RATIONAL DESIGN AND SYNTHESIS OF INHIBITORS FOR H1N1 NEURAMINIDASE AND DENGUE PROTEASE ENZYMES

by

MAYWAN HARIONO

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LIST OF SYMBOLS

Å	Angstrom
Fit	Fit value
∑	Sum
ΔG	The Free Energy
ΔS	The entropy
r	Correlation coefficient
K_i	Inhibition Constant
K_m	Michaelis Menten Constant
nM	nanoMolar
μM	microMolar
R	*Rectus* configuration
S	*Sinister* configuration
evals	Evaluation
[S]	Substrate concentration
J	Coupling constant
s	Singlet
d	Doublet
t	Triplet
q	Quartet
m	Multiplet
δ_C	Chemical shift of carbon-13
1/V	The reciprocal of reaction assay velocity
[E]	Enzyme concentration
r^2 adj	Adjusted quadratic correlation coefficient
r^2 pred	Predicted quadratic correlation coefficient
F	Variance
δ+	Positive partial
δ-	Negative partial
AlogP	Partition coefficient based on Ghose and Crippen's method

LIST OF ABBREVIATIONS

act	Actual
AMC	Aminomethyl coumarine
br	Broad
Boc	Butoxycarbonyl
Bz	Benzoil
C	Capsid
DANA	2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid
DIPEA	*N*, *N*-diisopropylethylamine
E	Envelope
ECFP	Extended Connectivity Finger Print
EDG	Electron Donating Group
ER	Endoplasmic Reticulum
EWG	Electron Withdrawing Group
GFA	Genetic Function Approximation
GTP	Guanine Triphosphate
GA	Genetic Algorithm
FEB	Free Energy of Binding
HA	Hemagglutinin
HBA	Hydrogen Bond Acceptors
HBD	Hydrogen Bond Donors
ITC	Isothermal Titration Calorimetry
kb	Kilobase
kDa	KiloDalton
L.O.F	Lack of Fit
M2	Membrane2
MES	2-(*N*-morpholino)ethanesulfonic acid
MLR	Multiple Linear Regression
MTase	Methyl Transferase
NCI	National Cancer Institute
Neu5Ac	N-Acetylneuraminic Acid
NS	Non Structural

NTPase	N Triphosphatase
P	Peptide
PDB	Protein Data Bank
pred	Predicted
prM	preMembrane
PTC	Phase Transfer Catalyst
RFU	Relative Fluorescence Unit
RMSD	Root Mean Square Deviation
RTPase	RNA Triphosphatase
SA	Sialic Acid
sNS	Secreted Non Structural
S.O.R	Significant of Regression
TBAI	Tetrabutylammonium Iodide
WNV	West Nile Virus

REKABENTUK SECARA RASIONAL DAN SINTESIS PERENCAT UNTUK ENZIM NEURAMINIDASE H1N1 DAN PROTEASE DENGGI

ABSTRAK

Influenza dan denggi adalah dua daripada penyakit berjangkit yang disebabkan oleh virus. Rintangan virus terhadap ubat influenza komersial dan ketiadaan ubat untuk merencat virus denggi menggalakkan usaha untuk mencari perencat virus yang berpotensi. Setakat ini, enzim neuraminidase H1N1 ialah satu daripada sasaran utama dalam pencarian perencat influenza A manakala enzim protease NS2B-NS3 DENV2 pula merupakan sasaran utama dalam penemuan ubat denggi. Kajian sebelum ini mendapati bahawa asid ferulik yang dipencilkan daripada kulit buah manggis dapat merencat aktiviti neuraminidase H1N1 dengan nilai IC_{50} = 200 μM. Struktur aromatiknya yang sederhana menarik perhatian untuk dikembangkan sebagai perencat neuraminidase H1N1. Sebanyak 20 terbitan asid ferulik telah pun direkabentuk dan disintesis. Kajian asai atas sebatian tersebut menunjukkan nilai IC_{50} daripada 50 sehingga > 1000 μM. Rekabentuk hubungan kuantitatif struktur dan aktiviti menggunakan kaedah *Multiple Linear Regression* dilaksanakan untuk menghasilkan model yang menentukan hubungan positif daripada penderma dan penerima ikatan hidrogen dengan keputusan statistik yang baik (r^2 = 0.758; r^2 (adj) = 1.185; *Least-squared error* = 0.189). Dua model lagi direkabentuk berasaskan kajian hubungan kuantitatif struktur-aktiviti ini dan ianya diramalkan mempunyai IC_{50} lebih rendah daripada sebatian sebelumnya. Kajian sebelum ini mengenai penskrinan virtual daripada pangkalan data Institut Kanser Nasional terhadap protease NS2B-NS3 DENV2 menyarankan thioguanine sebagai perencat protease ini. Langkah penyelidikan yang sama juga dilaksanakan kepada

protease ini, menunjukkan nilai IC_{50} daripada 28 sehingga > 1000 μM. Kajian hubungan kuantitatif struktur-aktiviti pula dilaksanakan menggunakan kaedah *Genetic Function Approximation* dengan *Linear* terpilih sebagai model terbaik berasaskan keputusan statistik ((r^2 = 0.921; r^2 (adj) = 0.884; r^2 (pred) = 0.820; *RMS residual errors* = 0.258; Friedman L.O.F = 0.141 and S.O.R *p-value* = $1.919e^{-006}$). Model ini menentukan koefisien pembahagi sebagai deskriptor positif untuk rekabentuk seterusnya. Sebanyak empat ligan baru direkabentuk, disintesis dan ditentukan aktivitinya terhadap protease denggi. IC_{50} yang diramalkan dan ditentukan melalui aktiviti *in vitro* adalah saling setuju. Kesimpulannya, kaedah pemodelan *in silico* dalam kajian ini telah berjaya menunjukkan konsep merekabentuk perencat enzim secara rasional.

RATIONAL DESIGN AND SYNTHESIS OF INHIBITORS FOR H1N1 NEURAMINIDASE AND DENGUE PROTEASE ENZYME

ABSTRACT

Influenza and dengue are two of infectious diseases which caused by viruses. The viral resistances towards commercial anti-influenza as well as no drug available to combat dengue infection have prompted the search for potential inhibitors. Currently, H1N1 neuraminidase is one of the major targets in searching for inhibitor of influenza A as well as DENV2 NS2B-NS3 protease in dengue drug discovery. In a previous study, ferulic acid from *G. mangostana* pericarps has been isolated and showed an inhibition against H1N1 neuraminidase *in vitro* with IC_{50} = 200 μM. Its simple aromatic structure was attractive to be developed as H1N1 neuraminidase inhibitor. Twenty ferulic acid derivatives were designed *in silico* and synthesised, respectively. The *in vitro* assay showed the inhibitory activity with IC_{50} from 50 to >1000 μM. The Quantitative Structure-Activity Relationship (QSAR) modelling using Multiple Linear Regression was then carried out to produce the model defining a positive correlation of number of hydrogen bond donor as well as hydrogen bond acceptor with a good statistical results (r^2 = 0.758; r^2 (adj) = 1.185; Least-squared error = 0.189). Two further models were designed based on this QSAR equation and they were predicted to have lower IC_{50} values. On the other hands, previous study on virtual screening of National Cancer Institute database against DENV2 NS2B-NS3 protease suggested thioguanine as a potential inhibitor for the enzyme. The same approach carried out on DENV2 protease inhibitor design demonstrated the inhibitors possessing IC_{50} in the range of 28 to >1000 μM. QSAR models were also generated using Genetic Function Approximation selecting a Linear model as the best QSAR equation upon statistical results (r^2 = 0.921; r^2 (adj) = 0.884; r^2 (pred) =

0.820; *RMS residual errors* = 0.258; Friedman L.O.F = 0.141 and S.O.R *p-value* = $1.919e^{-006}$). The model defined partition coefficient as the positive descriptors for further design. Four more ligands were then modeled, synthesised and tested their activity *in vitro*. The results showed a good agreement between its predicted and experimental $IC_{50.}$ In general, the *in silico* modelling used in this study successfully proved the concept of the rational enzyme inhibitor design.

CHAPTER ONE
INTRODUCTION

1.1 Statement of the Problem

In recent years, world is threatened with the emergence of pandemic and endemic infections of viruses such as influenza A and dengue. The news of highly pathogenic influenza (H5N1) transmission from birds to human that resulted in 53 deaths in Vietnam, Cambodia and Thailand shocked the world in 2005 (WHO, 2005). More deaths were reported in the subsequent years and the threat of H5N1 now being compounded by the emergence of H1N1 pandemic in 2009. The World Health Organization (WHO) confirmed that the pandemic was spread to over 220 countries with more than 39 million cases and 15,417 deaths worldwide (see Figure 1.1) (CDC, 2010a; Fajardo-Dolci *et al*., 2010). Compared to H5N1 avian influenza which emerged in 2005, H1N1 swine virus is less virulent but it is more prevalent than that of avian influenza (Salaam-Blyther, 2009).

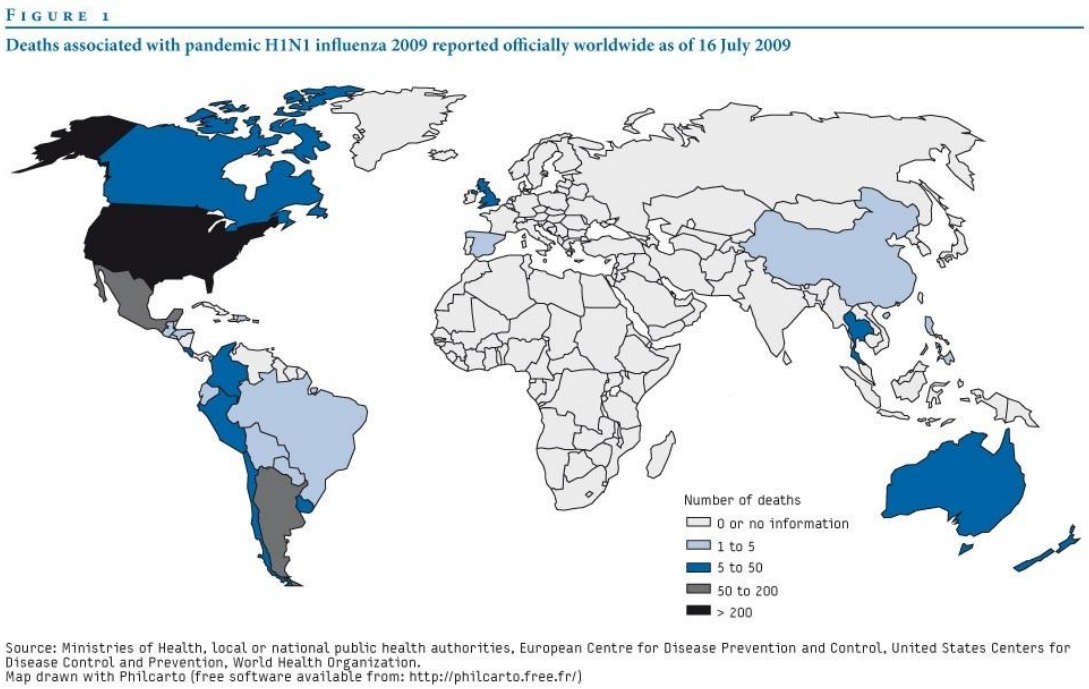


Figure 1.1 The death number of pandemic H1N1 influenza 2009 (CDC, 2010a).

Four years later in February 2013, a rare H7N9 subtype of avian influenza was isolated for the first time from the patient with pneumonia and acute respiratory distress syndrome in China. The infection showed alarming mortality rate (28% as of May 2013). Although H7N9 subtype is considered a low pathogen, the possibility of this subtype becomes resistant towards either vaccine or drug should be anticipated (Baranovich, 2013; Chen *et al*., 2013).

Vaccines are available to prevent the infection of influenza. However, the existing vaccines have been mostly ineffective due to the emergence of rapidly variable mutation (Kim *et al*., 1999; Zhang *et al*., 2008a). Thus, the development of effective and safe anti-influenza becomes an urgent need (Gong *et al*., 2007; Zhang *et al*., 2008b).

Historically, the adamantane-based M2 ion channel protein inhibitors (amantadine and rimantadine) were the first drugs available for the treatment of influenza. Such drugs had only been useful in the treatment of Influenza A infection due to the fact that only the A strain of the virus has M2 ion channel protein (von Itzstein, 2007). The drugs inhibit the virus replication by blocking this ion channel via binding at the allosteric site which triggers a conformational change in the pore region. This action causes interfering proton transfer through the ion channel across the membrane of the virus or endosome. Therefore, the virus is unable to penetrate the host cell of membrane and the replication being stopped (Sandrock, 2010; Tisdale, 2009). Several toxic effects (CNS toxicity can manifest dizziness, nervousness and insomnia and also gastrointestinal effect such as nausea,

constipation and lost of appetite) have been reported along with rapid emergence of drug-resistant variants. Studies between 1994 and 2005 showed the increase of worldwide amantadine and rimantadine-resistance from 0.4% to 12.3%. Thus, the use of these drugs have been discouraged (Moscona, 2008).

Due to those disadvantages of M2 ion channel inhibitors, there was a shift to drug design targeting the other two surface glycoproteins; hemagglutinin (HA) and neuraminidase (NA), which are expressed by both influenza A and B. The HA plays as the receptor-binding and membrane fusion glycoprotein whilst the NA has a role as a receptor-destroying enzyme (An *et al*., 2009). So far, no HA inhibitors have been clinically approved for influenza treatment (Meshram and Jungle, 2009).

Neuraminidase (NA), also known as sialidase, is the major surface glycoprotein that shows an important role in the viral replication, thus, has become an attractive target for anti-influenza drug. Zanamivir and oseltamivir are two examples of drugs that are effective for either A or B types of neuraminidase. Studies on NA active site and Structure-Activity Relationships (SARs) of published NA inhibitors disclose that the relative positions of the substituents (carboxylate, glycerol, acetamido, and hydroxyl) of the central ring mainly determine inhibition of the NA (Zhang *et al*., 2008b).

Although zanamavir is highly effective, its inhalational delivery (D'Souza *et al*., 2009; Hammad and Taha, 2009; Sun *et al*., 2010) is not very attractive as oral delivery (via capsule/tablet) is much preferred. Oseltamivir overcomes this limitation, but the production cost is quite expensive as it relies on the expensive

starting material shikimic acid (Chand *et al*., 1997). Although there have been a lot of efforts to discover new NA inhibitors with various scaffold, including aromatic (Chand *et al*., 1997; Luo *et al*., 1997), dihydropyrane (Taylor *et al*., 1998), cyclopentene (Babu *et al*., 2009), cyclohexene (Lew *et al*., 2000) and pyrrolidine (Zhang *et al*., 2008b), the currently circulating clinical H274Y H1N1 mutant is quite resistant to oseltamivir (Yen *et al*., 2006). Therefore, there is a need for new drugs that are cheaper and more effective against the influenza virus.

Next to another infectious disease, dengue has become a major disease burden for the South East Asian countries. Although the scale of dengue infection has not reached pandemic proportion, WHO currently estimates that there may be 50 million dengue infections worldwide every year (Murray *et al*., 2013), with the infection endemic in South East Asian Regions. During 2008, in Indonesia, there were 17,604 cases reported and 10,000 to 12,000 of them concentrated over East and Central Java (WHO, 2014a). Furthermore, it was reported that in India, Indonesia, Sri Lanka and Thailand, the epidemic season started up to 4 weeks earlier than normal, in part due to the heavy rainy season in 2010. In the same year, Vietnam has occurrence rates 10 times greater than in 2009 (CDC, 2010). In Malaysia itself, this deadly disease had killed 107 people in 2005 and 102 people in 2004 (Chen *et al*., 2006). In 2014, Bernama reported that dengue fever striked 17 deaths and 9,453 cases during January up to 2 February 2014, an increase over 100% of previous year (Bernama, 2014). It was reported that the case increased up close to 90,000 cases in December 2014. Figure 1.2 shows the dengue cases statistic in Malaysia from 1995-2014 ("Development of dengue vaccine: status sreview and future considerations," 2014). As of February 2015, the cases reached up to 59% more as compared to the

previous year, with 15,039 cases and 44 deaths as reported by Malaysian Department of Health (WHO, 2015).

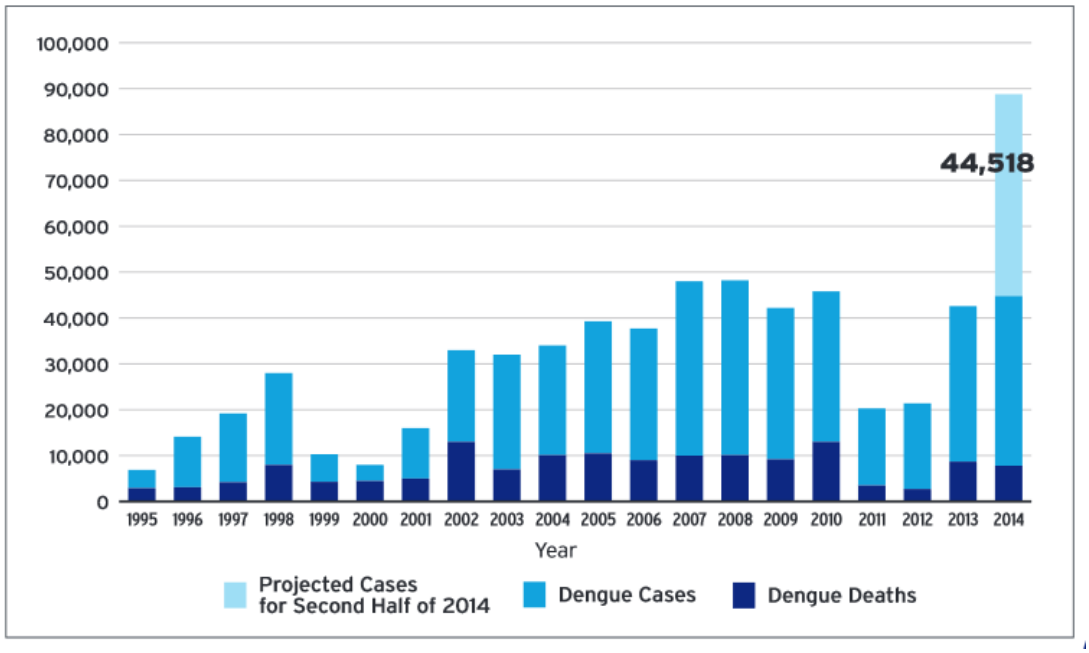


Figure 1.2 The dengue case statistic in Malaysia from 1995-2014 ("Development of dengue vaccine: status sreview and future considerations," 2014).

Dengue virus carries a positive single strand RNA in its genome. Serologically, this virus is divided into four serotypes, i.e. DENV1, DENV2, DENV3 and DENV4 (Jitendra and Vinay, 2011). Among those serotypes, DENV2 is the most prevalent type in dengue epidemic, especially in the South East Asian region. The virus genome is encoded by three structural proteins (C, prM, E) as well as seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Chambers *et al*., 1990; Henchal and Putnak, 1990). Understanding of the virus life cycle promotes the clearer key targets of gene/protein in the replication of the virus, thus, is important in a drug design study. Currently, the serine protease of dengue virus has been a major target in dengue drug discovery (Luo *et al*., 2008; Mueller *et al*., 2008).

Presently, available vaccines e.g. life recombinant, DNA and subunit vaccine are limited by their ability to protect the body system from the viral infection on one serotype only, e.g. if the vaccine is sensitive against DENV1 only, thus the patient is still exposed to the dangers of being infected by other serotypes (DENV2-4). Therefore, the discovery of antiviral drug is of utmost concern in the treatment of dengue and its related diseases (Nair *et al*., 2009; Tambunan *et al*., 2011). Up to now, there is no established antiviral drug able to inhibit the dengue virus replication. There are only symptomatic treatment administered to the dengue patient to relieve the symptoms such as analgesic-antipyretic for fever and blood transfusion to recover the thrombolytic level in hemorrhagic patient (Kumar *et al*., 2012; Zhou *et al*., 2008). One of antiviral drugs, ribavirin, successfully inhibited the virus replication *in vitro* but it is limited in *in vivo* study due to the fact that the sugar moiety of this compound contributes to its poor bioavaibility during oral administration (Sampath and Padmanabhan, 2009; Takhampunya *et al*., 2006).

NS3 protease (NS3pro) is a trypsin like serine protease which plays a role in a post-translation from the genome to its proteins as well as its maturation. This enzyme has a catalytic triad made up by His51, Asp75 and Ser135 and enhanced by another non-structural protein named NS2B as an enzyme cofactor (Frimayanti *et al*., 2011/2012; Yin *et al*., 2006b; Yin *et al*., 2006a). This cofactor activity is due to its hydrophilic region which is responsible for holding and promoting the activation of NS3 while the hydrophobic part playing around the membrane association upon the cleavage process (Chanprapaph *et al*., 2005; Kee *et al*., 2007; Niyomrattanakit *et al*., 2004; Steuer *et al*., 2009).

In most cases, the discovery of dengue antivirus by targeting NS2B-NS3pro activity was based on the non-prime substrates which were identified by profiling dengue virus using tetrapeptides (Frecer and Miertus, 2010; Yang *et al*., 2011). This peptidomimetic compound is able to inhibit the enzyme activity in a nanomolar concentration; however it is devoid of drug-like structure which can create a problem in physicochemical stability either during pharmaceutical preparation or in further pharmacokinetic step. As such, no peptidomimetic compound has been clinically used as dengue antivirus (Katzenmeier, 2004; Steuer *et al*., 2011).

Within the last decade, the discovery of NS2B-NS3pro inhibitor have been made possible by small molecules design (Kiat *et al*., 2006; Sidique *et al*., 2009; Tomlinson and Watowich, 2011), assisted by Computer Aided Drug Design (CADD). These virtual experiments are very helpful to avoid the trials and errors when the tested compound examined its activity *in vitro* as well as *in vivo* (Acharya *et al*., 2011; Korb *et al*., 2009; Tang and Marshall, 2011). There are various methods such as structure based drug design (docking and molecular dynamics) and ligand based drug designs (pharmacophore and quantitative structure-activity relationship (QSAR)).

To date, there is no effective vaccine or antiviral drug available to protect against dengue diseases (Nair *et al*., 2009; Tambunan *et al*., 2011). Thus, as mortality and economic burden of this disease are quite high, major effort must be put in developing effective antiviral for the treatment against dengue.

Recently, a current unpublished study from Pharmaceutical Design and Simulation Laboratory, School of Pharmaceutical Sciences, USM, found that ferulic acid which was isolated from *Garcinia mangostana* pericarps showed a reasonable inhibition toward H1N1 neuraminidase with IC_{50} = 200 µM. Although this activity is still far away from the commercial drug (oseltamivir, K_i = 3.78 nM) but ferulic acid structure can be used as a scaffold for NA inhibitor. Looking at the structure of ferulic acid, there are three functional groups identified probably contributing to H1N1 neuraminidase inhibition, i.e. carboxylic acid, hydroxyl, and methoxy groups. Furthermore, the ring system of aromatic compound is more planar than shikimic acid derivatives which showed bound to the NA active site and had high anti-neuraminidase activity (Chand *et al*., 1997). Ferulic acid has a highly correlated structure with vanillin (see Figure 1.3). Ferulic acid can be prepared synthetically by reacting vanillin and malonic acid; and vice versa, vanillin can be produced by hydrolyzing ferulic acid at a certain condition.

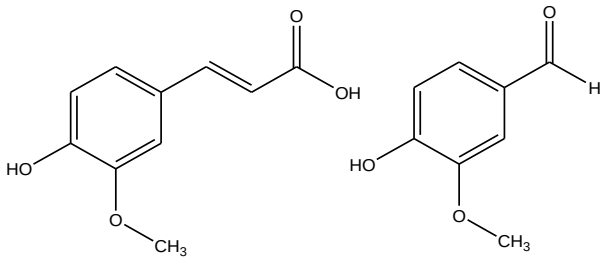


Figure 1.3 The structure of ferulic acid and vanillin.

On the other hand, also unpublished previous works from Pharmaceutical Design and Simulation (PhDs) laboratory, USM, found four National Cancer Institute (NCI) compounds possessed bioactivity *in vitro* against DENV2 NS2B-NS3pro from their virtual screening. Those four NCI compounds showed some hydrogen bond interactions to amino acid residues which are important for DENV2

NS2B-NS3pro activities. The docking results corresponded well with the *in vitro* study. These four compounds, however are not typically classified as peptidomimetic, therefore, there is an opportunity to develop small molecules to be the lead compound of dengue antivirus. Among those NCI compounds, the diversity0713 (see Figure 1.4) which has a thioguanine scaffold is considered chemically accessible in term of synthetic route as well as the availability of starting materials.

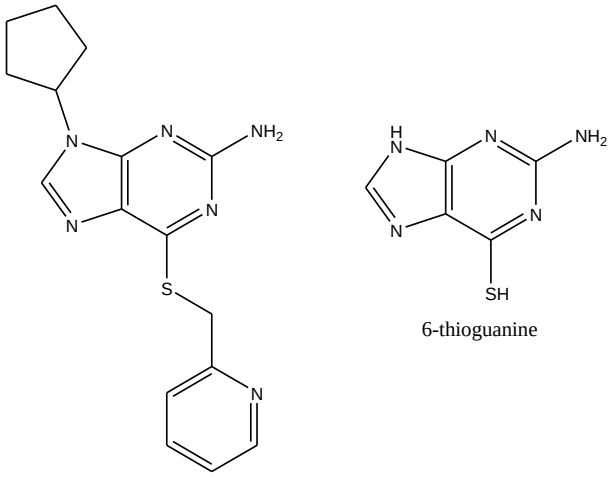


Figure 1.4 The structure of diversity0713 (www.pubchem.com) and thioguanine.

1.2 Objectives

The goal of this study is to discover novel neuraminidase and dengue antivirus. Specifically the objectives are:

1. To model and design a series of H1N1 NA and DENV2 NS2B-NS3pro inhibitors bearing ferulic acid and thioguanine scaffold respectively, using *in silico* methods.
2. To synthesise the designed H1N1 NA and DENV2 NS2B-NS3pro inhibitors.

3. To evaluate the *in vitro* activities of the synthetic compounds (H1N1 NA and DENV2 NS2B-NS3pro inhibitors) against H1N1 NA and DENV2 NS2B-NS3pro, respectively.
4. To study the quantitative structure-activity relationship (QSAR) of the synthetic compounds, ferulic acid and thioguanine derivaties as the H1N1 NA and DENV2 NS2B-NS3pro inhibitors, respectively.

CHAPTER TWO
LITERATURE REVIEW

2.1 Influenza A and Its Drug Treatment

2.1.1 Influenza A virus

Influenza A virus is a pathogen which commonly infects the upper respiratory tract causes several symptoms such as myalgia, malaise, and fever for a few days as common respiratory symptoms. The condition can be more complicated when it affects other cells or organs causes pneumonia and myocardial infection (Kuszewski and Brydak, 2000; Svetlikova *et al*., 2010). The major pandemics were reported in three periods during 20th centuries: 1918-1921 (H1N1"Spanish" Influenza), 1957-1958 (H2N2"Asian" Influenza) and 1968 (H3N2"Hongkong"Influenza) (Gautret *et al*., 2010). Since 1997 to 2005, H5N1 influenza became dominant in virus circulation among birds but later on, it kills hundreds people worldwide (Saif and Espinoza, 2006). The swine flu (H1N1) virus was firstly found having human to human transmission in Mexico (2009) but luckily, this virus is less pathogenic than H5N1 virus (Simonsen *et al*., 2011). The updated status of H1N1 reported by WHO in 2009 indicated this influenza virus found in 381 human specimens in Washington DC (WHO, 2014a).

Influenza A virus is a negative-strand RNA virus belonging to orthomyxo viruses. The shape of the virus is a pleomorphic particle, typically having a spherical or long 'lollypop' like filament shape. The enveloped-negative RNA segmented genomes are packed into a nucleocapsid which complexes with protein polymerase. This RNA-protein (RNPs) complex is packed in a lipoprotein envelope lined by three

surface proteins: hemagglutinin (HA), neuraminidase (NA), and the M2 proton ion channel (Magano, 2009; Shtyrya *et al*., 2009).

Figure 2.1 shows the schematic diagramme of Influenza A virus. Hemagglutinin (HA) is a glycoprotein which recognizes the cell targets by binding to sialic acid receptor on the host cell membrane. This protein binds to the receptor via α-ketosidic-linked terminal that are followed by fusion of the viral through the endosomal membranes succeeding endocytosis. After endocytosis, the pH of the cell changes and triggers the prolongation of the central coiled at the N-terminus thus drives out the fusion peptide and penetrates into a cellular membrane (Isin *et al*., 2002; Sollner, 2004; von Itzstein, 2007). There are 15 subtypes of HA (H1-H15); three of them: H1, H2 and H3 attack humans by binding at the sialic acid receptor in the respiratory tract; while another subtype, H5, invades protein in avian digestive enzyme (Fouchier *et al*., 2005). The swine is susceptible toward both human and avian influenza virus, thus the novel strain of H1N1can be generated by those two virus reassortment in this species leading to the theory called as “mixing vessel” (Ma *et al*., 2007).

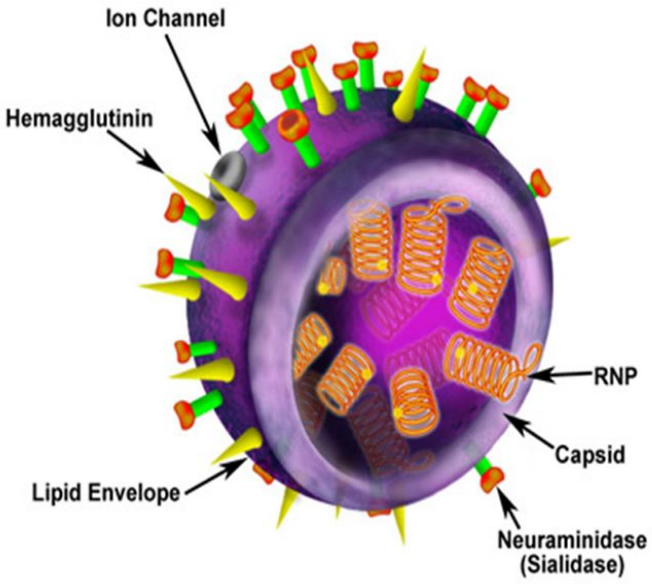


Figure 2.1 The influenza viral lipid envelope with a nucleocapsid containing three surface proteins: hemagglutinin (HA), neuraminidase (NA), and the M2 proton ion channel. The genome of the viral RNAs are presented as red coils bound to Ribonuclear Proteins (RNPs) (Betakova, 2007).

Neuraminidase (NA), also known as sialidase, exists as a tetramer of identical subunits. It is normally attached to the virus surface through a long protein stalk. The active sites are in a deep depression on the upper surface. They bind to polysaccharide chains of the sialic acid receptor and clip off the sugars (sialic acid) at the terminal end. The surface of neuraminidase is decorated with several polysaccharide chains (seen extending upwards and downwards in this structure, Figure 1.1) that are similar to the polysaccharide chains that decorate our own cell surface proteins. NA can be divided into 9 subtypes (N1-N9) and it is important for viral replication and infection (Du *et al*., 2007; Tisoncik *et al*., 2011). This enzyme cleaves the terminal sialic acid moieties from the receptors to facilitate release of the virion progeny from the infected cell. NA is able to facilitate the early processing of influenza virus infection in the lung epithelial cells. Due to these essential roles, NA

has been an attractive target in anti-influenza discovery (Du *et al*., 2007; Gong *et al*., 2007; Platis *et al*., 2006; Wang *et al*., 2010).

M2 ion protein channel is the third membrane protein which provides the virus structural integrity by permitting the proton to enter the virus particle during un-coating of the virion in the endosome (Bauer *et al*., 1999). It is a homotetramer consisting of four polypeptide chains from 96 amino acids, with the structural domains: an amino-terminal extracellular domain (comprising 23 residues), as a single internal hydrophobic domain that acts as a trans-membrane domain (19 residues) and 54-residue cytoplasmic tails. This membrane protein is important to prevent inactivation of progeny virus as well as premature acid activation of the newly synthesised HA (Betakova, 2007; Rossman *et al*., 2010).

2.1.2 Influenza A neuraminidase

The crystal structure of neuraminidase first solved in 1983 (Varghese *et al*., 1983) has given a glimpse on the mechanism of action of this enzyme at a molecular level. This structure was of N2 subtype at a resolution of 2.9 Å, showing that the enzyme adopts a tetramer shape (see Figure 2.2) with the total molecular weight of 240 kDa. Each monomer is formed by six 4-stranded of anti-parallel β-sheets which is arranged as blade propellers around the central of pseudo six fold. The first strand of each sheet is parallel to the central of the propeller and the outer strand is vertical toward it, causes each strand being twisted. The outermost strand of the first sheet is linked to the central of strand for the next sheets. Loops connecting these strands contain a diverse important amino acids and form enzymatic site on its upper surface.

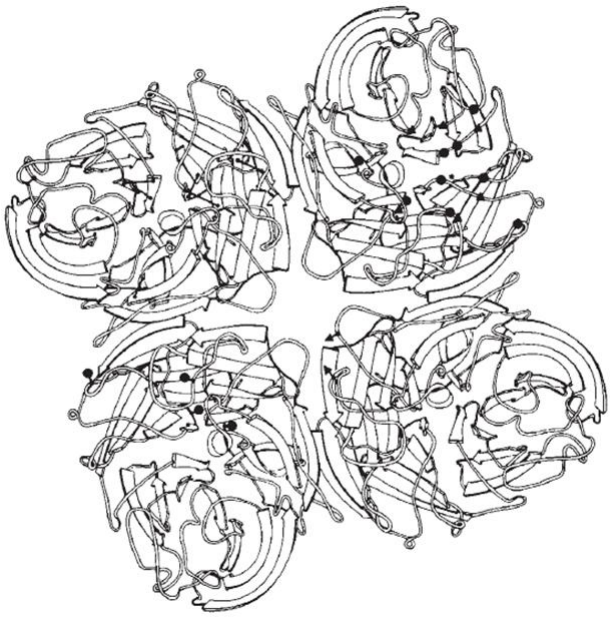


Figure 2.2 The tetramer shapes of neuraminidase N2 (Varghese *et al*., 1983).

Phylogenetic studies have divided the neuraminidase influenza virus into two groups, Group 1 contains subtypes N1, N4, N5 and N8 and Group 2, subtypes N2, N3, N6, N7 and N9 (Gong, 2007; Tisdale, 2009). The main difference between these two groups is the presence of a loop consisting amino acids 147 to 151 which is also named as 150-loop (Xu *et al*., 2008). For example, the 150-loop of the N1 has Val149 while for N9 it is formed by Ile149. For both groups, the 150-loop contains an amino acid Asp151 as shown in the superposition of N1 and N9 in Figure 2.3. However, in N1, its side chain has a carboxylic acid steering away from the active site of the enzyme in an open conformation, while in N9, it is a close form (Du *et al*., 2007).

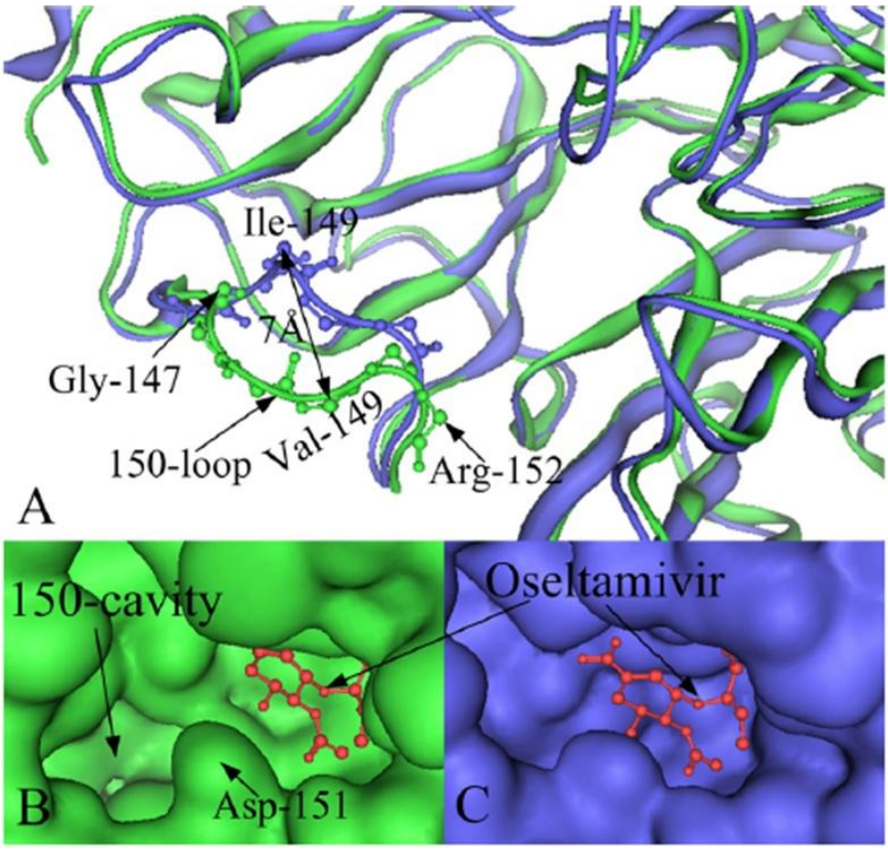


Figure 2.3 The comparison of crystal structures between N1 and N9. (A) A superposition of N1 (PDBID 2HTY) and N9 enzyme (PDBID 1F8B) where the key residues are shown in ball-and-stick forms. (B) Ligand in the active cavity of 2HTY with adjacent 150-loop. (C) Ligand in the active cavity of 1F8B (Du *et al*., 2007).

The function of NA is to facilitate mobility of the virus, both to and from the site of infection. NA catalyzes the cleavages of (2-6) - or α (2-3)-ketosidic linkage between a terminal SA and inward-face sugar residue. This broken bond facilitates to spread the virus in the respiratory tract and allows the elution of progeny virus from the infected cells. The removal of sialic acid from the oligosacharide moiety of HA and NA also helps to prevent the virus self-aggregation after leaving the host cells (Gong *et al*., 2007). The mechanism of neuraminidase activity that facilitates the viral replication is shown in Figure 2.4.

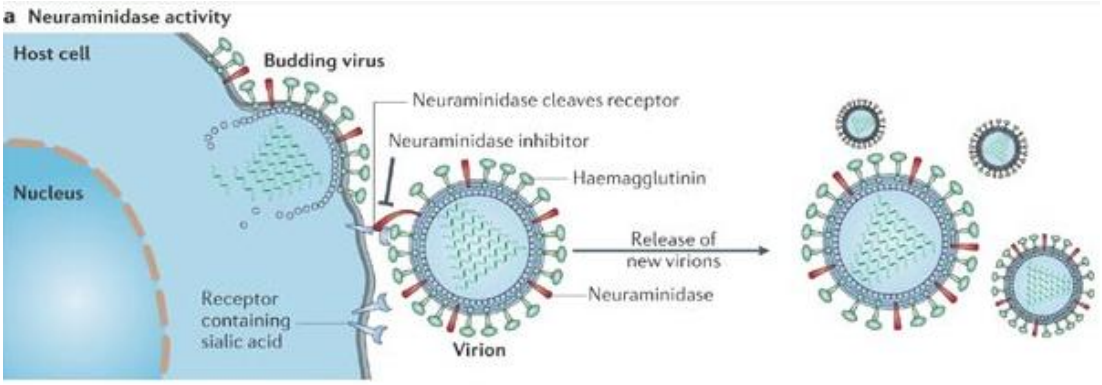


Figure 2.4 The cleavage of the new synthesised virus from its sialic acid receptor by neuraminidase (Clercq, 2006).

The availability of X-ray crystal structures of neuraminidase with high-resolutions complexed with sialic acid (SA, N-acetylneuraminic acid, Neu5Ac) has improved the understanding of the action mechanism of this enzyme. Figure 2.5 described the active binding site of neuraminidase when co-crystallized with Neu5Ac. This active site is highly conserved and presents a rigid catalytic center (Gong *et al*., 2007; von Itzstein, 2007; Yen *et al*., 2006). There are eight amino acids which directly make contact with Neu5Ac which provide conserved binding by the charge-charge interaction between the carboxylate group and three positively charged arginine residues (Arg118, Arg292 and Arg371). The NH group of 5-N-Acetyl interacts with the active site cavity via hydrogen bonding with water molecule while the oxygen carbonyl on the same 5-N-Acetyl moiety of Neu5Ac bonds to N of Arg152 via direct hydrogen bonding while its two hydroxyl groups of glycerol side chain are bonded to the carboxylate oxygens of Glu276. In addition, the 2-hydroxyl of Neu5Ac makes a direct contact with the carboxylate oxygen of Asp151 (Varghese, 1999; von Itzstein, 2007).

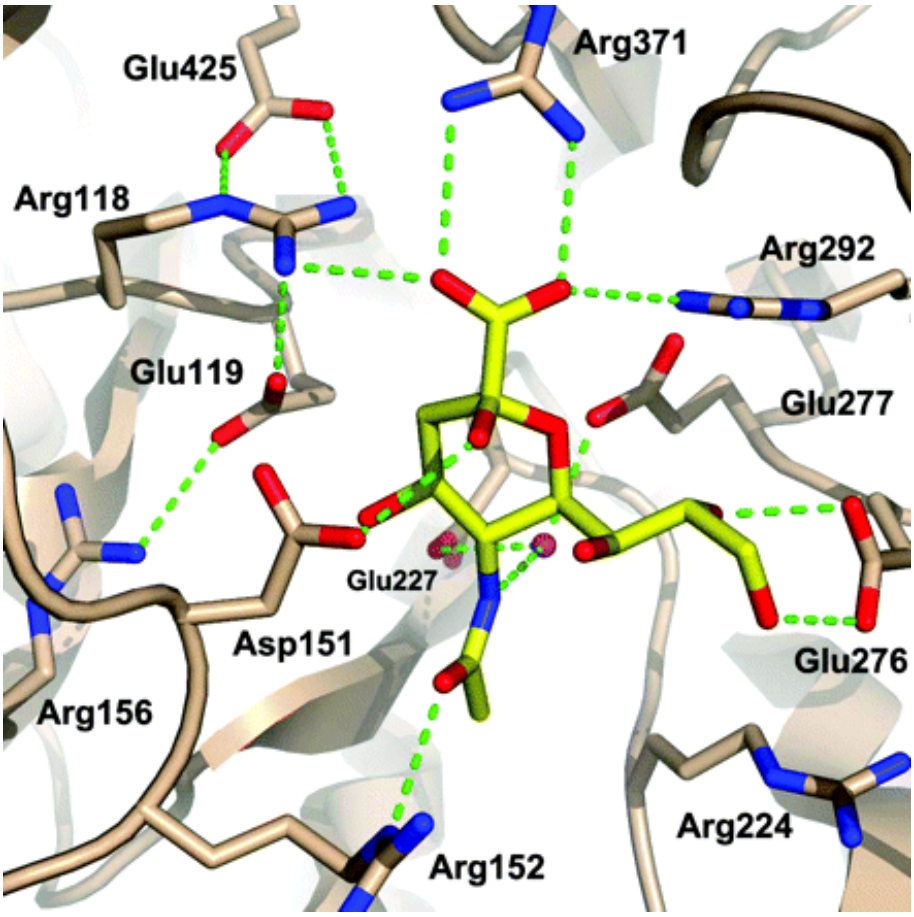


Figure 2.5 The binding site of A/Tokyo/3/67 (H2N2) influenza virus NA and sialic acid (PDBID 2BAT). The green dot lines describe the hydrogen bonding between ligand and specific amino acid residues of NA's active site (Yen *et al*., 2006).

2.1.3 Neuraminidase Inhibitors

Most potent NA inhibitors were developed based on the structural information of the N2 NA conserved active site and its complex with sialic acid. Figure 2.6 shows the two-dimensional 'airplane' model to illustrate the NA active site (Wang *et al*., 2010). This 'airplane' model was used to define the Structure-Activity Relationships (SARs) of NA inhibitors, which is mainly determined by the relative positions of the substituents (carboxylate, glycerol, acetamido and hydroxyl) in the central ring of the inhibitor. There are four well conserved active site domains which consist of Site 1 (Arg118, Arg292, Arg371), Site 2 (Asp151, Glu119,

Glu227), Site 3 (Ile222, Trp178) and Site 4 (Glu276, Glu277) (Gong *et al*., 2007; Zhang *et al*., 2008a).

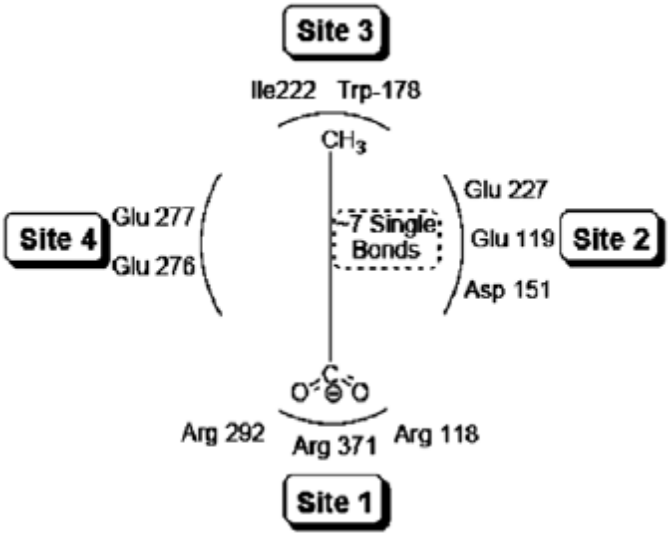


Figure 2.6 'Airplane' model of NA active site (Zhang *et al*., 2008a).

The elucidation of the three-dimensional structure of influenza neuraminidase located the catalytic site, which makes the design of highly effective inhibitors became feasible. The first NA inhibitor is 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA (**1**), Figure 2.7) synthesised in 1969 (Chen *et al*., 2013). It was designed as a mechanism-based inhibitor and was proposed to be a transition state analogue with K_i ~1pM. Compound **1** showed a good activity *in vitro* but was found inactive as antivirals in an animal model (Laver and Elspeth, 2002). Earlier structure based drug design work, showed the availability of space between **1** and the enzyme in the vicinity of the 4-hydroxyl binding pocket, and thus, it was desirable to fill that space with basic substituents on the sugar (Smith *et al*., 2001). Upon this information, the compounds targeted for synthesis were 4-amino-Neu5Ac2en (**2**) and 4-guanidino-Neu5Ac2en (**3**) (Figure 2.7). The K_i of these compounds are 20 and 5000 times respectively improved over that for **1** when

assayed against the neuraminidase of the viral isolate from which the structure had been determined.

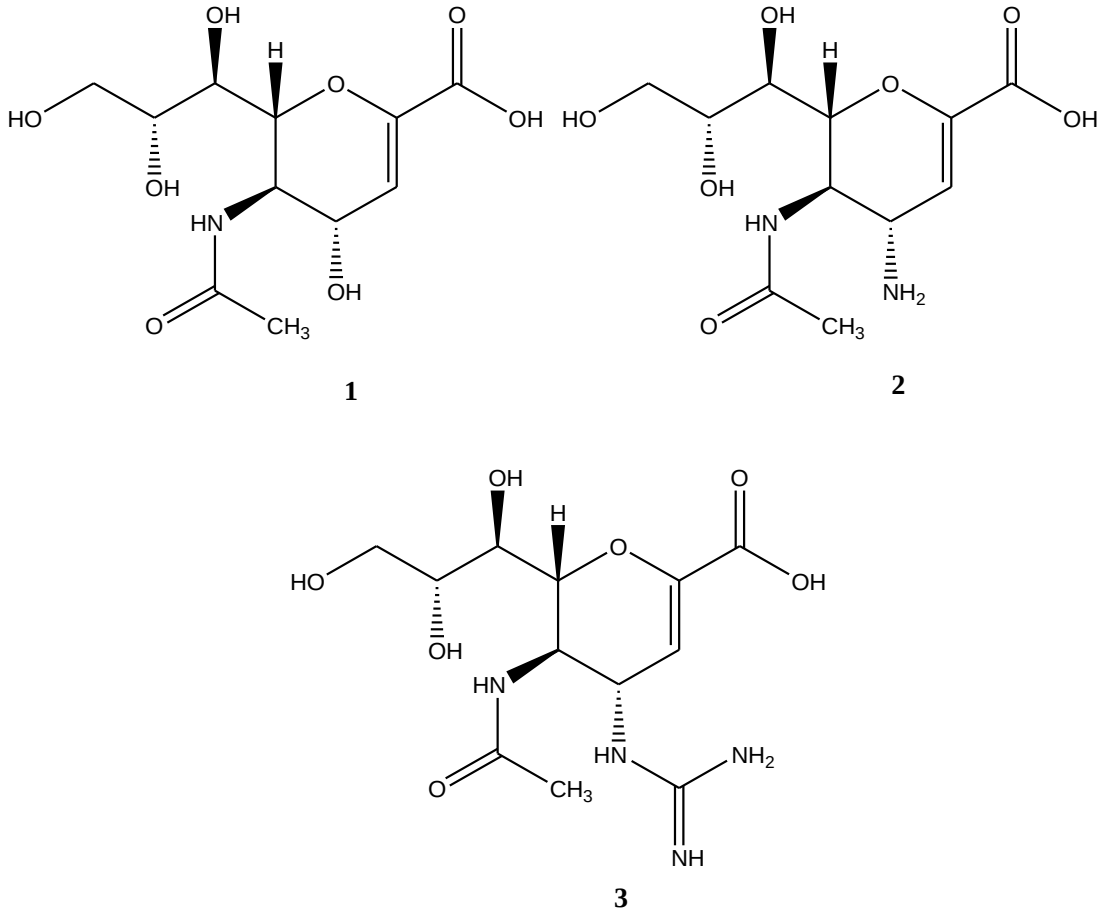
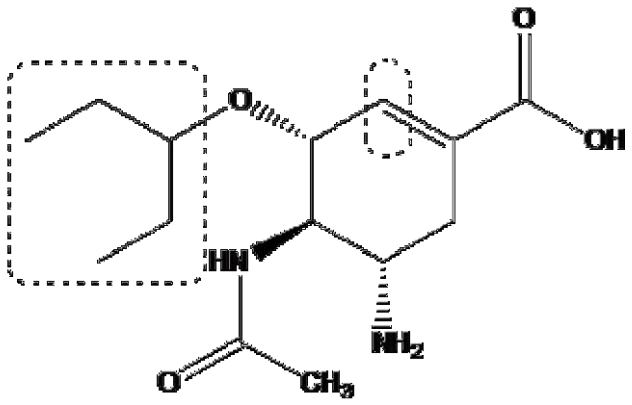


Figure 2.7 The structure of NA inhibitors from sialic acid derivatives (Tapar *et al*., 2011).

Compound **3** or its drug name Zanamivir has an oral inhalation administration not only due to its high polarity, but also consciously intended for direct delivery to the respiratory tract, the principal site of the viral replication (Gupta *et al*., 2011; Sun *et al*., 2010). This sialic acid derivative showed a potent antiviral activity *in vitro* against influenza A as well as B viruses, including amantadine-and rimantadine-resistant isolates. Further study also showed that **3** is active against avian influenza A viruses, including influenza A H5N1, H6N1, H7N7 and H9N2 as well as some influenza strains resistant to oseltamivir (Elliot, 2001).

A new orally active neuraminidase inhibitor, coded by GS4071 (**4**) was designed to overcome the problem of oral bioavailability of zanamivir (Zhang *et al*., 1997). The decrease in polarity contributed by carboxyl, hydroxyl as well as guanidine in zanamivir was achieved in **4** by removing the heterocyclic oxygen but augmenting the isopentyl as shown in Figure 2.8.



4

Figure 2.8 The structure of **4** with the dot boxes describes the nonpolar site of the particular structure.

The similarity of this compound to the compound **2** is evident especially their binding mode as demonstrated from the X-ray crystal structure of the complexes bound to neuraminidase (Figure 2.9). The carboxylate, acetyl as well as amino group belongs to the compound **4** were shown to bind to the conserved amino acid residues of neuraminidase's active site. In addition, the isopentyl group makes several favorable hydrophobic contacts with amino acid residues Ile222, Arg224, and Ala246, increasing the binding affinity (Lew *et al*., 2000).

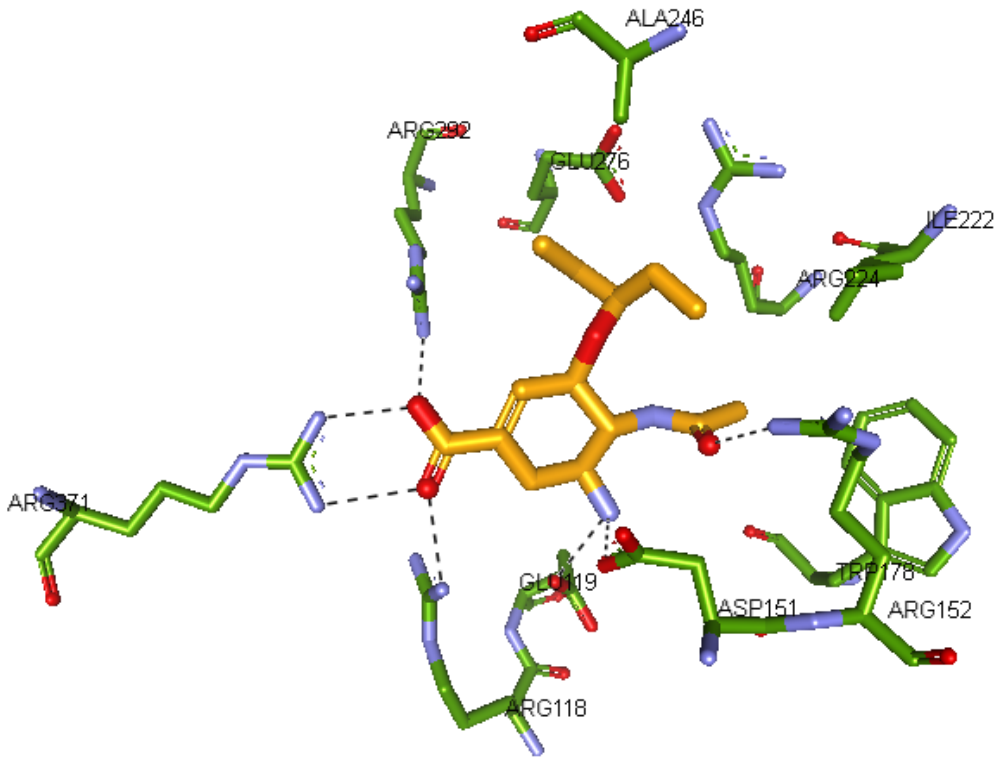


Figure 2.9 The X-ray crystal structure of neuraminidase complex with **4**. The hydrophobic interactions are indicated by the closeness between isopentyl group and amino acid residues Ile222, Arg224 and Ala246 (Lew *et al*., 2000).

In pharmacokinetic experiments, **4** demonstrated only ~5% bioavailability in rats which is similar to **3**. Somehow, the conversion of carboxylic acid to its ester form, oseltamivir, provides five folds higher bioavailability than **4** when it is administered orally. It was found that the ethyl ester form of this compound metabolized via hydrolysis reaction to become carboxylate, the active metabolite which is shown to have poor bioavailability. Therefore, the phosphate salt of oseltamivir was developed to overcome the bioavailability problem thus, the compound can be administered orally (D'Souza *et al*., 2009; Sun *et al*., 2010).

The recent X-ray crystallographic study reveals that influenza A virus of the group-1 NA (N1) contains a larger cavity adjacent to the active site, formed by

residue 147-152 (150-loop) which is not found in the group 2 NAs. The group 1 NAs can bind ligands in the open as well as in the closed conformation. However, only the close conformational state was found in the group 2 NAs. The flexible 150-loop residues are occupied on the vicinity by the ligand and interact mostly with the amino and acetamido groups of **4**. The active sites are still conserved at the triad arginines (Arg118, Arg292, and Arg371) that interact with the carboxylate group. Moreover, the affinity is increased by the interaction between the acetamido group and Glu276 that forms hydrogen bonds with the substrate of hydroxyl groups (Collins *et al*., 2008; Rungrotmongkol *et al*., 2009).

A new scaffold containing five members of alicyclic compound was introduced to generate the antiviral activity against influenza virus via neuraminidase inhibition (Bianco *et al*., 2005). Peramivir (**5**) with the cyclopentane core attached with carboxylic acid as well as acetylamino group and showed to possess *in vitro* activity against H1N1 virus with IC_{50} = 0.38 μM (Ikematsu *et al*., 2012). The crystal structure of N8 complex with Peramivir (PDBID 2HTU) shows the conserved binding mode of this compound to the enzyme (see Figure 2.10).

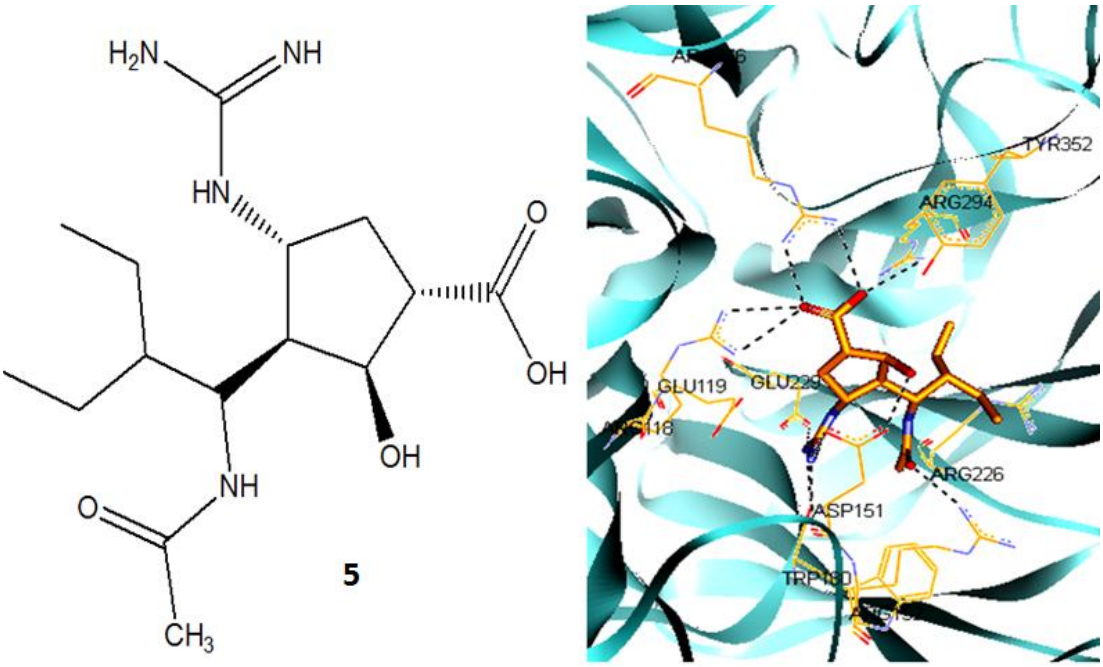


Figure 2.10 The structure of **5** in 2D as well as its 3D structure complex to N8 NA (PDBID 2HTU) was generated using Discovery Studio 2.5.

The latest NA inhibitor clinically approved in Japan in 2010 is Laninamivir which is proven effective against oseltamivir-resistant mutant. This drug is also prepared in octanoate ester form in the major structure of **3**. This laninamivir ester (**6**) is unique in its ability to rotate the Glu276 to form a salt bridge with Arg224, thus giving more space for Asn294 to interact with the the 9-ester-O of Laninamivir when it was crystallized with 2009 H1N1 NA (p09N1) (see Figure 2.11) (Ikematsu and Kawai, 2011; Vavricka *et al*., 2011).