] Su XC, Ozawa K, Qi R, Vasudevan SG, Lim SP, Otting G. NMR analysis of the dynamic exchange of the NS2B cofactor between open and closed conformations of the West Nile virus NS2B-NS3 protease. PLoS Negl Trop Dis. 2009;3(12):e561.

[24] Tomlinson SM, Watowich SJ. Substrate inhibition kinetic model for West Nile virus NS2B-NS3 protease. Biochemistry. 2008 Nov 11;47(45):11763-70.

[25] Uchil PD, Kumar AV, Satchidanandam V. Nuclear localization of flavivirus RNA synthesis in infected cells. J Virol. 2006 Jun;80(11):5451-64.

[26] Satchidanandam V, Uchil PD, Kumar P. Organization of flaviviral replicase proteins in virus-induced membranes: a role for NS1' in Japanese encephalitis virus RNA synthesis. Novartis Found Symp. 2006;277:136-45; discussion 45-8, 251-3.

[27] Kummerer BM, Rice CM. Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. J Virol. 2002 May;76(10):4773-84.

[28] Cahour A, Falgout B, Lai CJ. Cleavage of the dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junctions is mediated by viral protease NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. J Virol. 1992 Mar;66(3):1535-42.

[29] 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 Nov;65(11):6042-50.