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ANTIVIRAL STUDY OF SCHIFF BASE VANILLIN DERIVATIVES AGAINST NS2B-NS3 PROTEASE OF ZIKA VIRUS BASED ON PHARMACOPHORE MODELLING AND MOLECULAR DOCKING

(Kajian Antiviral Terbitan Vanillin Bes-Schiff Terhadap NS2B-NS3 Protease Virus Zika Berdasarkan Pemodelan Farmakofor dan Dok Molekul)

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Abstract

The Zika virus (ZIKV) is a mosquito-borne virus spread by the bite of *Aedes aegypti* and *Aedes albopictus* mosquitoes. The outbreak of the virus resulted in the 2015-2016 ZIKV epidemic, in which later Public Health Emergency of International Concern was declared by the World Health Organization (WHO). Despite the complications following the infection of ZIKV, clinically approved therapeutic agents and vaccines are still unavailable for the treatment of ZIKV. Schiff base vanillin derivatives, derived from vanillin and primary amines, were reported for their potential antiviral activity against a several viruses, including influenza virus and SARS coronaviruses. Therefore, they were aimed to be tested for their *in silico* antiviral activity against ZIKV NS2B-NS3 protease. In this research, ligand-based pharmacophore modelling was employed to analyse the antiviral activity of Schiff base vanillin derivatives. They were imported as test sets in the pharmacophore model generated from a list of training sets, which are reported drugs against ZIKV. Furthermore, structure-based molecular docking was also performed to analyse the docking performances of the Schiff base vanillin derivatives in the crystal structure of ZIKV NS2B-NS3 protease in a complex with a boronate inhibitor (PDB: 5LC0). The analyses were based on pharmacophore scores, binding affinities and matching interactions in comparison with the 5LC0 ligand in the active site. Based on the findings *via* ligand-based pharmacophore modelling and structure-based molecular docking, it was discovered that a number of Schiff base vanillin derivatives showed potential antiviral activity against ZIKV, thus being promising drug candidates and bringing futuristic *in vitro* and *in vivo* tests.

Keywords: Zika virus, Schiff base vanillin derivatives, pharmacophore modelling, molecular docking, computer-aided drug design

Abstrak

Virus Zika (ZIKV) adalah virus bawaan nyamuk yang disebarkan melalui gigitan nyamuk *Aedes aegypti* dan *Aedes albopictus*. Wabak virus ini menyebabkan epidemik ZIKV 2015-2016, di mana kemudian Darurat Kesihatan Awam Keprihatinan Antarabangsa telah diisytiharkan oleh Pertubuhan Kesihatan Sedunia (WHO). Walaupun komplikasi setelah jangkitan ZIKV, agen terapeutik dan vaksin yang diluluskan secara klinikal masih belum tersedia untuk rawatan ZIKV. Terbitan vanillin bes-Schiff, yang diperolehi daripada vanilin dan amina primer, telah dilaporkan aktiviti antiviral berpotensi terhadap beberapa virus, termasuk virus influenza dan koronavirus SARS. Oleh itu, mereka akan diuji untuk aktiviti antiviral secara *in silico* terhadap protease NS2B-NS3

ZIKV. Dalam penyelidikan ini, pemodelan farmakofor berasaskan ligan digunakan untuk menganalisis aktiviti antiviral terbitan vanillin bes-Schiff. Mereka diimport sebagai set ujian terhadap model farmakofor yang dihasilkan daripada senarai set latihan, iaitu ubat-ubatan dilaporkan terhadap ZIKV. Selain itu, dok molekul berasaskan struktur juga dilakukan untuk menganalisis prestasi dok terbitan vanillin bes-Schiff di dalam struktur kristal ZIKV NS2B-NS3 protease dalam kompleks dengan perencat boronat (PDB: 5LC0). Analisis dijalankan berdasarkan skor farmakofor, afiniti dok, dan interaksi yang sepadan dengan ligan 5LC0 di tapak aktif. Berdasarkan hasil pemodelan farmakofor berasaskan ligan dan dok molekul berasaskan struktur, adalah didapati bahawa beberapa terbitan vanillin bes-Schiff menunjukkan aktiviti antiviral yang berpotensi terhadap ZIKV, oleh itu menjadi calon ubat yang menjanjikan dan mampu membawa ujian *in vitro* dan *in vivo* pada masa depan.

Kata kunci: Virus Zika, terbitan vanillin bes-Schiff, pemodelan farmakofor, dok molekul, reka bentuk ubat bantuan komputer

Introduction

The Zika virus (ZIKV) is a mosquito-borne viral disease spread by the bite of *Aedes aegypti* and *Aedes albopictus* mosquitoes, which belongs to the *Flaviviridae* virus family (Figure 1). The virus belongs to the Flavivirus genus, which is the same genus as dengue, yellow fever, Japanese encephalitis, and West Nile viruses [1]. ZIKV was first discovered in 1947 in the Ziika forest of Uganda, which was identified in a Rhesus macaque monkey [2]. Infections among humans have since been detected in Africa and Asia from the 60s to 80s, which then relentlessly spread over the Western hemisphere in the past decade [2]. Major outbreaks were recorded in 2007 in Africa, which was in the Yap Islands, Micronesia [3], and later in 2013 in French Polynesia [4]. In 2015 in Brazil, ZIKV infection was detected in association with microcephaly, a condition where the size of head is smaller than the normal size [2]. Consequently, the World Health Organization (WHO) declared a Public Health Emergency of International Concern (PHEIC) from February to November 2016 [2].

ZIKV is highly infectious among pregnant women, not only it can lead to microcephaly among infants, but as well as congenital malformations, such as limb contractures, high muscle tone, eye abnormalities and hearing loss, also known as congenital Zika syndrome [2]. Other than that, other complications such as preterm birth and miscarriage might follow as well [5]. On top of that, Zika infections may also increase the risk of developing a rare autoimmune disorder, known as the Guillain–Barré syndrome [5]. The virus is not only transmitted by the bite of *Aedes aegypti* and *Aedes albopictus* mosquitoes, as well as mother-to-fetus virus transmission, sexual transmission, transfusion of blood and organ transplantation [2]. Despite numerous complications, licensed and approved drugs and vaccines are still unavailable from the treatment of Zika infections [2], which resulted in great deal of active research in different fields in search of drugs and vaccines for the medication of Zika infections.

ZIKV is a small enveloped, positive-sense single-stranded RNA virus [6]. The opening frame (ORF) encodes one polyprotein made up of 3419 amino acids, which is cleaved by the host of viral proteins into 3 structural proteins and 7 non-structural proteins [7]. The structural proteins consist of activating capsid, membrane, and envelope, while the non-structural proteins consist of NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 proteins [8] (Figure 2). Among all non-structural proteins, NS2B-NS3 protease serves a very crucial role in replication of virus and maturation of non-structural viral proteins, hence activating the virus [7]. NS3 protease belongs to the trypsin/chymotrypsin protease superfamily, whereby the protease domain is found at the N-terminus, and the RNA helicase domain is found at the C-terminus [9]. The NS3 helicase, which plays an imperative role in the replication and capping of RNA, is encoded by the NS3 C-terminal 440 residue region [10]. However, in order for the activation of NS3 protease, NS2B protease must be present in complex with NS3 protease [11]. Thus, the transmembrane NS2B protease combines with the N-terminal 170-residues of NS3 to form NS2B-NS3 protease, which has excellent proteolytic activity, making it an attractive and promising target for the design and development of drugs to inhibit the viral activity of NS2B-NS3 protease, which in turn to search for the medication of Zika infection [12].

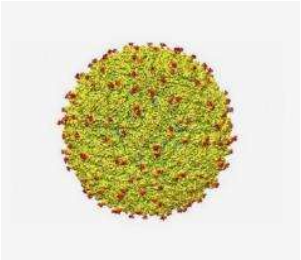


Figure 1. Representation of the surface of ZIKV [13]

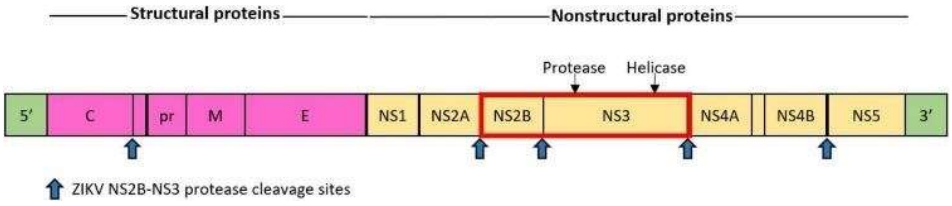


Figure 2. Representation of ZIKV genome [12]

Heretofore, clinically approved therapeutic agents and vaccines are still unavailable not only for Zika infection, but for most of the viral infections. Affected patient from ZIKV are only prescribed pain and fever reliever such as paracetamol and acetaminophen, and recommended staying hydrated, as well as getting enough rest. However, the complications as elaborated above might still follow, which describes the severity of the condition and the necessity to search for the clinically approved and licensed drugs and medications. Nevertheless, the design and discovery of drugs, medicines or novel compounds as inhibitors of viral proteases has always been a very complicated and challenging field among researchers. Not only being expensive, laborious and time-intensive, it was reported that in the past, but the process of drug discovery was also done based on serendipitous discovery from the isolation of crude extracts from different natural products, such as *Aphloia theiformis*, an edible endemic plant from Indian Ocean islands [14]. On top of that, these crude extracts and chemicals were only tested for their biological activity but not based on the biological target [15]. Fortunately, following the advance of modern technology, computer-aided drug design (CADD) allows the process of drugs discovery to be more efficient and time-saving at the cost of minimum expense. On top of that, in combination with laboratory processes, the mechanism of drug against the biological target, and the design of novel drugs or inhibitors based on the active sites of biological target can be illustrated well. Therefore, a great deal of research and efforts have been performed *via* CADD which resulted in the disclosure of numerous compounds possessing great potential as inhibitors of NS2B-NS3 protease of ZIKV (Figures 3-5).

Myriad studies shed light on the analysis of protein-ligand interaction. 2 drugs approved by Food and Drug Administration (FDA), which are asunaprevir and simeprevir, were discovered as potential NS2B-NS3 inhibitors *via* structure-based molecular docking of CADD, therefore possess potent anti-ZIKV activity [16]. Similarly, Meewan et al. reported their work in analysing 7 million compounds against the NS2B-NS3 protease through virtual screening, whereby out of all, 6 of the compounds which contain phenylquinoline and aminobenzamide groups showed positive docking result in the active site of the protease, therefore exhibiting potential antiviral activity to suppress the viral replication [12]. Furthermore, in another reported research of virtual screening of 1861 FDA approved drugs in Drug Bank against the crystal structure of NS2B-NS3 protease, antihistaminic chlorcyclizine displayed the most important interaction with the active site, thus suggesting itself for being a promising drug against ZIKV [17]. Moreover, a few clinically approved drugs showed great binding affinity in the active site of ZIKV, which includes sofosbuvir, hydroxychloroquine, azithromycin and novobiocin [9].

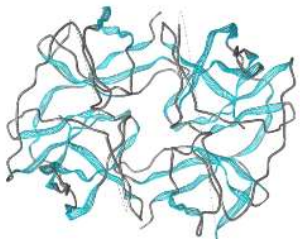


Figure 3. Crystal structure of ZIKV NS2B-NS3 Protease Shown in Structure-Based Perspective of LigandScout 4.4 [18]

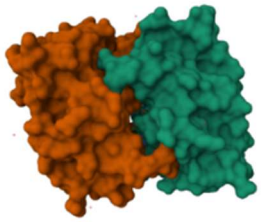


Figure 4. Crystal structure of ZIKV NS2B-NS3 Protease in Complex with Boronate Inhibitor [18]

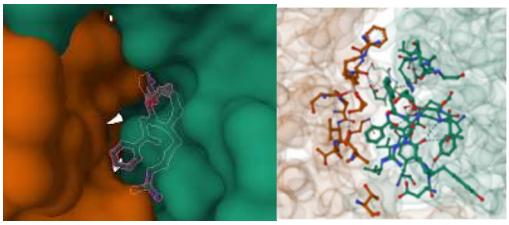


Figure 5. The boronate inhibitor in the active site of 5LC0 [18]

Among all the significant efforts of researchers, one of the class of compounds which potentially spike an interest in the Schiff base vanillin derivatives. Schiff base vanillin derivatives, which are synthesised from the Schiff base reaction between vanillin and primary amines, were reported for their potential antiviral activity against distinct types of viruses. In an earlier *in silico* research, the vanillin derivatives were reported to be able to inhibit the neuraminidase protease of influenza virus, which was later proven again by the performance of 2′-(4-Methylumbelliferyl)-*α*-*D*-*N*-acetylneuraminic acid (MUNANA) assay [19]. Similarly, the vanillin derivatives were also reported to show inhibitory activity against the main protease of severe acute respiratory syndrome coronavirus-2 (SARS-CoV 2) by the performance of both ligand-based pharmacophore modelling and structure-based molecular docking [20]. For having great interest and significant potential as antiviral compounds, but being limited in terms of the amount of research simultaneously, the present study focuses on the analysis and development of Schiff base vanillin derivatives as ZIKV NS2B-NS3 inhibitors *via in silico* methods, which are structure-based molecular docking and ligand-based pharmacophore modelling by using LigandScout 4.4.

Materials and Methods

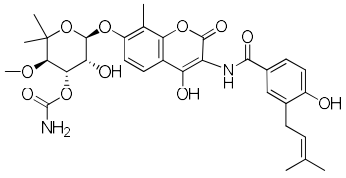
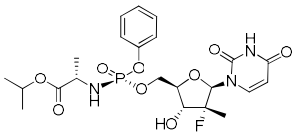
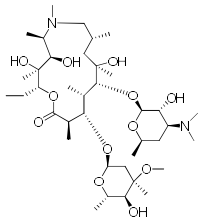
Ligand-based pharmacophore modelling was performed by using LigandScout 4.4 software. Chemical structures of training sets were downloaded from ChemSpider in the MDL-mol format, while all the chemical structures of Schiff base vanillin derivatives were drawn by using ChemDraw Ultra 12.0 and downloaded in MDL-mol format as well. Structure-based molecular docking was performed by both LigandScout 4.4 and Autodock Vina. All Schiff base vanillin derivatives were converted from the MDL-mol format to the MDL-sdf format by using

Open Babel GUI.

Ligand-based pharmacophore modelling

3 drugs which were reported in previous research for their inhibitory activity against ZIKV NS2B-NS3 protease were selected as training sets. The 3 training sets novobiocin [21], sofosbuvir [22] and azithromycin [23] were selected due to the depiction of a lot of common pharmacophore features by their molecular structures, hence being able to generate a model with as many pharmacophore features as possible. Their chemical structures are shown in Table 1 below:

Table 1. 2D and 3D chemical structures of training sets: novobiocin [21], sofosbuvir [22] and azithromycin [23]

Training Sets	2D Chemical Structure	3D Chemical Structure
Novobiocin		
Sofosbuvir		
Azithromycin		

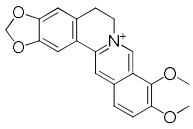
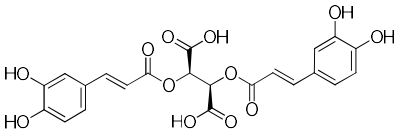
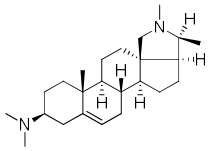
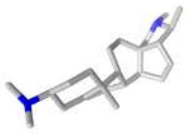
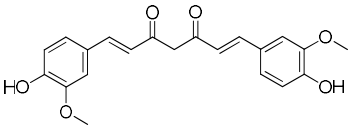
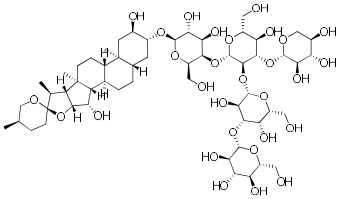
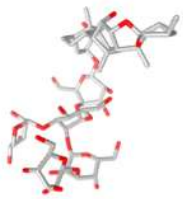
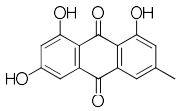
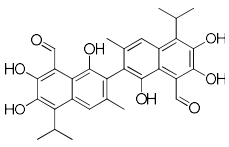
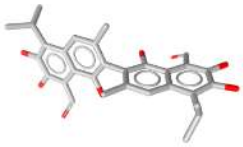
The chemical structures of the training sets were downloaded from ChemSpider in the MDF-mol format and imported into the ligand-based perspective of LigandScout 4.4 to generate the pharmacophore model based on common feature hypotheses. Test sets played imperative role in validating the reliability of the generated pharmacophore model. Hence, chemical compounds from natural products that were reported in previous literatures to possess inhibitory activity against NS2B-NS3 protease were selected as test sets to evaluate and validate the reliability of the pharmacophore model. A total of 12 chemical compounds from natural products include berberine [24], chicoric acid [25], conessine [26], curcumin [26], digitonin [26], emodin [24], gossypol [26], luteone [25], pedalitin [27], quercetin [27], reserpine [25] and rosmarinic acid [25]. These chemical compounds were downloaded from ChemSpider and imported as test sets in the ligand-based perspective of LigandScout 4.4 in the MDF-mol format (Table 2).

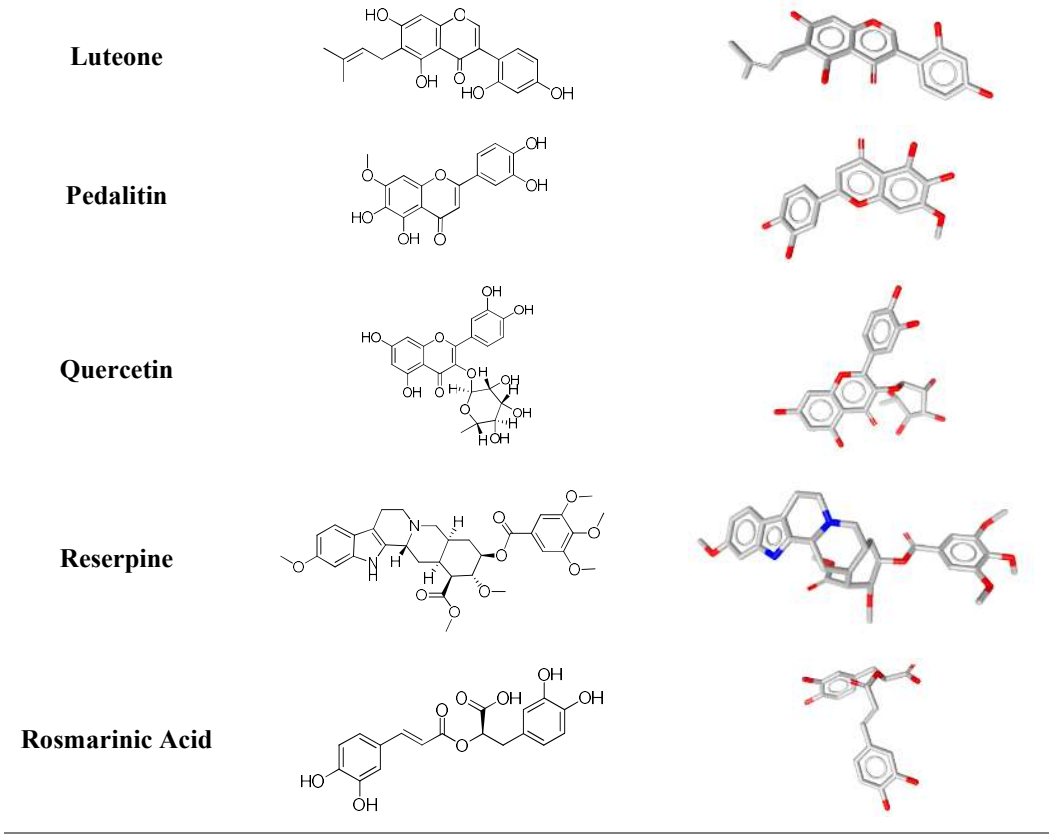
After the import of training sets and test sets, all the MMFF94 energies of the chemical structures were first minimised to ensure closest local minimum and maximum stability. Conformations of ligand sets were generated, clustered based on pharmacophore RDF-code similarity and average cluster distance calculation, and ligand-based pharmacophore modelling was performed based on pharmacophore fit. The 3 training sets generated a pharmacophore model, in which evaluations and analyses were based on the test sets' matching pharmacophore features and pharmacophore fit values in the generated pharmacophore model. The selection of test sets was based on compounds from natural products with wide difference of chemical structures and functional groups. The greater the difference in the chemical structures of the test sets, the higher the accuracy of these test sets to validate the

reliability of the pharmacophore model. If test sets were able to show pharmacophore fit values similar to or greater than the values of the training sets, as well as depicting as many matching pharmacophore features as possible, the pharmacophore model is said to be reliable for the assessment of the Schiff base vanillin derivatives as ZIKV NS2B-NS3 protease inhibitors.

The chemical structures of Schiff base vanillin derivatives were drawn and downloaded in the MDL-mol format by using ChemDraw Ultra 12.0. The Schiff base synthesis mechanism was generally as shown in Figure 6:

Table 2. 2D and 3D chemical structures of test sets from natural product

Test Sets	2D Chemical Structure	3D Chemical Structure
Berberine		
Chicoric Acid		
Conessine		
Curcumin		
Digitonin		
Emodin		
Gossypol		



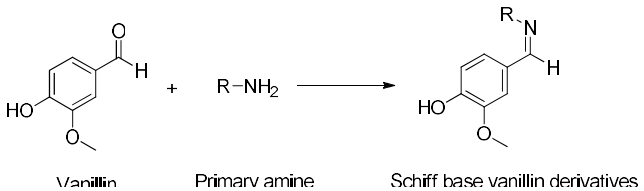


Figure 6. Schiff base synthesis mechanism

Schiff base vanillin derivatives were drawn based on the general mechanism between vanillin and primary amines, in which the list of primary amines was as follow:

(1) 1-piperidinecarboxamide
(2) 4-dinitrophenylhydrazine
(3) 2,6-dibromo-4-nitroaniline
(4) 2-amino-3-bromo-5-methylbenzoic acid
(5) 2-amino-6-bromo-4-chlorophenol
(6) 2-aminopyridine
(7) 4-bromo-3-methyl-1-phenyl-1H-pyrazol-5-ylamine
(8) 5-aminoisatoic anhydride
(9) Acetyl-L-asparagine
(10) Allylurea
(11) Anilline
(12) Benzaldehyde semicarbazone
(13) Benzohydrazide
(14) Benzotriazole-1-carboxamide
(15) Ethyl 2-aminothiazole-5-carboxylate
(16) Indole-3-acetamide
(17) Levetiracetam
(18) Methyl 2-amino-5-bromo-4-isopropylbenzoate
(19) Nicotinic hydrazide
(20) Phenylhydrazine

(21) Thiobenzamide

After all Schiff base vanillin derivatives were drawn and downloaded, they were imported as test sets into the ligand-perspective of LigandScout 4.4, together with novobiocin, sofosbuvir and azithromycin as training sets. Similarly, MMFF94 energies were minimised, conformations of all ligands were generated and clustered, and ligand-based pharmacophore modelling was run. The results were also obtained based on the vanillin derivatives' matching pharmacophore features with the features of the generated pharmacophore model, as well as their pharmacophore fit values in the model. Schiff base vanillin derivatives that displayed satisfied pharmacophore fit values and matching features were recorded and brought forward to the analyses in structure-based molecular docking.

Structure-based molecular docking

Crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor (PDB code: 5LC0) was retrieved from Protein Data Bank (PDB) and imported in the structure-based perspective of LigandScout 4.4, as shown in Figures 7 and 8.

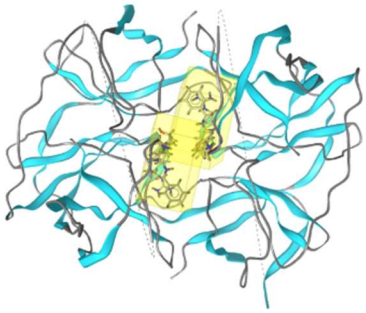


Figure 7. Crystal structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor (PDB code: 5LC0) [18]

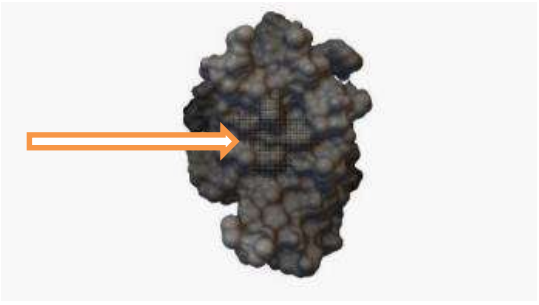


Figure 8. 3D cloud structure of Zika virus NS2B-NS3 protease in complex with a boronate inhibitor (PDB code: 5LC0) viewed by Autodock Vina

The boronate inhibitor, which is the ligand in the active site of 5LC0, shows inhibitory activity against the NS2B-NS3 protease of ZIKV (Figure 9). Therefore, evaluations and analysis were based on the alignments of the Schiff base vanillin derivatives with the pharmacophore model of the ligand to show their matched and aligned interactions with the active sites. Furthermore, the Schiff base vanillin derivatives were docked into the active sites of 5LC0 to evaluate their pharmacophore scores and binding affinities. The ligand was first selected, and the pharmacophore model of the ligand was generated. The ligand and generated pharmacophore model were then copied to the alignment perspective. The first Schiff base vanillin derivative, which is vanillin + 2-Amino-3-bromo-5-methylbenzoic acid was inserted as a single compound in the MDL-sdf format into the structure-based perspective. Again, all ligands and pharmacophore model were copied into the alignment perspective (Figure 10).

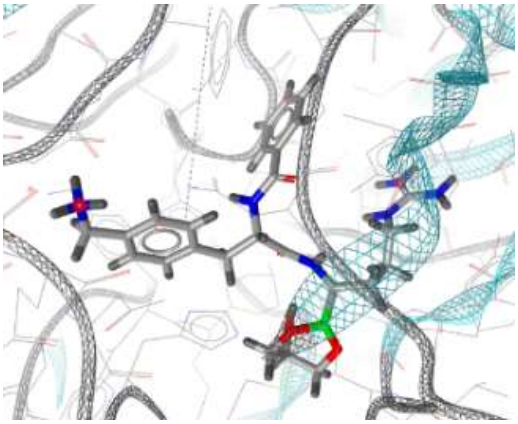


Figure 9. The boronate inhibitor in the active site of 5LC0 [18]

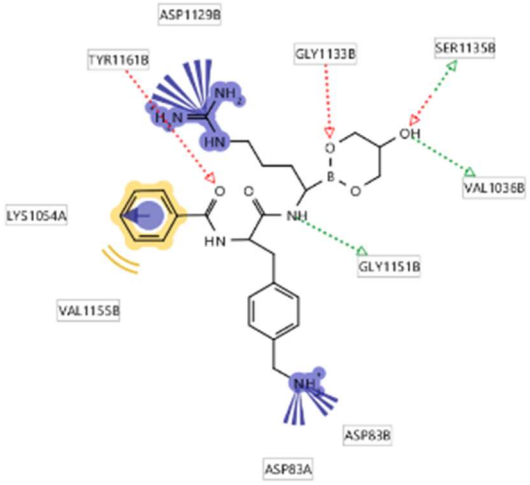


Figure 10. The protein-ligand interactions of boronate inhibitor against the NS2B-NS3 protease [18]

In the alignment perspective, the MMFF94 energy of the first Schiff base vanillin derivative was first minimized to represent minimum energy and maximum stability of the conformation. Alignment was then performed between the boronate inhibitor, and the Schiff base vanillin derivative based on common pharmacophore feature. The yellow spheres, blue features, green and red arrows were the pharmacophore features of boronate inhibitor, while the orange spots showed the common pharmacophore feature shared between the two ligands as depicted in Figure 11.

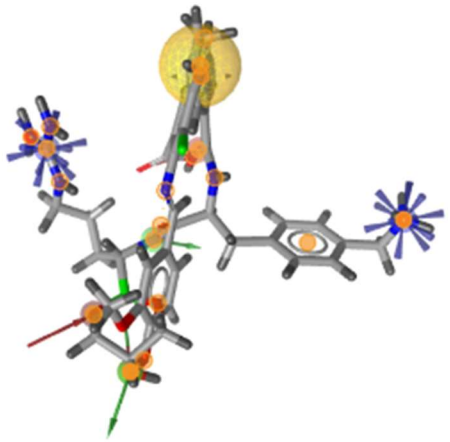


Figure 11. Alignment between pharmacophore model of boronate inhibitor and Schiff base vanillin derivative

Shared pharmacophore model was then generated, followed by merging the pharmacophores and interpolating the overlapping features. The result was as displayed in Figure 12 below.

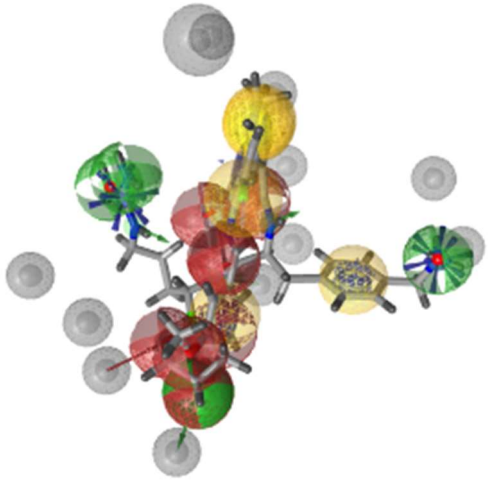


Figure 12. Shared pharmacophore featured, merged pharmacophores and interpolated overlapping features

The ligand was then injected back into the active site of 5LC0 to show the interactions of Schiff base vanillin derivatives inside the active site of NS2B-NS3 protease. From here, the interactions shown by Schiff base vanillin derivatives in compared to those depicted by boronate inhibitor in the active site of 5LC0 is analysed as portrayed in Figure 13 below.

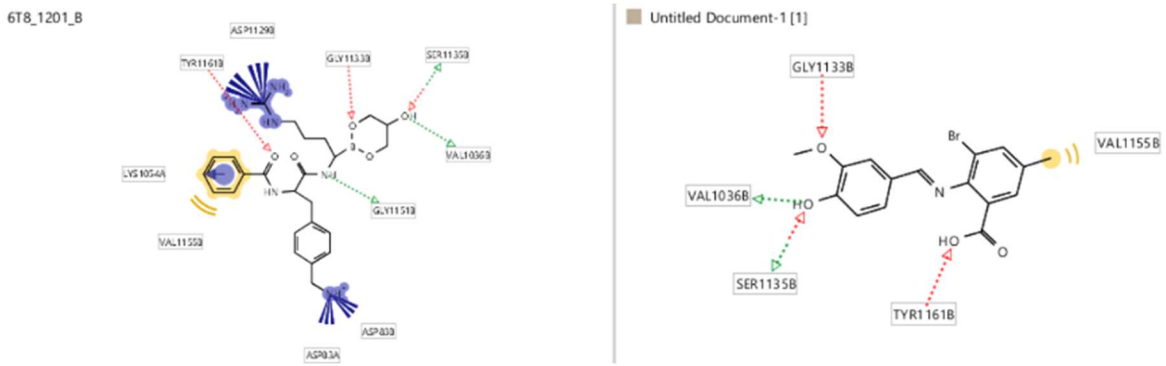


Figure 13. Protein-ligand interactions of Vanillin + 2-Amino-3-bromo-5-methylbenzoic acid, in which out of 10 interactions by boronate inhibitor, the vanillin derivative successfully matched 5 interactions

After the evaluation of interactions between the Schiff base vanillin derivative and the active sites of NS2B-NS3 protease, the vanillin derivative was then docked into the active site of the protease by using AutoDock Vina. 9 conformations of the vanillin derivatives with the best docking result obtained, and from here, the conformation with the best pharmacophore score and binding affinity was analysed. All the above steps are repeated for all drawn Schiff base vanillin derivatives, whereby the derivatives that showed the best results together with ligand-based pharmacophore modelling were recorded, evaluated and discussed.

Results and Discussion

Ligand-based pharmacophore modelling

A pharmacophore model is successfully generated from the training sets, which were novobiocin, sofosbuvir and azithromycin. The model (Figure 14) was composed of 11 pharmacophore features, which are 8 hydrogen bond acceptors (HBA), 2 hydrogen bond donors (HBD) and 1 hydrophobic interaction (HI).

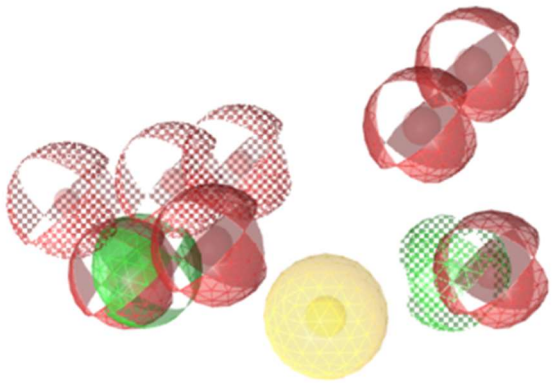


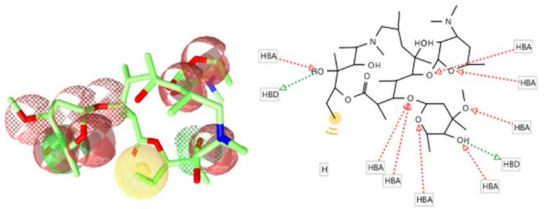
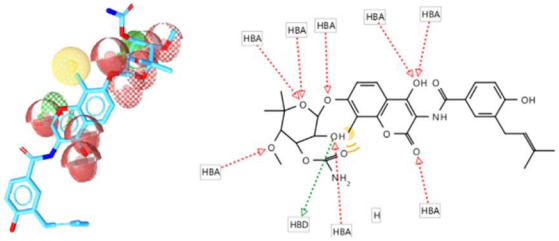
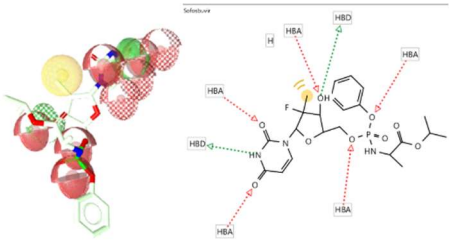
Figure 14. Generated pharmacophore model with 11 pharmacophore features

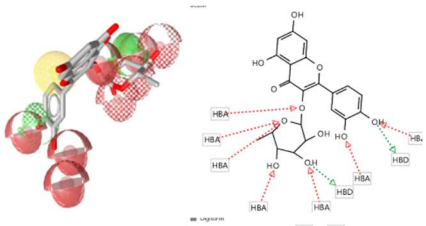
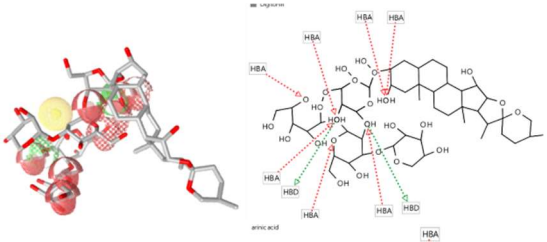
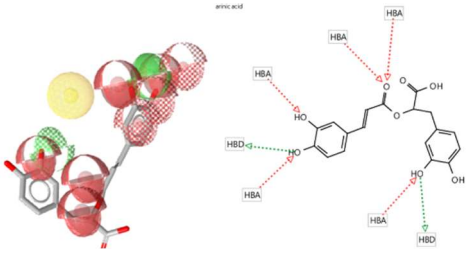
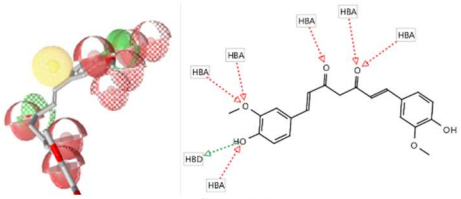
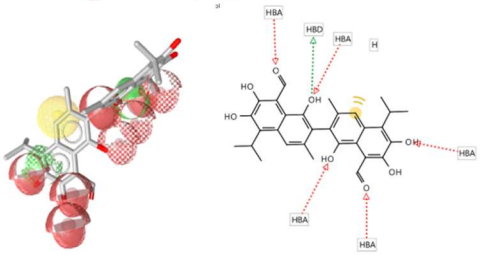
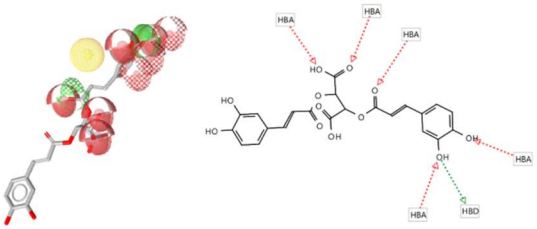
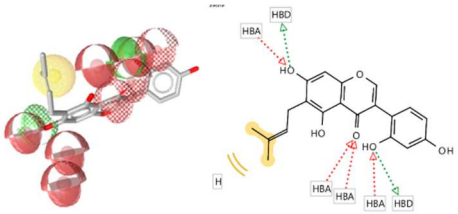
Out of 12 test sets, which were the chemical compounds from natural products for the validation of pharmacophore model, 11 test sets were able to illustrate 4 to 11 matching pharmacophore features, as well as good pharmacophore fit values. 2 test sets, which were quercetin and digitonin, showed higher fitting values than the training set. Thus, the pharmacophore model is justified for its reliability in evaluating the inhibitory activity of Schiff base vanillin derivatives against ZIKV NS2B-NS3 protease. Table 3 shows the pharmacophore fit values and matching features of test sets in the pharmacophore model, and Table 4 shows their matching features with the pharmacophore features depicted by the model.

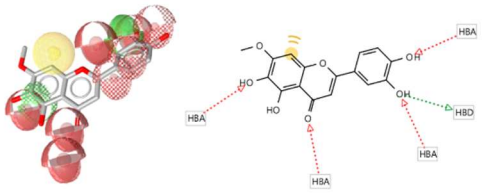
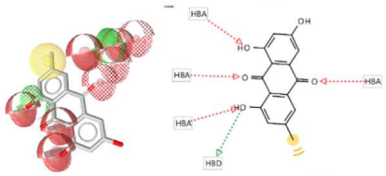
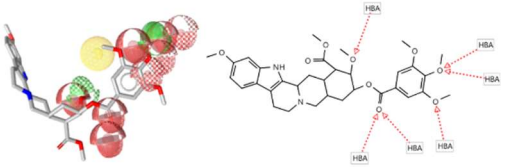
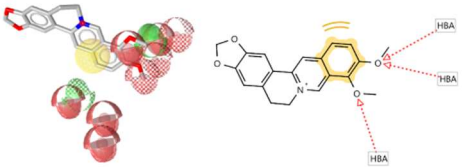
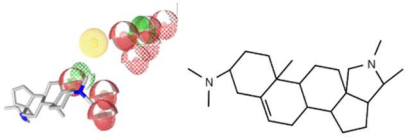
Table 3. Pharmacophore fit values and matching features of test sets in the pharmacophore model

Name of Compounds	Type	Matching Features	Pharmacophore Fit Values
Azithromycin	Training		103.19
Novobiocin	Training		95.84
Quercetin	Test		83.07
Digitonin	Test		82.53
Sofosbuvir	Training		82.21
Rosmarinic acid	Test		72.23
Curcumin	Test		72.16
Gossypol	Test		71.97
Chicoric acid	Test		66.03
Luteone	Test		65.75
Pedalitin	Test		62.69
Emodin	Test		59.30
Reserpine	Test		58.97
Berberine	Test		38.51
Conessine	Test		0.00

Table 4 Matching features of test sets with the pharmacophore features of the pharmacophore model

Name of Compounds	Matching Features	Pharmacophore Fit Values
Azithromycin		103.19
Novobiocin		95.84
Sofosbuvir		83.07

Quercetin		82.53
Digitonin		82.21
Rosmarinic acid		72.23
Curcumin		72.16
Gossypol		71.97
Chicoric acid		66.03
Luteone		65.75

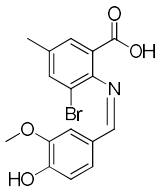
Pedalitin		**62.69**
Emodin		59.30
Reserpine		58.97
Berberine		38.51
Conessine		0.00

Schiff base vanillin derivatives were drawn by using ChemDraw Ultra 12.0 based on the Schiff base synthesis mechanism between vanillin and the listed 21 primary amines, as shown in Table 5 below.

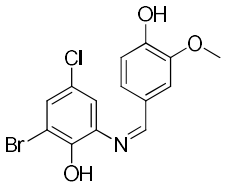
Table 5. List of Schiff base vanillin derivatives

Vanillin Associated With	Chemical Structures	Vanillin Associated With	Chemical Structures
(1) 1-piperidinecarboxamide		(12) Benzaldehyde semicarbazone	
(2) 2,4-dinitrophenylhydrazine		(13) Benzohydrazide	
(3) 2,6-dibromo-4-nitroaniline		(14) Benzotriazole-1-carboxamide	

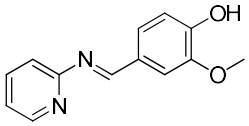
(4) 2-amino-3-bromo-5-methylbenzoic acid



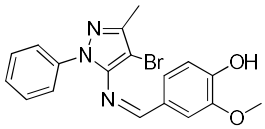
(5) 2-amino-6-bromo-4-chlorophenol



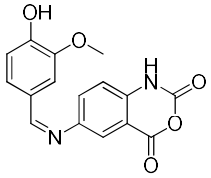
(6) 2-aminopyridine



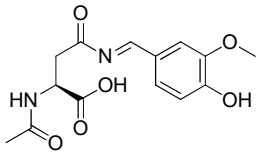
(7) 4-bromo-3-methyl-1-phenyl-1H-pyrazol-5-ylamine



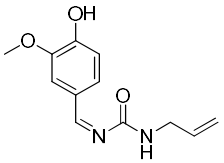
(8) 5-aminoisatoic anhydride



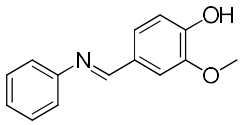
(9) Acetyl-L-asparagine



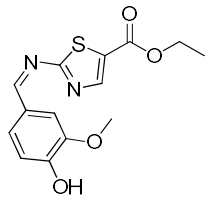
(10) Allylurea



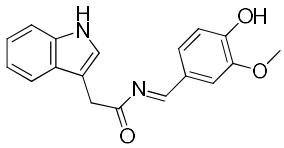
(11) Anilline



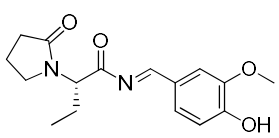
(15) Ethyl 2-aminothiazole-5-carboxylate



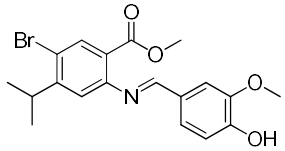
(16) Indole-3-acetamide



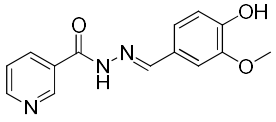
(17) Levetiracetam



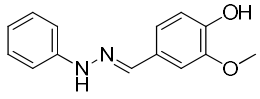
(18) Methyl 2-amino-5-bromo-4-isopropylbenzoate



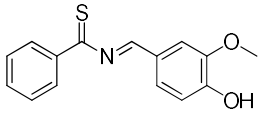
(19) Nicotinic hydrazide



(20) Phenylhydrazine



(21) Thiobenzamide



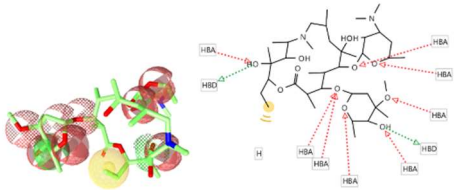
Similarly, the training sets generated a pharmacophore model with 11 features, which are 8 hydrogen bond acceptors (HBA), 2 hydrogen bond donors (HBD) and 1 hydrophobic interaction (HI). Out of 21 vanillin derivatives, a total of 19 compounds showed good pharmacophore fit values and 6 to 9 matching

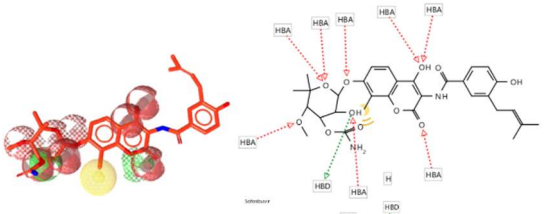
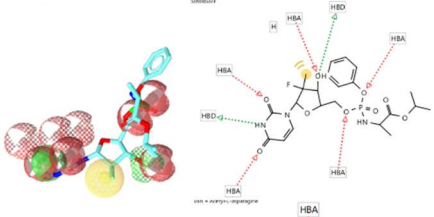
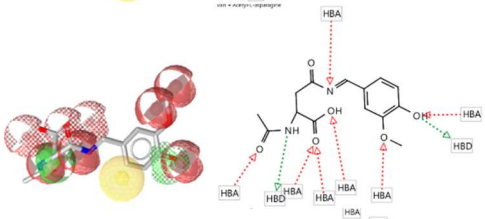
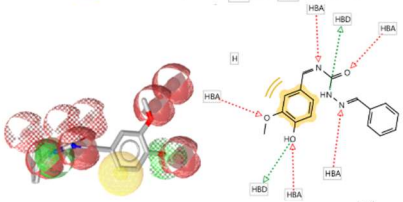
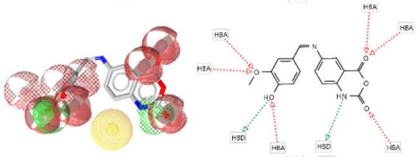
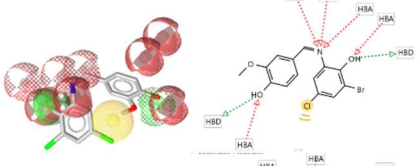
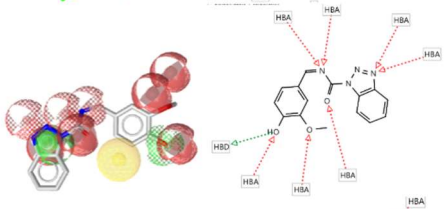
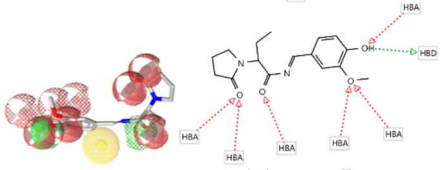
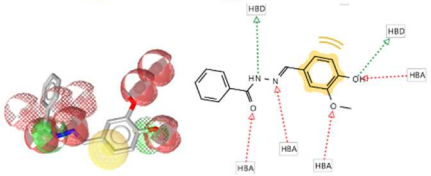
pharmacophore features out of the 11 features from the model. On top of that, compound (9) showed a fit value higher than the training set and the majority number of matching features, depicting immense potential as ZIKV NS2B-NS3 inhibitor. Nonetheless, (1) and (11) are unable to depict good matching features and pharmacophore fit values. The results of ligand-based pharmacophore modelling of Schiff base vanillin derivatives in the generated pharmacophore model were shown in Table 6, while Table 7 displayed their matching features with the pharmacophore features of the model.

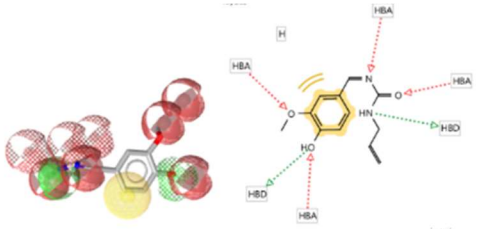
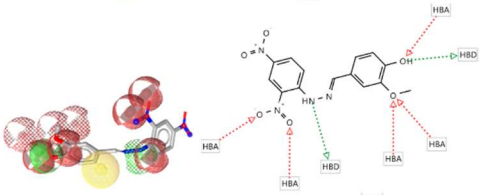
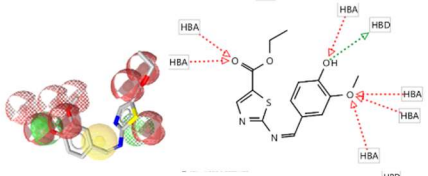
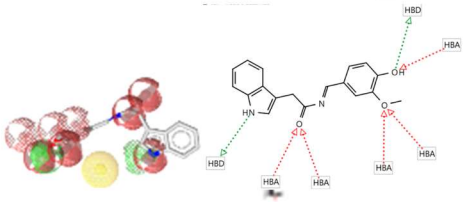
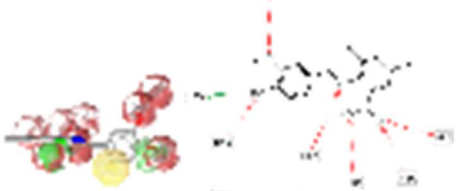
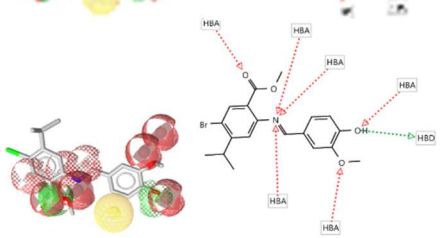
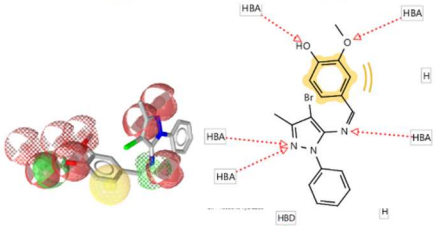
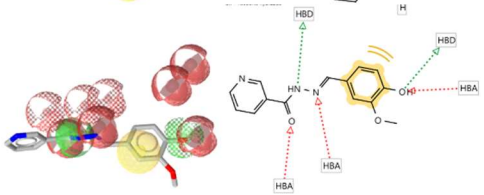
Table 6. Pharmacophore fit values and matching features of Schiff base vanillin derivatives in the pharmacophore model

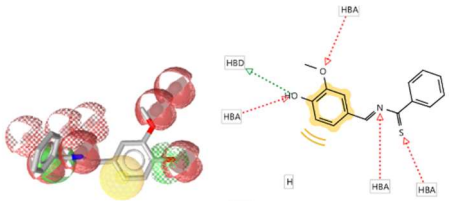
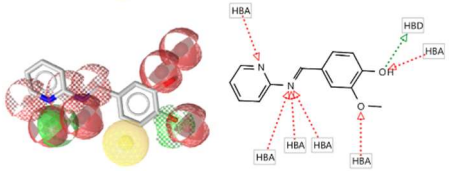
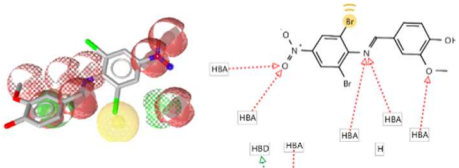
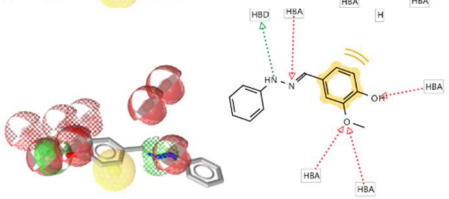
Name of Compounds	Type	Matching Features	Pharmacophore Fit Values
Azithromycin	Training		103.19
Novobiocin	Training		95.84
(9)	Test		82.40
Sofosbuvir	Training		82.21
(12)	Test		79.31
(8)	Test		79.20
(5)	Test		75.54
(14)	Test		72.38
(17)	Test		72.12
(13)	Test		71.81
(10)	Test		69.29
(2)	Test		69.17
(15)	Test		68.57
(16)	Test		68.41
(4)	Test		66.08
(18)	Test		65.32
(7)	Test		62.50
(19)	Test		62.47
(21)	Test		62.46
(6)	Test		62.15
(3)	Test		58.61
(20)	Test		55.53

Table 7. Matching features of Schiff base vanillin derivatives with the pharmacophore features of the pharmacophore model

Name of Compounds	Matching Features	Pharmacophore Fit Values
Azithromycin		103.19

Novobiocin		95.84
Sofosbuvir		82.21
(9)		82.40
(12)		79.31
(8)		79.20
(5)		75.54
(14)		72.38
(17)		72.12
(13)		71.81

(10)		69.29
(2)		69.17
(15)		68.57
(16)		68.41
(4)		66.08
(18)		65.32
(7)		62.50
(19)		62.47

(21)		**62.46**
(6)		62.15
(3)		58.61
(20)		55.53

Based on Table 8, HBA and HBD were found mostly in both hydroxyl group, OH and ether group, C-O-C of vanillin part. Similarly, HI interaction was mostly found in the benzene ring of vanillin. This depicted the importance of vanillin in showing matching interactions with the generated pharmacophore model, hence showing vanillin's potential as ZIKV NS2B-NS3 inhibitors as the training sets. On top of that, HBA and HBD were also discovered in the imine linkage, C=N of Schiff base vanillin derivatives. Hence, this explains the importance and capability of Schiff base vanillin derivatives to show similar and matching interactions with the training sets as ZIKV NS2B-NS3 inhibitors. For the primary amine part, most HBA was shown by O atom in carbonyl, C=O groups, hydroxyl, OH groups and even nitro, NO_2 group, as well as depicted by N atoms. Due to the great amount of HBA and HBD involved, the selection of primary amines in reaction with vanillin to form Schiff base vanillin derivatives should consider most of the O and N atoms. This explains why compound (9) can show exceedingly high pharmacophore fit values, even higher than one of the training sets. The primary amine of (9), which is acetyl-L-aspargine, consists of 1 OH group, 3 O of C=O and 1 N group. Therefore, in combination with the OH and C-O-C of vanillin part, (9) depicted most of the pharmacophore features by the model, as well as having suitable molecular orientation. In some of the Schiff base vanillin derivatives, HI interaction was discovered in Br and Cl atoms. Thus, due to the presence of HI interaction in the model, primary amines with group 17 atoms or aromatic rings are preferably selected. The primary amines of compound (1) and (11) lack of the required functional groups to be aligned with the pharmacophore model. 1- piperidinecarboxamide in (1) has only 1 C=O and N group, while aniline in (11) has only 1 N group and 1 benzene ring. Due to the shortage of common pharmacophore features as well as suitable alignment orientation in the pharmacophore model, (1) and (11) are unable to display good fitting results in the model. Based on the results and explanations, all 19 Schiff base vanillin derivatives possessed potential inhibitory activity against ZIKV NS2B-NS3 protease, just as the interactions shown by the training sets. Therefore, all are subjected to structure-based molecular docking for further evaluation as ZIKV NS2B-NS3 inhibitors.

Structure-based molecular docking

Based on the downloaded PDB crystal structure, the pharmacophore model and interactions of boronate inhibitor with the active sites of NS2B-NS3 protease were shown in Figure 15 below. The inhibitor showed 11 interactions, which were 3 HBA, 3 HBD, 3 positive ionisable area (PI), 1 aromatic ring (AR) and 1 HI.

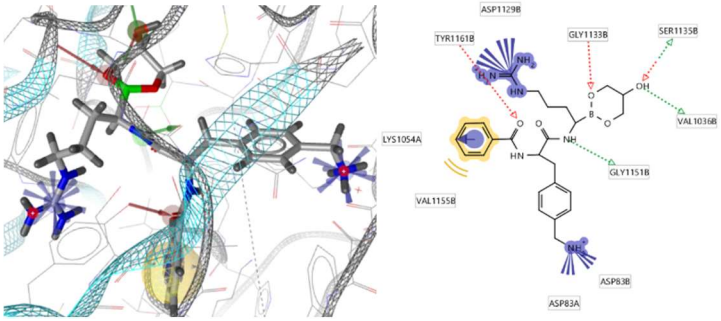


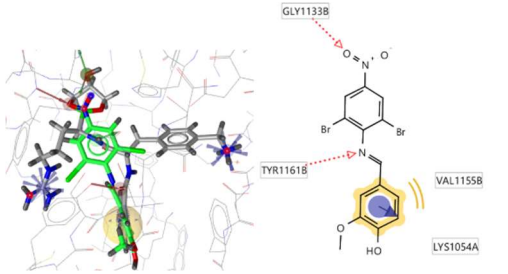
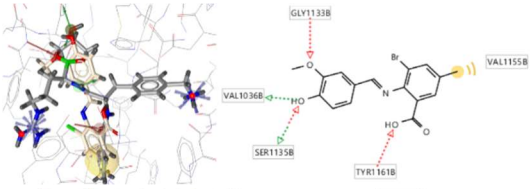
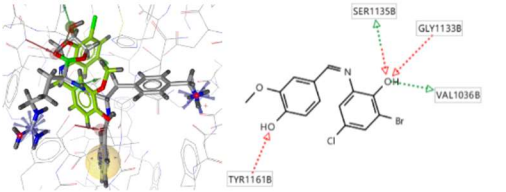
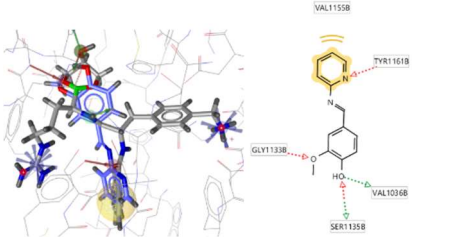
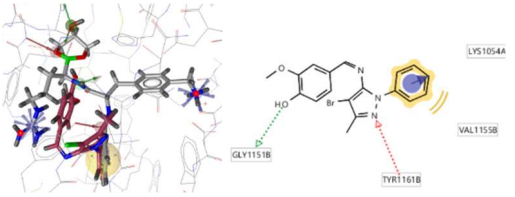
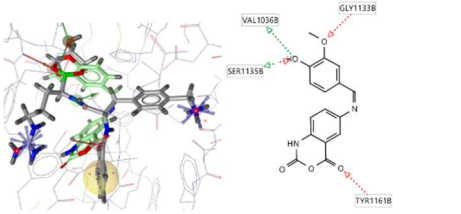
Figure 15. Pharmacophore model and interactions of boronate inhibitor with the active sites of 5LC0

Upon alignment and docking of the 19 Schiff base vanillin derivatives from ligand-based pharmacophore modelling results, 17 of them can depict 4 to 7 matching interactions with the active sites of 5LC0. On top of that, they are also able to show good pharmacophore scores and binding affinity values, hence having great inhibitory potential against NS2B-NS3 protease. Nevertheless, (1), (2), (9) and (11) were unable to show enough matching features in interaction with ZIKV NS2B-NS3 protease. This is due to the lack of enough common pharmacophore features to be aligned with the boronate inhibitor, as well as in the active site of 5LC0. Table 8 showed the pharmacophore scores and binding affinity values of the Schiff base vanillin derivatives upon docking *via* AutoDock Vina in the active site of ZIKV NS2B-NS3 protease, whereby the shaded columns, which were derivative (14), (15) and (19) showed the highest pharmacophore scores. Table 9 depicts their aligned interactions with the boronate inhibitor in the active site of ZIKV NS2B-NS3 protease.

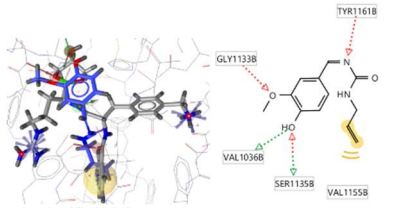
Table 8. Pharmacophore scores and binding affinity values (kcal mol^{-1}) of Schiff base vanillin derivatives upon docking by AutoDock Vina in the active site of ZIKV NS2B-NS3 protease

Schiff Base Vanillin Derivatives	Pharmacophore Scores	Binding Affinity Values (kcal mol^{-1})
(3)	46.95	-8.20
(4)	36.49	-8.60
(5)	45.23	-7.70
(6)	35.49	-7.40
(7)	37.73	-7.70
(8)	44.57	-8.10
(10)	35.93	-6.20
(12)	36.95	-8.50
(13)	37.36	-8.30
(14)	55.99	-8.90
(15)	56.30	-7.20
(16)	37.66	-8.90
(17)	35.83	-7.40
(18)	36.33	-8.50
(19)	47.07	-7.60
(20)	35.09	-7.40
(21)	46.56	-8.00

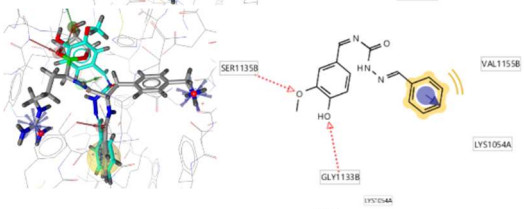
Table 9. Aligned interactions of Schiff base vanillin derivatives with boronate inhibitor in the active site of ZIKV NS2B-NS3 protease

Schiff Base Vanillin Derivatives	Aligned Interactions
(3)	
(4)	
(5)	
(6)	
(7)	
(8)	

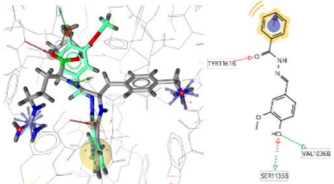
(10)



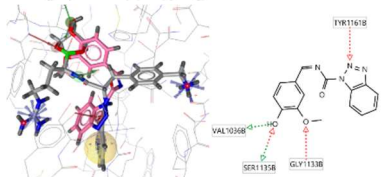
(12)



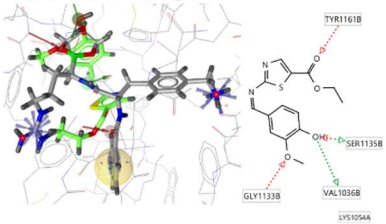
(13)



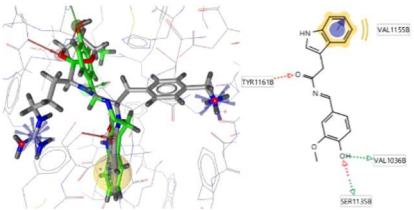
(14)



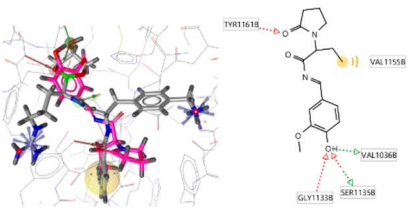
(15)



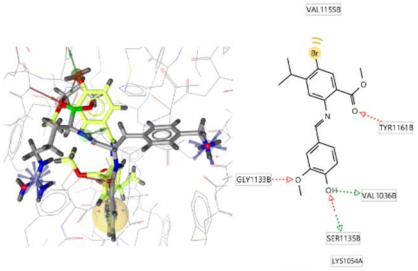
(16)



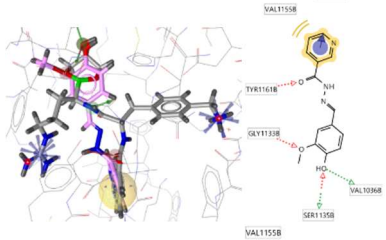
(17)



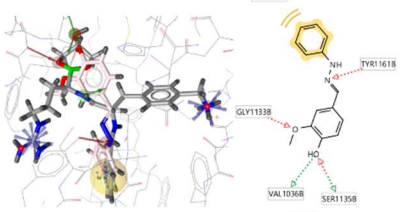
(18)



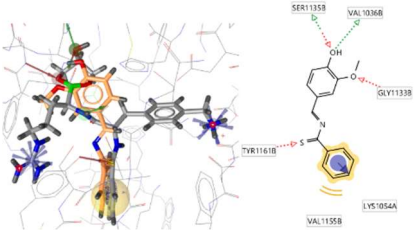
(19)



(20)



(21)



The 17 Schiff base vanillin derivatives were able to depict at least 4 matching interactions with satisfying pharmacophore scores, hence showing their potential inhibitory activity against NS2B-NS3 protease. Other than that, the molecular docking results of the Schiff base vanillin derivatives were also expressed based on their binding affinity values, which is a function of Gibbs free energy [28]. Binding affinity refers to the strength of binding interaction between a protein molecule to its ligand, which is between the 5LC0 protease with the Schiff base vanillin derivatives in this study. Being represented by the equilibrium dissociation constant, the more negative the binding affinity values, the greater the binding interaction and affinity [29]. Since binding affinity values are a function of Gibbs free energy, therefore affinity values less than -6.0 kcal mol^{-1} are considered active molecules, while affinity values greater than -6.0 kcal mol^{-1} are considered inactive molecules [29]. Based on the obtained results, the Schiff base vanillin derivatives showed binding affinity values less than -6.0 kcal mol^{-1}, ranging from -6.20 kcal mol^{-1} to -8.90 kcal mol^{-1}, indicating their property as active cluster molecules.

The interactions of the boronate inhibitor with the active sites of NS2B-NS3 protease are HBA towards TYR161B, GLY133B and SER135B, HBD towards SER135B, VAL36B and GLY151B, PI towards ASP129B, ASP83A and ASP83B, AR towards LYS54A, as well as HI towards VAL155B. The Schiff base vanillin derivatives mostly depict HBA towards TYR161B, GLY133B and SER135B, HBD towards SER135B and VAL36B, AR towards LYS54A and HI towards VAL155B. Most of the HBA and HBD were found to be depicted by the hydroxyl and ether group of the vanillin part, showing that vanillin itself plays an

imperative role in interactions with the active sites of ZIKV NS2B-NS3 protease. Moreover, the Schiff base vanillin derivatives displayed their inhibitory action against LYS54A and VAL155B, which are AR and HI through the benzene and pyridine rings of the primary amine part. This shows the potential of aromatic primary amines as NS2B-NS3 protease inhibitors, to be utilised as a starting product for the synthesis of Schiff base vanillin derivatives to inhibit NS2B-NS3 protease. This may explain the lack of potential of (9), as the primary amine of (9) is an aliphatic primary amine. Even though compound (2) shows reliable results in ligand-based pharmacophore modelling, due to the lack of enough pharmacophore features, as well as unable to depict suitable orientation in the active site of 5LC0, compound (2) is unable to align with boronate inhibitor. In another crystal structure of ZIKV NS3 protease in complex with peptidomimetic inhibitor, which is 4-guanidinomethyl-phenylacetyl-Arg-Arg-Arg-4-amidinobenzylamide, the inhibitor also showed HBA against TYR161B, GLY133B and SER135B, thus depicting the importance of interactions with these active sites [30]. Similarly, the crystal structure of ZIKV NS2B-NS3 protease with compound MI2219 (PDB code: 7VLH), and the crystal structure of Zika NS2B-NS3 protease with compound MI2220 (PDB code: 7VLI) showed HBA and HBD interaction with TYR161B, showing the imperative role of interaction with TYR161B in inhibition activity against the NS2B-NS3 protease [31], which is depicted by 16 out of 17 of the Schiff base vanillin derivatives. Compounds (1) and (11) have been shown to lack HBA and HBD interactions in ligand-based pharmacophore modelling due to the lack of required functional groups, hence they are unable to depict any interaction with these important active sites, especially TYR161B, GLY133B and SER135B. In conjunction with the results obtained from ligand-based pharmacophore modelling, all the 17 Schiff base vanillin derivatives above showed a great deal of potential as ZIKV NS2B-NS3 protease inhibitors *via in silico*, being promising drug candidates and bringing futuristic *in vitro* and *in vivo* tests to confirm the inhibitory activities.

Lipinski's rule of 5

Lipinski's rule of 5 or known as RO5 is a guideline rule which analyse and evaluate the drug likeness, and pharmacological and biological activity of chemical compounds as orally active drug for human consumption. The rule described the molecular properties crucial for a drug's pharmacokinetics in humans, known as 'ADME': absorption, distribution, metabolism, and excretion. RO5 states that for a chemical compound to be an orally active drug, the chemical compound should have:

- Not more than 5 hydrogen bond donors (HBD) (nitrogen-hydrogen and oxygen-hydrogen bonds)
- Not more than 10 hydrogen bond acceptors (HBA) (nitrogen and oxygen atoms)
- Molecular weight less than 500 g/mol
- Partition coefficient log P not greater than 5 [32]

Based on RO5, all 17 Schiff base vanillin derivatives that showed potential inhibitory activity against ZIKV NS2B-NS3 protease fulfilled all rules, having less than 5 HBD, less than 10 HBA, molecular weight less than 500 g/mol and partition coefficient log P less than 5. Therefore, they are considered to possess drug likeness as potential orally active drugs, thus supporting their role as potential NS2B-NS3 inhibitors. Table 10 summarised the RO5 results of the Schiff base vanillin derivatives.

Conclusion

From ligand-based pharmacophore modelling to structure-based molecular docking, a total of 21 Schiff base vanillin derivatives were evaluated for the ZIKV NS2B-NS3 protease inhibitory activity. Out of 21 derivatives, 17 showed potential results as NS2B-NS3 inhibitors. In ligand-based pharmacophore modelling, 19 of them showed good pharmacophore fit values, and 6 to 9 matched pharmacophore features out of 11 features depicted by pharmacophore model of training sets. In structure-based molecular docking, 17 of them showed 4 to 7 matching interactions with the 11 interactions shown by boronate inhibitor ligand in the active site of 5LC0, as well as good fit values and binding affinity values upon docking. As a result, the Schiff base vanillin derivatives that passed both ligand-based pharmacophore modelling and structure-based molecular docking are **(3), (4), (5), (6), (7), (8), (10), (12), (13), (14), (15), (16), (17), (18), (19), (20)** and **(21)**. The 17 Schiff base vanillin derivatives also fulfilled Lipinski's Rule of 5, depicting their drug likeness

properties and being potential orally active drugs. Being proven as promising drug candidates through *in silico* study, further analysis and research *in vivo* and *in vitro* becomes interesting and potential research in future to ensure the safety and lethality of these derivatives, and to bring more opportunities to more research in the study of ZIKV NS2B-NS3 inhibitors.

Table 10. Lipinski's Rule of 5 of Schiff base vanillin derivatives

Vanillin Derivatives	c log P	Hydrogen Bond Donors (HBD)	Hydrogen Bond Acceptors (HBA)	Molecular Weight (g/mol)
(3)	3.730	1	7	396.159
(4)	3.689	1	5	364.201
(5)	4.273	2	4	356.609
(6)	2.546	1	4	228.251
(7)	4.408	1	4	386.255
(8)	1.946	2	5	312.281
(10)	1.715	2	4	234.255
(12)	2.563	2	5	297.314
(13)	1.933	2	4	270.288
(14)	1.631	1	6	296.286
(15)	2.553	1	5	306.340
(16)	3.070	2	4	308.337
(17)	1.747	1	5	304.346
(18)	4.592	1	4	406.282
(19)	1.328	2	5	271.276
(20)	2.847	2	3	242.278
(21)	3.195	1	4	271.338

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