NEURAMINIDASE INHIBITION AND *IN VITRO* ANTI INFLUENZA A (H1N1) ACTIVITIES OF *BRUCEA JAVANICA* (L.) MERR

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UNIVERSITI SAINS MALAYSIA

2013

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by

NENY PURWITASARI

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

APRIL 2013

ACKNOWLEDGMENT

Praise to Allah Alone, the Lord of the worlds. I wish to express my deepest gratitude to my principal supervisor Assoc. Prof. Dr. Nornisah Muhamed, my co-supervisors Prof. Dr. Habibah A.Wahab and Prof. Dr. Chairul Anwar Nidom, DVM, for their encouragement to finish this work, endless support, guidance and constructive criticism during these years. I consider myself very fortunate for being able to work with them.

I owe my thanks to the Dean of School of Pharmaceutical Sciences, Universiti Sains Malaysia for providing the necessary facilities to enable me to complete this study.

I wish to extend my thanks to Directorate General of Higher Education, Ministry of Education and Culture of the Republic of Indonesia, for granting me financial support and study leave to pursue this study.

I appreciate the help of Pharmaceutical Design and Simulation (PhDS) laboratory members, especially N. Kusaira K. Ikram, Pak Muchtaridi, Muhamad Yusuf, for helping me a lot carried out this study. I thankfully acknowledge Maywan Hariono and Dr. Choi Sy Bing for their inspiring discussion and proof reading of this thesis.

I would like to thank to Rector of Airlangga University, and to all members of Avian Influenza and zoonotic Research Center (AIRC), Airlangga University for providing the facility. I thank to Reviany Vibriaanita Nidom for her valuable help and discussion regarding the virus bioassays.

I would like to express my appreciation to my dear parents who always give me prayer and blessing for my study.

Finally, my deep and special thanks are addressed to my husband, Mat Sjafii, and our wonderful and active son Rasyad for love and joys they share to me and for their patience and encouragement during the hard times of our life.

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LIST OF ABBREVIATIONS AND SYMBOLS

AA	Amino Acid
AIRC	Avian Influenza and zoonotic Research Center
Ala	Alanine
Arg	Arginine
Asn	Asparagine
BSA	Bovine serum albumine
BSC	Bio-Safety cabinet
BSL-3	Bio-Safety Level-3
BuOH	Butanol
$CaCl_2$	Calcium chloride
CPE	Cythophatic effect
DANA	2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulfoxide
dpf	Docking parameter file
ESI-MS	Electron Spray Ion-Mass Spectrometry
EtOAc	Ethyl acetate
FBS	Fetal Bovine serum
FCS	Fetal Calvin serum
FDA	Food Drug Association
FEB	Free energy of binding
FT-IR	Fourier Transform Infra Red
g	grams
Glu	Glutamic acid
Gly	Glycine
GM	Growth medium
HA	Hemaggutinin
His	Histidine
IC_{50}	Half maximum inhibitory concentration
Ile	Isoleusine
L	liter
Lys	Lisyne
M1,M2	Matrix protein
MDCK	Madin Dabin Canin Kidney
MeOH	methanol
MES	(2-Morpholino) ethanesulfonic acid
MEM	Minimum Essential Medium
M	molar
MM	Maintenance medium

mg	miligrams
mL	mililiter
mm	milimeter
mM	milimolar
mU	miliunit
MOI	Multiplicity of Infection
MNTD	Maximum non-toxic dose
MUNANA	2'-(4-methylumbelliferyl)-a-D-acetylneuraminic acid
m.p	melting point
Na	Sodium
NA	Neuraminidase
NaCl	Sodium chloride
NADI	Natural Drug Discovery
NANA	N-Acetyl Neuraminic Acid
NaOH	Sodium Hydroxide
NAI/NI	Neuraminidase Inhibitors
NeuAc	*N*-acetylneuraminic
NP	Nucleoprotein
PBS	Phosphate buffer saline
PDB	Protein Data Bank
PFU	Plaque Forming Unit
PD	Proportionate distance
Pen-strep	Penicillin- streptomycin
ppm	Part per million
RBC	Red blood cell
RMSD	Root Mean Square Deviation
RNA	Ribbo Nucleic Acid
SA	Sialic acid
S-OIV	Swine Origin Influenza Virus
$TCID_{50}$	Half Tissue Culture Infectious Dose
TLC	Thin Layer Chromatography
TPCK	Tosylsulfonyl phenylalanyl chloromethyl ketone
Tyr	Tyrosine
UV	Ultra Violet
V	Volume
WHO	World Health Organization
^{1}H-NMR	Proton-Nuclear Magnetic Resonance
^{13}C-NMR	Carbon-Nuclear Magnetic Resonance
µL	micro liter
Å	Angstrom
ΔG	Free energy of binding
ºC	Degree centigrade
%	percentage

®	registered trade mark
J	coupling constant
δ	Chemical shift
s	singlet
d	doublet
t	triplet
br	broad
3D	Three dimension
2D	Two dimension

APPENDICES

AKTIVITI PERENCATAN NEURAMINIDASE DAN ANTI SELESEMA A(H1N1) SECARA *IN VITRO BRUCEA JAVANICA* (L.) MERR

ABSTRAK

Wabak virus selesema A (H1N1 and H5N1) telah menimbulkan kebimbangan global terhadap risiko pandemik di masa akan datang. Oseltamivir, perencat neuraminidase semasa , tidak dapat memenuhi permintaan jika terdapat wabak utama. Oleh itu, terdapat keperluan untuk mencari rawatan alternatif untuk influenza A. Kerja pendokan molekul ke atas neuraminidase telah membawa kepada pemilihan tumbuhan untuk dikaji iaitu, *Brucea javanica* (L.) Merr (Simaroubaceae). Objektif kajian ini adalah untuk menentukan perencatan neuraminidase dan menentukan secara *in vitro* aktiviti antivirus influenza A *Brucea javanica* (L.) Merr. Dalam usaha untuk mendapatkan maklumat mengenai sebatian aktif daripada tumbuhan ini, buah-buahan kering telah diekstrak dengan menggunakan metanol dan difraksinasi menggunakan n-heksana, etil asetat dan n-butanol. Satu sebatian telah diasingkan daripada fraksi etil asetat, yang dikenalpasti sebagai β-sitosterol 3-*O*-*β*-*D*-glukopiranosida atau daukosterol. Ekstrak mentah metanol, fraksi *n*-heksan, fraksi etil asetat, fraksi *n*-butanol serta daukosterol telah tertakluk kepada asai perencatan neuraminidase (asai MUNANA) terhadap *Clostridium perfringens*, H5N1 dan H1N1 enzim neuraminidase dan asai antivirus *in vitro* terhadap virus A/Indonesia/Unair/2011 (H1N1) pada sel-sel MDCK. Pengurangan titer hemaglutinin telah dijalankan menggunakan asai penghemaglutinan (HA), manakala pengurangan titer jangkitan virus dijalankan menggunakan asai setengah Dos Jangkitan Kultur Tisu ($TCID_{50}$). Fraksi etil asetat menunjukkan perencatan yang paling tinggi terhadap *Clostridium perfringens* neuraminidase, dengan IC_{50} sebanyak 136,46 μg / mL,

dan fraksi *n*-heksana mengurangkan titer hemagglutinin hampir sifar dalam asai penghemagglutinan. Manakala, daukosterol telah mengurangkan 99.9% titer jangkitan virus H1N1 dan menunjukkan aktiviti perencatan tertinggi terhadap neuraminidase H1N1 dan neuraminidase H5N1 masing-masing dengan IC_{50} 117.45 μg/mL dan 148.93 μg/mL. Menariknya, struktur kimia daukosterol adalah berbeza dengan perencat neuraminidase biasa, yang lazimnya dibangunkan berdasarkan analog-analog asid sialik. Oleh itu, pendokan molekul (Autodock 3.0.5) telah dijalankan untuk menentukan interaksi daukosterol pada tapak pengikat H1N1 neuraminidase (id PDB: 3TI6). Keputusan ini telah menunjukkan bahawa daukosterol mempunyai keafinan ikatan yang baik pada neuraminidase H1N1 dengan tenaga pengikat bebas yang rendah (ΔG = -15,29 kkal / mol). Kesimpulannya, *Brucea javanica* (L.) Merr mempunyai aktiviti perencatan virus berpotensi, bukan sahaja dengan menghalang virus neuraminidase tetapi juga oleh dengan merencat aktiviti penghemagglutinan virus dan mengganggu replikasi virus pada sel MDCK.

Kata Kunci : *Brucea javanica* (L.) Merr, Selesema A, Daukosterol, Assai MUNANA, Assai HA, $TCID_{50}$, Pendokan Molekul.

NEURAMINIDASE INHIBITION AND *IN VITRO* ANTI INFLUENZA A(H1N1) ACTIVITIES OF *BRUCEA JAVANICA* (L.) MERR

ABSTRACT

The outbreak of influenza A viruses (H1N1 and H5N1) has raised a global concern on the future risk of a pandemic. Oseltamivir, the current neuraminidase inhibitor, could not meet the demand if there is a major outbreak. Thus, there is a need to find alternative treatment for influenza A. Our molecular docking work on neuraminidase has led to the selection of plant to be studied, *Brucea javanica* (L.) Merr (Simaroubaceae). The objectives of this study were to determine the neuraminidase inhibition and *in vitro* influenza A antiviral activities of *Brucea javanica* (L.) Merr. In order to obtain information about the active compounds from this plant, the dried fruits were extracted using methanol, and fractionated using n-hexane, ethyl acetate and *n*-butanol. One compound was isolated from ethyl acetate fraction, identified as β-sitosterol 3-*O*-*β*-*D*-glucopyranoside or daucosterol. Crude methanol extract, *n*-hexane fraction, ethyl acetate fraction, *n*-butanol fraction as well as daucosterol were subjected to the neuraminidase inhibition assay (MUNANA Assay) against *Clostridium perfringens*, H5N1 and H1N1 neuraminidase enzymes and *in vitro* antiviral assay against A/Indonesia/Unair/2011 (H1N1) virus on MDCK cells. The reduction of hemagglutinin titre was performed in hemagglutination assay (HA Assay), while the reduction of virus infectious titre was performed in Half Tissue Culture Infectious Dose ($TCID_{50}$) assay. The ethyl acetate fraction showed highest inhibition towards *Clostridium perfringens* neuraminidase, with IC_{50} of 136.46 µg/mL and *n*-Hexane

fraction reduced the hemagglutinin titre near to zero in hemagglutination assay. Whilst daucosterol has shown to reduce 99.9 % of H1N1 virus infectious titre and exhibited the highest inhibition activity against H1N1 and H5N1 neuraminidases with IC_{50} of 117.45 µg/mL and 148.93 µg/mL, respectively. Interestingly, the chemical structure of daucosterol is different with the common neuraminidase inhibitors, which are usually based on sialic acid analogues. Thus, molecular docking (Autodock 3.0.5) was carried out in order to determine the interaction of daucosterol on the binding site of H1N1 neuraminidase (PDB id: 3TI6). The result indicated that daucosterol has good binding affinity with H1N1 neuraminidase with low free energy of binding (ΔG = - 15.29 kcal/mol). In conclusion, *Brucea javanica* (L.) Merr has a promising viral inhibition activity, not only by inhibiting viral neuraminidase but also by inhibiting the viral hemagglutination activities and interfere to the viral replication on MDCK cell.

Keywords: *Brucea javanica* (L.) Merr, daucosterol, influenza A, MUNANA assay, HA assay, $TCID_{50}$, Molecular Docking.

CHAPTER ONE

INTRODUCTION

1.1 Statement of Problem

In April 2009, the World Health Organization (WHO) announced global pandemic of new strain of influenza A virus called „swine flu" pandemic. It spread rapidly all over the world, causing hundreds of more reported cases within days. The new variant has been identified as originating of H1N1 form of swine influenza origin (S-OIV in short), a progeny strain that caused the huge and deadly flu pandemic in 1918-1920 (WHO, 2009a).

This was the first global influenza pandemic during 41 years. Starting from 1918-1919, the Spanish flu that similar with 2009 H1N1 infecting 5% of world population causing 20-50 millions of death, the Asian flu (H2N2) (1957-1958) causing 1- 4 millions deaths and in 1968, the Hongkong flu (H3N2) also causing 1- 4 millions deaths worldwide (Ebrahim, 2010).

In addition, the novel swine (H1N1) 2009 virus began spreading globally with unpredicted speed that WHO increased its pandemic alert phase from Phase 4 to Phase 5 on 29 April 2009. On 11 June 2009, the pandemic level was increased further from phase 5 to 6, the highest level, which has made the health authorities aware of its future potentials as the killer disease (WHO, 2009a).

As 1st August 2010, there were over then 18,449 deaths in more than 214 countries that have reported laboratory confirmed cases of pandemic influenza H1N1 2009, for the period April 2009 to August 2010. However, WHO stated that the total deaths (including unreported deaths) from the H1N1 strain were actually higher (WHO, 2010a). In fact, Dawood (2012) reported that the estimated global deaths associated with H1N1 pandemic 2009 influenza were 15 times higher, which were 284,400 deaths (Dawood *et al*., 2012).

Even in 10 August 2010, WHO has announced that the world were in post pandemic, it did not mean that the virus has moved out. The rapid change of the genetic mutation of the virus is expected to continue to circulate into seasonal influenza and cause another pandemic in the future. It is also feared that another high mortality causing virus might arise at anytime (WHO, 2010b). Two years after the post pandemic was announced, there are still sporadic cases reported. In August 2012, during weeks 30 to 31 (22 July 2012 to 4 August 2012), WHO reported that from 67 countries, there were 2,168 influenza cases, 8.5 % of them (172) related to influenza A (H1N1) pandemic 2009 virus (WHO, 2012).

Influenza is one of severe viral infections which is highly transmittable affecting severe upper respiratory illness, causes substantial morbidity and mortality (Zhang *et al*., 2008; Sundarajan *et al*., 2010). This virus belongs to Orthomyxoviridae family of RNA viruses, which divided into A, B and C types. Type A is more virulent then other influenza virus B and C. Among of them, only influenza virus A and B seem like pathogen to human life while influenza virus C has never been considered causing a

large-scale epidemic. The common species which are infected by Influenza A are avian, swine, equine, pigs, horse and human. Influenza B and C are mostly found in humans, even though a few evidence showed the ability of these virus infect to humans (Gong *et al*., 2007; von Itzstein, 2007).

Fortunately the majority of 2009 influenza A viruses were sensitive to oseltamivir, the current neuraminidase inhibitor that has been approved by FDA. However, as the frequent change of antigenic character of virus is emerging fast, some drug resistance strains are now beginning to develop. In 15 December 2010, WHO reported that more than 314 cases of oseltamivir resistant virus carrying the H275Y substitution have been detected worldwide (WHO, 2009a). At the end of 2011, there were 29 cases of oseltamivir resistant carrying H275Y neuraminidase substitution reported in Australia (Hurt *et al*., 2011).

The existed vaccines against seasonal influenza virus to control this disease have been ineffective due to its rapid variable mutation (Kim *et al*., 2003a; Zhang *et al*., 2008). In the period of pandemic, vaccine supplies would not be adequate (Moscona, 2005). Thus, the development of effective and safe anti-influenza becomes a matter of certainty in drug discovery (Zhang *et al*., 2008; Gong *et al*., 2007).

Our work on virtual screening of natural plant compounds toward neuraminidase enzyme resulted plant species to be studied. In this study, 3000 natural compounds in NADI database were screened by molecular docking using Autodock 3.0.5 software. The natural compounds were docked rapidly to the neuraminidase enzyme (PDB id:

3B7E). The top 100 compounds were ranked by the lowest energy binding affinity of the ligand to the protein binding site and compliance to Lipinski"s rule of five (drug likeness) i.e hydrogen bond donors less than 5, hydrogen bond acceptor less than 10, molecular weight less than 500 g/mol, and a partition coefficient less than 5 (Lipinski *et al*., 1997). The top 100 of compounds were clustered according to the plants from where the reported compounds have been isolated. One of the plants that contain compounds which were predicted to be active inhibiting neuraminidase enzyme was *Brucea javanica* L. (Merr) (Unpublished paper of Prof. Habibah A.Wahab).

1.2 H1N1 virus

1.2.1 Definition

Influenza viruses are negative segmented strand RNA, divided in types A, B and C, with only type A and B causing severe disease. The virus types can be classified for their antigenic characteristics of the core protein. One of virus that belongs to type A virus is H1N1. The H1N1 influenza virus (family: *Orthomyxoviridae*) commonly called "swine flu" is a strain of virus made up of swine, human and avian genes, and preferentially infects younger people with no or lack of immunity (Kiely *et al*., 2009). Influenza A often causes a global pandemic, while influenza B and C only causes regional and local endemic (Alexander and Brown, 2000).

The H1N1 pandemic 2009 virus contains arrangement of gene segments of human, swine and avian influenza viruses. The repeated chance of interspecies contact between swine, human and bird lead to materialize evolution of new genetic mutation to "avian-like" and "human-like" swine lineages (Naffakh and van der Werf, 2009). The

unique genetic combination is leading to the possibility of increased human-to-human transmission of H1N1 virus.

1.2.2 Anatomy of the Virus

The influenza A virus looks like a sphere, round or oval shape, with many spikes and mushroom shape object on its surface and approximately 90 nm in diameter. The A influenza virus containing two functional surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA) and also transmembrane proteins: M2 channels (WHO, 1980; Wei *et al*., 2006). Refer to Figure 1.1, the spikes-shaped is known as hemagglutinin (HA) and the mushroom-shaped is considered as neuraminidase (NA). Both of them recognized the same host cell in the host respiratory membrane, sialic acid, that contains proteins with enzymatic functions that are needed for genome replication (Oxford and Lambkin, 1998).

In 1980, virologist announced a simple formula for identifying influenza A virus, H_xN_y (x for the molecule type of HA, and y for the molecule type of NA). H1N1 is influenza A virus with hemagglutinin molecule type 1 and neuraminidase molecule type 1 (WHO, 1980). So far, there are 16 different molecules of HA and 9 different molecules of NA have been identified (Liu *et al*., 2009a). The schematic diagram of influenza A virus was represented in Figure 1.1.

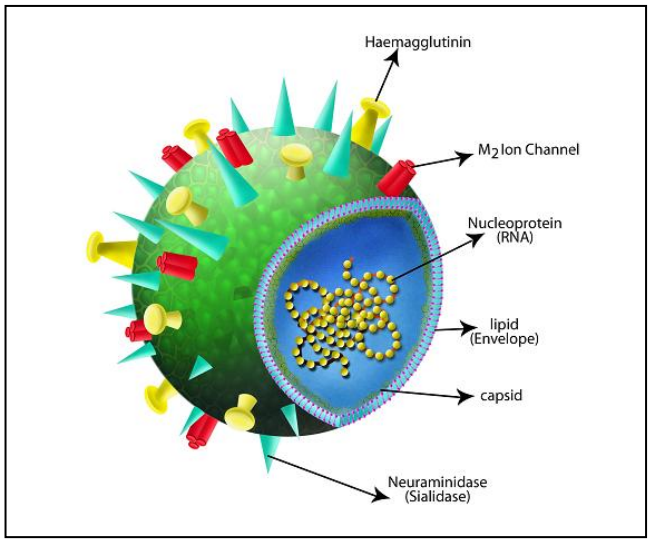

Figure 1.1 Schematic diagram of influenza A virus

1.2.2.1 Neuraminidase (NA)

A neuraminidase enzyme is a large family, found in a range of organisms. The scientists have found nine different types of NA, (N1-N9) but only N1 and N2 are important in human infection, while seven additional of NAs are infected only in animals. Neuraminidase is the major protein surface of influenza virus which acts extracellularly by cleaving the terminal neuraminic acid from cellular receptors recognized by HA. Based on phylogenetic studies, influenza virus neuraminidase has been divided into two groups. Group one contains N1, N4, N5 and N8, and the other group contains N2, N3, N6, N7 and N9 (Gong *et al*., 2007). Neuraminidase plays an important role in the pathogenesis of virus infection. It is facilitating the release of newly formed virions from the host cell surface to the neighbourhood cells. By removing terminal sialic acid of receptor of the virus, NA acts as a receptor destroying enzyme in influenza virus (Clercq, 2006; Lew *et al*., 2000).

The function of NA is to facilitate the mobility of the virus from the site of infection. It catalyzes the cleavage of α (2,6)- or α (2,3)-ketosidic linkage between a terminal sialic acid and inward-face sugar residue. This broken bond facilitates to spread the virus in the respiratory tract and allows elution of progeny virus from the infected cells. Lastly, this enzyme will prevent viral inactivation and stimulate viral penetration into respiratory epithelial cells (Gong *et al*., 2007).

1.2.2.2 Active Site of Neuraminidase

The discovery of three-dimensional structure of influenza neuraminidase in the early 1990 provides a tremendous prospect for drug design. There are eight amino acids of the active site which are group as basic residues (Arg118, Arg292, Arg371), acidic residue (Glu119, Glu227) and hydrophobic residues (Arg152, Trp178, Ile222) with kind of amino acid as mentioned in parentheses. These amino acids make direct contact with *N*-Acetylneuraminic acid (Neu5Ac), sialic acid and its derivatives at the distinct adjoining pockets. The active site is highly conserved and provides a rigid catalytic centre, with the presence of some highly specific hydrophobic area (Gong *et al*., 2007; von Itzstein, 2007).

It is well understood that the structure of NA complexed with Neu5Ac is mainly conserved by the charge-charge interaction between the carboxyl group and the pocket of three positively charge arginine residues (Arg118, Arg292, Arg371). The NH group of 5-*N* acetyl interacts with the floor of the active site cavity via hydrogen bonding with water molecule while the oxygen of the same group is bonded to N of Arg152 via

hydrogen bonding. The methyl group is attracted to the hydrophobic pocket near Ile222 and Trp178 while the two hydroxyl groups of glycerol side chain are bonded to carboxylateoxygens of Glu276. The 4-hydroxyl is directly in contact with carboxylate oxygen of Glu119 and the glycosidic oxygen interacts with carboxylate oxygen of Asp151 (Varghese, 1999; von Itzstein, 2007). The complex structure between neuraminidase and sialic acid is presented in Figure 1.2.

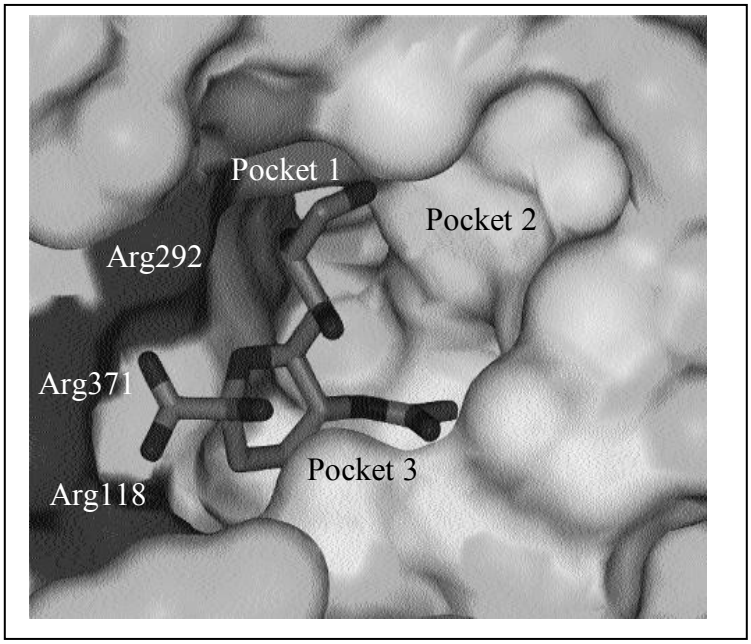

Figure 1.2 Complex structure of NA (Shown in surface representation) and sialic acid (in stick representation)(Kim *et al*., 1999).

The X-ray crystallographic study reveals that influenza A virus of the group 1-NA contains a larger cavity neighbouring to the active site, formed by residue 147-152 (150-loop) which is not found in the group 2-NAs. This cavity creates a much fewer polar environments, making it possible to hold the hydrophobic moiety of an inhibitor molecule. The group1-NAs can bind ligands in open conformation as well as in the

closed one. There is only closed conformational state found in the group 2-NAs. The flexible 150-loop residues are occupied on the area and interact mostly with the amino and acetamido groups of oseltamivir moiety. The active sites are still conserved at the triad amino acid residues, arginine triad (Arg118, Arg292, and Arg371) that interact with carboxylate groups. Moreover, the affinity is increased by the interaction between acetamido group and Glu276 that forms hydrogen bonds with the substrate of hydroxyl groups (Collins *et al*., 2008; Rungrotmongkol *et al*., 2009b).

Figure 1.3 shows the interaction of oseltamivir with active sites of neuraminidase. Green dashed arrows showed H-bonds between oseltamivir and amino acid residues of NA (Rungrotmongkol *et al*., 2009a).

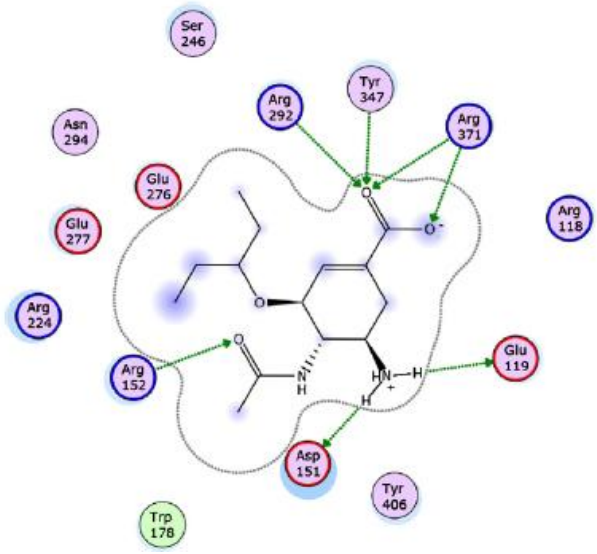

Figure 1.3 The interactions of oseltamivir active metabolite with the active site of neuraminidase. Green dashed arrows showed H-bonds between oseltamivir and amino acid residues of NA (Rungrotmongkol *et al*., 2009b).

1.2.2.3 Hemagglutinin (HA)

Hemaggglutinin (HA) is the spike shaped glycoprotein (refer to Figure 2.1). HA of influenza virus has a special role in virus entry. In the first initializing of virus infection to respiratory tract, HA mediates influx of proton into the infecting virion, which facilitates the dissociation of the ribbonucleuproteins from the virion interior and allows them to be released into the cytoplasm and transported into the cell nucleus (Moscona, 2005). The infected virus is able to attach the cell surface, sialic acid containing receptor mediated by HA. On the other hand, virus entry into cells was mediated by HA. Indeed, hemagglutinin is the major virulence (disease-causing) factor of the influenza virus (Klenk *et al*., 1977). Hemagglutinin named derives its activity. It can stick to, or agglutinate the red blood cells. So far, scientists have found 16 subtypes of HA (H1 to H16) but only H1, H2, and H3 sub types of HA are prevalent in human infection, while other additional of HAs are important in animal infection (Eisen *et al*., 1997; Liu *et al*., 2009a).

1.2.2.4 Matrix 2 (M2) Proton Channel

Matrix 2 (M2) of influenza virus is an interesting transmenbrane protein that forms tiny proton channel in the viral envelope. M2 ion protein channel is the third membrane protein which provides the virus structural integrity by permitting the proton to enter virus particle during uncoating of virions in endosome (Bauer *et al*., 1999.) It is a homotetramer consisting of four polypeptide chains of 96 amino acids, with the structural domains: an amino-terminal extracellular domain (comprising 23 residues), as single internally hydrophobic domain that acts as a transmembrane domain (19 residues) and 54-residue cytoplasmic tails. This membrane is important to prevent inactivation of

progeny virus as well as premature acid activation of newly synthesized HA which is cleaved extracellularly (Betakova, 2007; Rossman *et al*., 2010).

1.2.3 Transmitting of the Virus

Like other influenza viruses, the H1N1 viruses may transmit from human to human by aerosol route via inhalation of infected droplets from coughing or sneezing or through contamination of hands or surface. It can be easily transmitting by air (Kiely *et al*., 2009).

1.2.4 Treatment of the Virus

There are two ways to control the virus infection, chemotherapy and vaccination. Vaccination is targeting the variable antigenic surface glycoproteins of circulating strain. Vaccine is currently available in public health service as a preventive way in high risk population. Vaccination can prevent effectively 50% - 68% of acquiring influenza infection and minimize the complication and mortality. Vaccine is the best choice for the prophylaxis on control the pandemic. However, vaccine is providing very limited protection due to specificity of the strain of virus that could be prevented. New vaccine is needed annually, due to the rapid change of the virus glycoprotein character (He *et al*., 2011; Oxford and Lambkin, 1998).

In short period, chemotherapy using antiviral agent is vital to control the spread of the virus. There are only two classes of antiviral licensed globally; adamantane derivatives and neuraminidase inhibitor.

1.2.4.1 Adamantane Derivatives

Historycally, the adamantane-based M2 ion channel protein inhibitors are the first drugs available for the treatment of influenza. They are amantadine and rimantadine which have only been useful in the treatment of Influenza A infection due to only the A strains of the virus have M2 ion channel protein (Kiely *et al*., 2009). The drugs inhibit the virus replication by blocking M2 ion channel via its binding at allosteric site which triggers a conformational alteration in the pore region. This action causes interfering proton transfer during the ion channel across the membrane of the virus or endosome. Therefore, the virus is unable to enter the host cell of membrane and the replication is blocked (Tisdale, 2009; Sandrock, 2010).

Studies during 1994 to 2005 showed the worldwide increasing of amantadine and rimantadine-resistance from 0.4% to 12.3%. New strain of A virus has a resistance to amantadine and rimantadine due to the presence of S31N mutation that increase the M2 protein mobility. The adamantane derivatives have been reported having a poor of bioavailability in the human blood. They also have been reported having a side effect on gastrointestinal and central nervous system (Rungrotmongkol *et al*., 2009a). The chemical structures of adamantane derivatives were described in Figure 1.4

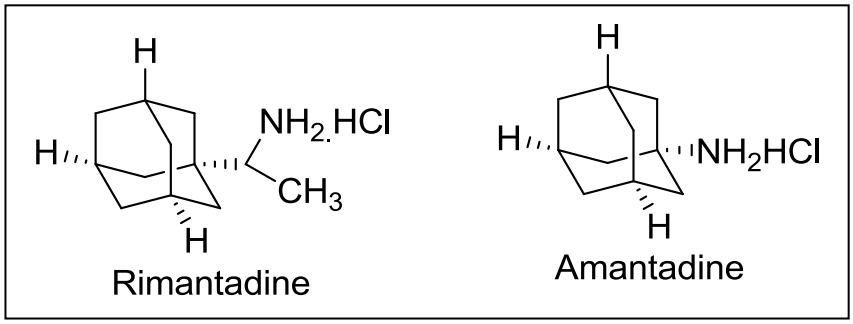

Figure 1.4 Chemical structures of adamantane derivatives (Clercq, 2006)

1.2.4.2 Neuraminidase Inhibitor

Neuraminidase inhibitor is a sialic acid of the surface of human epithelial cells compound analogue. The purpose of the inhibition is that the virus will bind to the inhibitor instead of the human epithelial cells so the inhibitor-viral complex can be removed from the body. The neuraminidase inhibitor has become a drug of choice in antiviral drug because of its important role of neuraminidase enzyme. If the enzyme is blocked, infection will be limited, and the spread the new virion will be disabled. NA has become the primary target for drug design against influenza virus because it is responsible toward influenza virus replication in the final cycle of virus‟s life (Wang *et al*., 2006; Wei *et al*., 2006).

The first NA inhibitor analogue of sialic acid is 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid or Neu5Ac2en (DANA) (Meindl *et al*., 1974). DANA showed a good activity *in vitro*. However, this compound was non-selective NA inhibitor and did not exhibit activity *in vivo* (Kim *et al*., 1999).

The discovery of complex 3D structure of NA and DANA, triggered the development of more potent anti neuraminidase. In 1993, Mark von Itzsein modified DANA by changing the OH in 4 position with guanidinyl group, made 2,4-dideoxy-2,3-didehydro-4-guanidinosialic acid or 4-guanidino-Neu5Ac2en (zanamivir) (von Itzstein *et al*., 1993). Zanamivir became one of the potent inhibitors which has a structure mimicking to sialic acid, fitting into the active site pocket and locking the protein in the lowest binding free energy (Moscona, 2005). Zanamivir (or often published as GG167), is a selective NA inhibitor which also has activity both *in vivo* and *in vitro*. Zanamivir

with the brand name Relenza®, due to its high polarity, it was reported to have a poor bioavailability in human blood. It was also excreted immediately by the kidneys. Thus, Zanamivir has never been advised to use via oral route but only via inhalation. Moreover, it is indicated only given for adults and children above 5 years old (Moscona, 2005).

In 2000, zanamivir structure was added by a lipophylic side chain in C6 position in order to increase the bioavailability. This modified compound was named oseltamivir (GS 4104) (Lew *et al*., 2000). This compound has emerged as a promising antiviral for the treatment and prophylaxis of human influenza infection. Oseltamivir acts as prodrug (ethyl ester) which is hydrolyzed *in vivo* to its active form, the carboxylic acid, after taken orally. The active form has a poor bioavailability, therefore, oseltamivir is prepared in phosphate salt and administered orally (D'Souza, 2009; Hammad and Taha, 2009). The chemical structure of current neuraminidase inhibitors were presented in Figure 1.5.

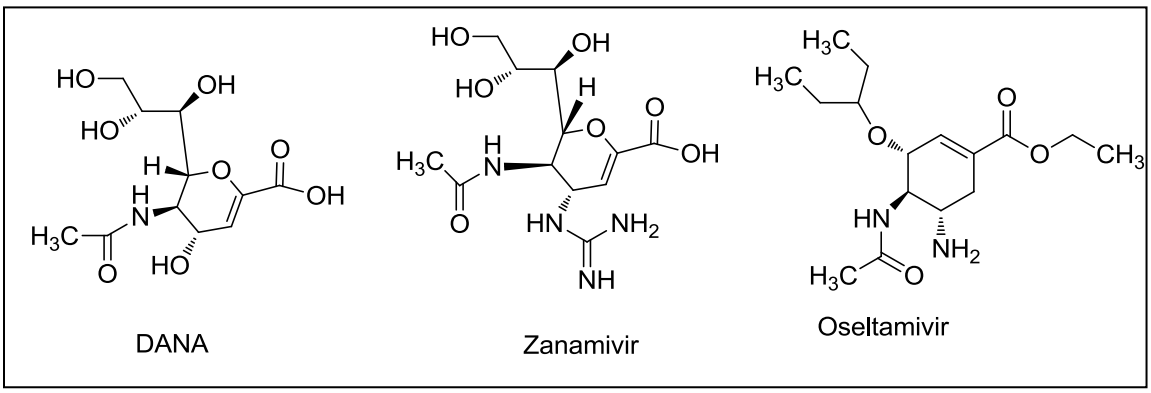

Figure 1.5 Chemical structure of neuraminidase inhibitors; DANA, Zanamivir and Oseltamivir (Clercq, 2006)

So far, only two kinds of neuraminidase inhibitor have been approved by FDA, oseltamivir and zanamivir (Lew *et al*., 2000; Dunn and Goa, 1999). They are active against all type of influenza virus, including type A and B influenza virus (Moscona, 2005).

Lately, new neuraminidase inhibitor, peramivir, an intravenous neuraminidase inhibitor has been approved in Japan and Korea. In October 2009, FDA issued an Emergency Use Authorization (EUA) which expired in June 2010. The EUA based on safety data from Phase 1, Phase 2 trials, and limited Phase 3 trial. Later on, laninamivir, a long-acting intranasal neuraminidase inhibitor which has stronger activity than zanamivir, has also been licensed for limited use in Japan, even the clinical publications were still limited (Kubo *et al*., 2010; Yamashita, 2011; WHO, 2010c).

However, as the widely used of anti influenza drug and the rapid change of the virus genetic, resistant strains of the virus can be expected to develop (Moscona, 2005). Oseltamivir with the brand name Tamiflu®, was reported to be resistant on several cases of Influenza A infection, and also had side effects such as nausea and vomiting to among patients given treatment with this drug (Meindl *et al*., 1974; Oxford and Lambkin, 1998).

1.2.5 Antiviral Analysis: Detection of Viral Presence in Samples.

Since the virus‟s attack was difficult to be detected, scientists tried hard to develop the methods to distinguish the efficacy of antiviral substances to limit the viral

replication. The general way of detecting the presence of virus in an infected cell is using two principal methods: CPE and hemadsorbtion (Knipe and Howley, 2007).

1. CPE (cytophathic effect), which is composed by two different phenomenon:
    (a) Morphological alterations induced in individual cells or groups of cell by viral infection which is identifiable under a light microscope.
    (b) Inclusion, defined the aggregates of stainable substances in biological cells.
2. Hemadsorbtion: refers to the ability of erythrocytes to stick to virus-infected cells. Influenza viruses produce cell attachment proteins (HA), which bring out their function totally or in part by binding substituent (neuraminic acid) that are plentiful on a wide diversity of cell types, including erythrocytes (Knipe and Howley, 2007).

1.2.6 General Methods of Anti-Viral Assay

Many viruses have distinctive character in their structure and replication cycles. Those make them possible to be the potential targets of inhibition. Since virus enzymes are essential in viral life for facilitating the viral replication and enhancing disease progression, inhibition of the enzymes become the most attractive strategic approach (Chattopadhyay *et al*., 2009). In influenza virus, common ways to limit the virus progression were by inhibiting the host cell binding (hemagglutinin inhibitor), uncoating the capsid (adamantine derivatives), DNA or RNA polymerase inhibitor (ribavirin) and neuraminidase inhibitor (zanamivir, oseltamivir) (von Itzstein, 2007). The mechanism scheme of viral inhibition was described in Figure 1.6.

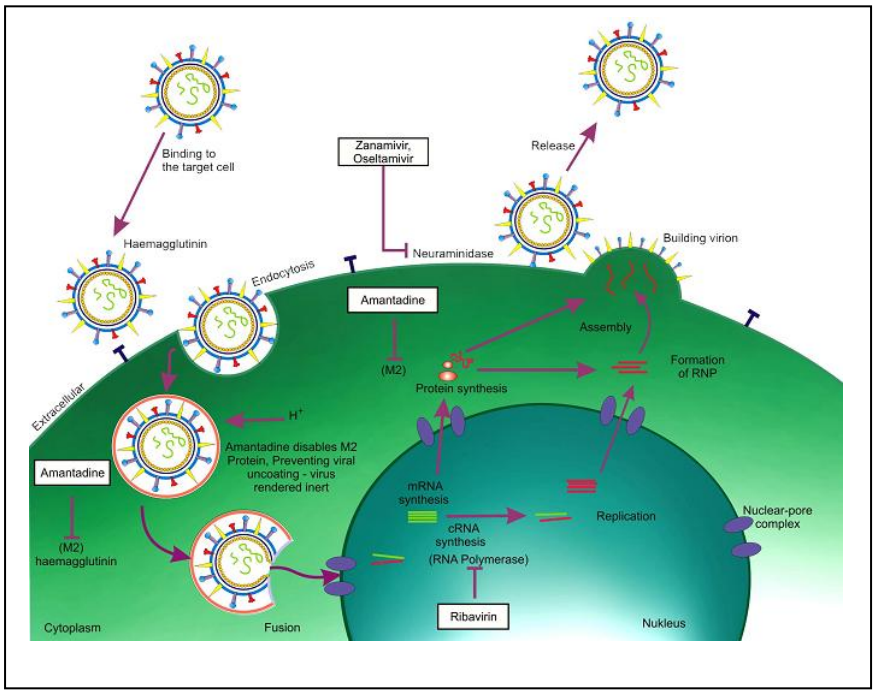

Figure 1.6 Inhibition Mechanism Schematic of Influenza Antiviral (Modified from von Itzstein (2007))

1.2.6.1 Hemagglutination Assay

The first opportunity to prevent influenza virus replication is when the virus attaches to the host cell membrane. Influenza virus hemagglutinin oligosaccharides attached to the host cell membrane associated with sialic acid terminal position. The agents act to inhibit the viral replication by interfering the first attachment step of the virus to the host cell, resulting the reducing of HA titre in culture specimen (Cross *et al*., 2001).

Hemagglutination assay is different from other forms of virus quantification such as a plaque assay or 50% Tissue Culture Infectious Dose ($TCID_{50}$). This method does not give any measure of viral infectivity, because no virus replication is required in this

assay. Influenza viruses have envelope surface HA protein that are identified by their ability to agglutinate (stick to) human or animal red blood cells (RBC). This activity (hemagglutination) can be tested by mixing virus dilutions with RBC in microtitre plates. The chosen of RBC depend on the type of influenza virus. H1N1 is more sensitive to guinea pig‟s RBC, while H5N1 is more sensitive to chicken‟s erythrocytes. For this assay, the guinea pig‟s red blood cell was used (WHO, 2002). If the tested compounds or extracts inhibit the virus replication thus the viral titre, HA titre will be reduced. This method can be used to examine the inhibitory effect of any drug onto the hemagglutination activity (Chattopadhyay *et al*., 2009). RBCs will form a button or a ring at the bottom of the wells and recorded as „-‟. If hemagglutination occurs, that is RBCs remain in suspension, it is recorded using a „+‟ symbol. The highest dilution of virus that causes complete hemagglutination was considered the endpoint in HA titration (WHO, 2002). The titre of a hemagglutination assay is determined by the last viable "lattice" or "sand" form. This is because it is at the point where, if diluted anymore, the amount of virus particles will be less than that of the RBCs and thus not be able to agglutinate them together (Chattopadhyay *et al*., 2009). So far, only few compounds have been tested and showed a good activity to reduce the HA titre, one of those compounds is Poloxometalate Derivative (POM-4960) (Hosseini *et al*., 2012).

Percentage of HA titre reduction:

HA titre reduction of drug treated virus compare to the untreated (control) virus can be performed using this following equation:

$$\text{Percentage of reduction} = \frac{\text{untreated HA titre (control)} - \text{treated HA titre}}{\text{untreated HA titre (control)}} \times 100\ \%$$

1.2.6.2 Infectious Titre Reduction Assay

Several methods have been developed to determine antiviral activity. Titre reduction assays to determine the efficacy of antiviral candidates is fundamental approach used by virologist. *In vitro* assay using cell lines or cell cultures generally exploit the ability of virus to infect and replicate in host cells. It is often performed using plaque reduction or cytophatic effect (CPE) reduction on appropriate cell lines in cell culture systems. As the development of CPE in this assay was indicating the presence of infectious virus, the CPE reduction in presence of test compound could be due to inhibition of virus replication. The quantitative statement of the virus titre reduction often performed by plaque forming unit (PFU) and $TCID_{50}$ (Half Tissue Culture Infectious Dose). The use of $TCID_{50}$ can be useful if the plaque was not forming. $TCID_{50}$ is a virus quantifies reflecting an end point dilution which can kill 50 % of cell culture or to produce 50% of CPE in infected cell. Fresh cells are used to evaluate the decreasing of infectious virus titre of drug-treated compare to untreated virus by measuring CPE induced by these viruses. To assist the investigation, this assay was performed by staining of surviving cells after being infected with drug-treated and free-drug virus in several days of incubation time depending on the type of virus. After incubation, the percentage of cell mortality was manually observed. $TCID_{50}$ was calculated using Reed-Muench method. Every method has certain limitation. Skilled personnel, cell culture reagents, time consuming and expensive are some of technical problems in antiviral assay using titre reduction assay (LaBarre and Lowy, 2001; Chattopadhyay *et al*., 2009).

To support the replication of the virus, the appropriate mammalian cell lines should be prepared firstly. MDCK cells are the preferred host for the influenza virus. Confluent monolayer of MDCK cells need to be prepared prior to the cytotoxity test, virus growth inhibition assay and $TCID_{50}$ assay in tissue culture plates as needed (WHO, 2002).

In order to obtain the Maximum Non-toxic Dose (MNTD), the cytotoxicity test should be performed before proceeding to Viral Growth Inhibition Assay. This assay was important to differentiate which activity was caused by the extract or compound toxicity to cells or due to the virus activity itself. Confluent MDCK cell monolayer in 24 well tissue culture plates (after 2-3 days of incubation in GM) were incubated with 10-750 μg/mL dilutions of extract and substance in 5 % of DMSO and Maintenance Medium. No virus was needed in this experiment. The plates were observed microscopically for any morphological changes at 24, 48 and 72 hours of incubation. The morphological changes can be granuling, enlargement, rounding and detachment of cells from bottom of plates. At the end of incubation day, the medium was removed carefully from each wells, the plate was stained with 2% of crystal violet, thus dye was took up by healthy and living cells, and the remaining cells were stained in violet. The maximum concentration of substances that did not cause any alterations on MDCK in the test condition cells was considered as Maximum Non-Toxic Dose (MNTD) (Chattopadhyay *et al*., 2009).

1.2.6.3 Neuraminidase Inhibition Assay.

Inhibitors of neuraminidase have been developed in an effort to prevent the viral infection. The inhibitors are structurally similar to the sialic acid on the surface of human epithelial cells. The rational hypothesis is that the virus will bind to the inhibitor rather than to the human cells, the inhibitor-viral complex can be removed from the body, thus the spread of infection can be limited (Oxford and Lambkin, 1998).

It has been well known that the specific one or more enzymatic systems present in the body were used by utilizing a small molecule. As the mechanism of enzyme being well understood, the drug design targets to enzyme were become highly developed resulting in more potent and selective enzyme inhibitors. The removing of N-acetyl neuraminic acid (NANA) from sugar chains of glycoproteins by NA is critical for its viral activity facilitation to spread of infection to new cells. The inhibition of this viral activity can be performed by specific antibodies forms the basis of the neuraminidase-inhibition (NAI) assay.

A few of neuraminidase assays are currently used for the NAIs assessment. Synthetic sialic acid derivatives are commonly used as a substrate for these assays based on its cleavage facilitated by NA produce convenient reporting signals for the measurement (Pedersen, 2008).

The compound 2‟-(4-methylumbelliferyl)-a-D-acetylneuraminic acid (MUNANA) (Figure 1.7) is widely used as a substrate of NA. The cleavage of this substrate produces fluorescence which can be detected at 460 nm in emission as well as

its excitation at 355 nm. The intensity of fluorescence can reflect the activity of NA sensitivity. The calculation of IC_{50} was done by plotting the percentage of inhibition versus the inhibitor concentration and determination of each point was performed in triplicate (Zhang *et al*., 2008). Neuraminidase inhibitory assay performed by enzymatic reaction using MUNANA was used in this experiment as described by Portier (1979). The activity of NA was measured fluorometrically by determination of the degradation product 4-MU from the substrate 4-MUNANA (Potier *et al.*, 1979).

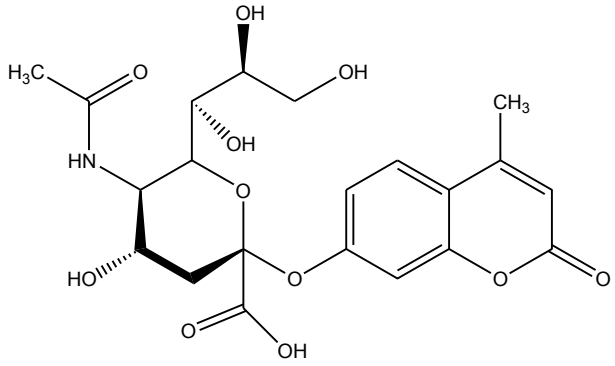

Figure 1.7 Chemical structure of 2‟‟-(4-methylumbelliferyl)-α-*D*-acetylneuraminic acid (MUNANA)

The principle of NA inhibitory assay by using MUNANA as substrate was described in Figure 1.8. In this reaction, NA catalyzes the hydrolysis of MUNANA by water to get umbeliferone and N-acetyl neuraminic acid (NANA) as products. The presence of umbeliferone can be detected by fluorometric method, which is the more activity of NA the more quantity of fluorescence absorbtion (Li *et al*., 2009).

The inhibition percentage can be counted by using this following equation:

$$\text{Inhibition ratio} = \frac{\text{F enzyme activity control} - \text{F test}}{\text{F enzyme control} - \text{F blank control}} \times 100\%$$

F : fluorescence absorption

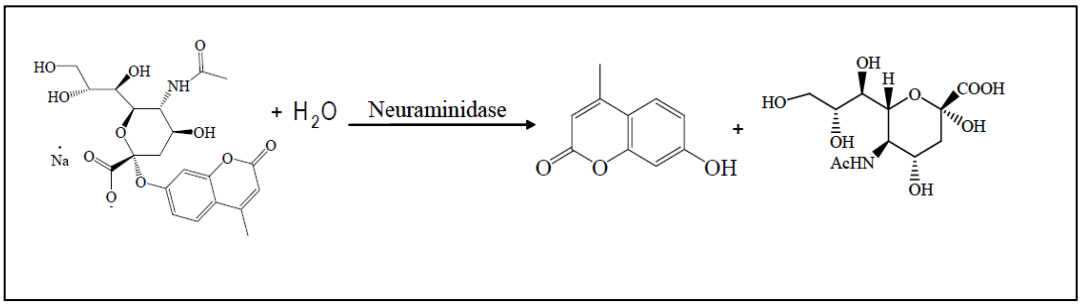

Figure 1.8 Reaction principle of microplate-based screening assay for NA inhibitors. In this reaction, NA catalyzes the hydrolysis of MUNANA by water to get umbeliferone and N-acetyl neuraminic acid (NANA) as products. The presence of umbeliferone can be detected by fluorometric method (Li *et al*., 2009)

1.3 Molecular Docking

Docking is a method used for computational studies that attempt to find the "best" matching between two molecules: a receptor and a ligand (Halperin *et al*., 2002). It is one of the methods in molecular modelling employed to calculate the bioactive conformation of a molecule in the binding site of the protein target. In general, it corresponds to find the local minimum of the binding free energy of ligand-protein system. Ligand is a tiny particle which interacts with the binding location of protein which is recognized as the area corresponding to its biological activity (Kaapro and Ojanen, 2002).

One of the docking methods developed by the Scripps Research Institute is Autodock 3.0.5 (Morris *et al*., 1998). In general, steps in docking by using AutoDock are divided into:

1. Ligand preparation.

Ligand is assigned with partial atomic charges (gasteiger charges) and the number of torsion was calculated. This is then written as pdbq file that recognized by AutoDock .

2. Protein preparation

For protein preparation, partial atomic charge (kollman charges) is assigned. In addition, the protein is also assigned with generalized salvation parameter. The protein structural file is then written in pdbqs file recognized by AutoDock.Grid

3 Parameter files preparation.

The grid parameter file describes the parameter that needed for the AutoGrid program to generate grid maps. This includes the location and extent of those maps and specifies pair-wise potential energy parameters. Figure 1.9 illustrates the main features of grid map. The centre of the grid map is fitted to the ligand, buried inside the active side of the protein. The grid spacing is the same in all three dimensions. The macromolecule (i.e protein) is embedded in a three-dimensional grid and a probe atom is placed at each grid point. The grid point makes a cubical box with 0.375 Å of grid spacing. An affinity grid is calculated for each atom type that present in ligand, typically carbon, oxygen, hydrogen and nitrogen. The probe atom stored the potential energy due to all the atoms in the macromolecule. Figure was taken from the Autodock *User's Guide* Version 3.0.5