

SYNTHESIS, *IN SILICO* STUDIES, AND BIOLOGICAL
EVALUATION OF CARVONE DERIVATIVES AS
POTENTIAL NEURAMINIDASE INHIBITORS

BY

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ABSTRACT

Current outbreaks of highly pathogenic influenza strains have shown that new anti-influenza drugs need to be developed. To date, four antiviral agents have been approved for the treatment of influenza infection; zanamivir (RelenzaTM), oseltamivir (TamifluTM), peramivir, and most recently, laninamivir. However, increasing reports of these drugs resistance and side effects lead researchers to discover novel inhibitors against influenza. Carvone, which naturally can be found in spearmint essential oil, was studied as antiviral agents for its property. To explore the potential of carvone as neuraminidase (NA) inhibitors, a series of fourteen carvone derivatives compounds have been successfully synthesised using several strategies including epoxidation, epoxide ring opening, aminolysis, reductive amination, and condensation reaction. All the synthesised compounds obtained were elucidated using FT-IR, ¹H NMR, ¹³C NMR, and ESI-MS. Molecular docking was conducted to gain insight into possible binding modes and preferred conformations of complex synthesised compounds in the NA active site. Based on the docking analysis, compound **3e** was found to have the lowest energy binding (ΔG_{bind}) value of -8.35 kcal/mol, which is closed to the reference drug oseltamivir (**OTV**) with ΔG_{bind} value of -8.58 kcal/mol. Molecular dynamics (MD) simulation was later performed to analyse the flexibility and stability of protein-ligand binding complex with NA protein. Our simulation study showed that the **3e-NA** complex is as stable as the **OTV-NA** complex during the MD simulation of 50 ns. Compounds with good solubility in 2.5% DMSO were further evaluated for neuraminidase inhibition assay. Among ten compounds tested, compound **3e** showed the highest inhibition activity of 60.95% inhibition with an IC₅₀ value of 44.13 μ M.

خلاصة البحث

أظهرت حالات التفشي الحالية لسلالات الإنفلونزا الممرضة بشدة أن العقاقير الجديدة المضادة للإنفلونزا بحاجة إلى تطوير. إلى الآن تمت الموافقة على أربعة عوامل مضادة للفيروسات لعلاج عدوى الأنفلونزا، وهي الزاناميفير (RelenzaTM)، والأوسيلتاميفير (TamifluTM)، والبيراميفير، ومؤخرا عقار اللانيناميفير. ومع ذلك فقد دفعت التقارير المتزايدة عن مقاومة الفيروسات لهذه الأدوية وآثارها الجانبية الباحثين إلى اكتشاف مثبطات جديدة ضد الأنفلونزا. تم دراسة مركب الكارفون (carvon) كعامل مضاد للفيروسات نظرا لخواصه، والذي يمكن العثور عليها بشكل طبيعي في زيت النعناع المدب. لاستكشاف إمكانات الكارفون كمثبط للنيورامينيداز (NA) تم بنجاح استحداث سلسلة من أربعة عشر مركبا مشتقا من الكارفون باستخدام العديد من الاستراتيجيات منها الإيبوأكسدة، وفتح حلقة الإيبوكسيد، والتحلل الأميني، إضافة الأمين الاختزالية، تفاعل التكثيف. تم عرض جميع المركبات المستحدثة باستخدام FT-IR، و ¹H NMR، و ¹²C NMR، و ESI-MS. تم إجراء الإرساء الجزيئي لفهم أوضاع الربط الممكنة والتوافقات المفضلة للمركبات المستحدثة في موقع NA النشط. استنادًا إلى تحليل الإرساء فقد وجد أن المركب 3e يحتوي على أقل قيمة للطاقة الرابطة (ΔG_{bind}) بقيمة -8.35 سعرة حرارية/مول، والذي يكون مغلقا لعقار الأوسيلتاميفير المرجعي (OTV) بقيمة ΔG_{bind} بلغت -8.58 كيلو كالوري/مول. تم إجراء محاكاة الديناميات الجزيئية (MD) في وقت لاحق لتحليل مرونة واستقرار مركب البروتين-الربطة الرابط مع بروتين ال NA. أظهرت دراسة المحاكاة أن مركب 3e-NA كان مستقرا مثل مركب OTV-NA خلال محاكاة ال MD البالغة 50 ns. تم إجراء التقييم الإضافي للمركبات ذات الذوبان الجيد في محلول 2.5% DMSO لاختبار تثبيط ال NA. من بين المركبات العشرة التي تم اختبارها، أظهر المركب 3e أعلى نشاط تثبيطي وذلك بنسبة تثبيط بلغت 60.95% وبقيمة IC_{50} بلغت 44.13 μ M.

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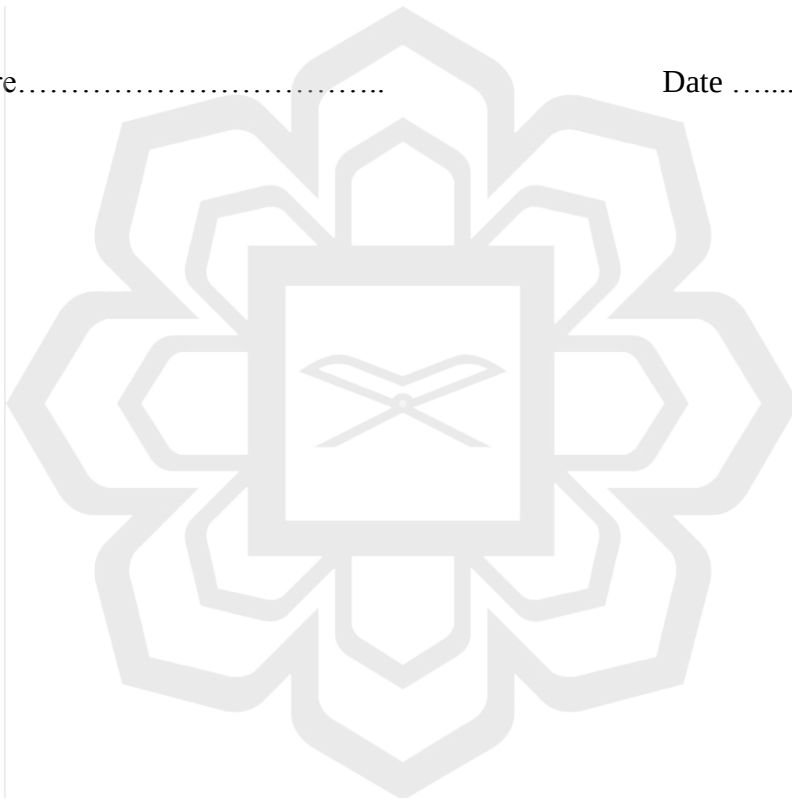
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LIST OF ABBREVIATIONS

COSY	Correlation Spectroscopy
d	Doublet
dd	Double doublet
dt	Double triplet
DCM	Dichloromethane
DMSO	Dimethyl sulphoxide
ESI-MS	Electrospray Ionization-Mass Spectroscopy
HMQC	Heteronuclear Multiple Quantum Coherence
HRMS	High Resolution Mass Spectrometer
Hz	Hertz
H	Hour
^1H - ^1H COSY	Correlated Spectroscopy
<i>J</i>	Coupling Constant
<i>K_i</i>	Inhibition Constant
MD	Molecular Dynamics
NA	Neuraminidase
NMR	Nuclear magnetic resonance
OTV	Oseltamivir
ppm	Part per million
qn	Quintet
QSAR	Quantitative Structure-activity-relationship
s	Singlet

t	Triplet
TLC	Thin layer chromatography
Δ bind	Energy binding



CHAPTER ONE

INTRODUCTION

1.1 RESEARCH BACKGROUND

Influenza is one of the deadliest infectious diseases causing significant fatality to the human population. In recent years, the world is threatened by the emergence of pandemics and epidemics of influenza infections such as H5N1 and more recently H1N1. The H5N1 threat was further compounded by the pandemic H1N1 emergence in 2009 (Patel et al., 2010). Highly pathogenic influenza H5N1 transmission from avian to human caused 43 deaths in the world in Thailand, Vietnam, China, Cambodia, and Indonesia in 2005 (WHO, 2012). More deaths were reported in 2006 and 2007 with 79 and 59 numbers of deaths, respectively. Even though the H1N1 swine influenza is less virulent than H5N1 avian flu, it is more prevalent compared to that of avian flu (Salaam-Blyther, 2009).

There are two major classes of antivirals used to combat the influenza virus, namely matrix-2 (M2) and neuraminidase (NA) inhibitors. M2 inhibitors such as amantadine and rimantadine, prevent the release of the virus by blocking the M2 ion channel proton, while NA inhibitors prevent the release of newly formed virions from the cell surface (McKimm-Breschkin, 2013). Historically, M2 inhibitors were the first drugs available for influenza treatment, however, these inhibitors are only effective against influenza A virus since no M2 protein exists in influenza B virus (Wang & Wade, 2001). Moreover, the use of these drugs was limited because of the rapid emergence of resistant virus in treated patients and in a single passage in tissue culture (McKimm-Breschkin, 2013). Research between the year 1994 and 2005 showed the

increase of worldwide M2 inhibitors-resistance from 0.4% to 12.3% (Dong et al., 2015). Thus, the use of amantadine and rimantadine drugs was no longer encouraged.

Due to the disadvantages of M2 protein inhibitors, many researchers focus on the drug design targeting neuraminidase (NA) proteins (Magano, 2009; Hanessian, Bayrakdarian, & Luo, 2002). This enzyme, which cleaves terminal sialic acid moieties from the receptors, is crucial for virus replication and infection. Zanamivir and oseltamivir are two effective drugs for both types A and B influenza. Zanamivir (trade name RelenzaTM) is the first potent inhibitor approved by U.S. Food Drug and Administration (FDA) in 1999 for the treatment of influenza A and B infections (von Itzstein & Thomson, 2009). However, its inhalational delivery is inconvenient compared to oral delivery (D'Souza et al., 2009). Oseltamivir (marketed as TamifluTM) overcomes this limitation, but it can cause nausea and vomiting. Moreover, the production cost is very high since it uses an expensive starting material of shikimic acid, which the price is about RM123/g (Chand et al., 1997). According to the study done by Kim & Park (2012), the synthesis of oseltamivir was accomplished in nine steps with a 27% overall yield from a readily available shikimic acid.

To date, researchers have designed the NA inhibitors with various scaffolds, including bicyclic molecules (Colombo et al., 2018), disulfide cyclic peptide (Putra et al., 2018), pyrimidine ring (Lou et al., 2014), tetrahydrofurans (Wang et al., 2005), pyrrolidines (Wang et al., 2001), cyclopentane (Sidwell et al., 2001; Young, Fowler, & Bush, 2001), aromatic (Chand et al., 1997), and sialic acid (von Itzstein et al., 1993). Although there have been a lot of efforts to discover new inhibitors, the current oseltamivir resistant mutant (H274Y H1NI) is quite resistant to oseltamivir (Yusuf et al., 2016). Therefore, there is a continuous need to search for more effective and inexpensive anti-influenza drugs.

Carvone is a monoterpene ketone showing antiviral properties that can be naturally found in spearmint essential oil, *Mentha spicata* (Souza et al., 2013). It can exist as enantiomers; *R*-(-)-carvone and its mirror image, *S*-(+)-carvone (Figure 1.1). Carvone can be synthesised from limonene via limonene nitrosochloride that can be formed in glacial acetic acid by treating limonene with isoamyl nitrite. It has high optical purity that make them much used chiral starting materials for enantioselective synthesis of various biological and natural active compounds. The several applications of carvone as antimicrobial agent, potato sprouting inhibitor, biochemical environmental indicator, fragrance and flavour, together with its relevancy in the medical field, further justifies the interest in this monoterpene (de Carvalho & da Fonseca, 2006).

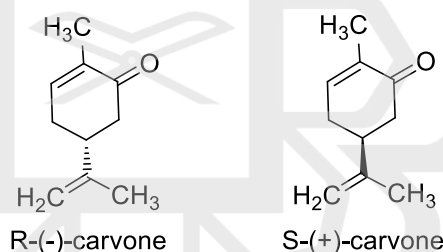


Figure 1.1 Enantiomers of Carvone

1.2 PROBLEM STATEMENT

Although several antiviral drugs are available to combat the influenza virus, more development on a new potential drug is needed due to increasing emergence of antiviral resistance of the existing drugs. The currently circulating clinical mutant of H274Y H1N1 is resistance to oseltamivir warrants urgency into more research into more effective and cheaper anti-influenza drugs. Here, carvone was chosen as starting material since it has similar scaffold with oseltamivir, which is cyclohexene scaffold.

It is also naturally found in spearmint oil and inexpensive than other starting materials. Computer-based modeling methodology has been widely used to design new targets for therapeutic agents. It improves the efficiency of detection and reduces the experimental cost. In this research, molecular docking and dynamics studies were explored to aid in design new inhibitors for the influenza virus. Therefore, *in silico* study was conducted to help in predicting the binding affinity of the synthesised compounds to the active site of neuraminidase enzyme.

1.3 RESEARCH OBJECTIVES

The general objective of this study is to synthesise a series of carvone derivatives as potential NA inhibitors. Specifically, the objectives are:

- i. To synthesise and characterise a series of organic compounds derived from carvone as potential NA inhibitors
- ii. To analyse the binding affinity between the synthesised compounds and NA active site through molecular docking and molecular dynamics (MD) simulation
- iii. To evaluate the enzyme inhibition activities of the compounds against NA

1.4 RESEARCH HYPOTHESIS

The study was conducted based on the hypothesis that carvone derivatives can be synthesised through several steps of reaction including epoxidation, epoxide opening, and reduction. In this study, carvone was synthesised through six steps of reaction, which are epoxidation, opening of epoxide, reduction, selective acetylation, amination, and condensation reaction. Most importantly, the proposed work is three steps fewer compared to current method to synthesise oseltamivir. Some of functional

groups modification was optimised in synthesising the compounds, resembling the available drug, oseltamivir. Each of the substituents might play a vital role in the interaction of the NA. In this study, the molecular docking and molecular dynamics simulation studies were employed to understand the binding interactions between the compounds and NA.

1.5 SIGNIFICANCE OF THE STUDY

Although many research have been done to find new inhibitors of neuraminidase, none of them use carvone scaffold as the core structure. In this study, carvone was chosen as starting material as it have similar scaffold with the current drug, oseltamivir. Other than that, it can be naturally found in spearmint oil and also cheaper than others. In addition, this study might help in contributing to the enhancement of knowledge in the avian flu research in Malaysia by understanding the structural design of the molecules.

CHAPTER TWO

LITERATURE REVIEW

2.1 INFLUENZA A VIRUS

Influenza viruses belong to the five genera of family *Orthomyxviridae*; *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Isavirus*, and *Thogotovirus* (de Groot et al., 2011). It is an enveloped (-) RNA that contains a virus with a segmented genome, and its genetic material is coded by eight segments of RNA (Shtyrya, Mochalova, & Bovin, 2009). There are three main types of human flu virus known as types A, B, and C, which are identified based on differences of antigenic in nucleoprotein and matrix protein. Among these types, only types A and B virus had been reported to cause virulent to human being (van Regenmortel & Mahy, 2004). Influenza C virus is less common and produces milder disease (Glezen, 2013).

Influenza A virus commonly infects the upper respiratory tract causes several symptoms such as malaise, fever, and myalgia. In serious condition, it can cause fatality when it affects other cells or organs causing pneumonia and myocardial infection (Louie et al., 2011). In Malaysia, influenza viruses circulated throughout the year with higher occurrences during the middle of the year (Saat et al., 2010). There are usually three to six strains of the influenza virus that simultaneously circulate every year.

The surface of influenza A virus consists of three proteins, namely neuraminidase or sialidase (NA), hemagglutinin (HA), and M2 ion channel protein. Figure 2.1 shows the schematic diagram of influenza A virus. HA is the receptor-binding and membrane fusion glycoprotein of influenza virus, and seems to have two vital roles. The first role is to provide the target host cell surface with an initial point

of contact for the virus, and the second role is to initiate the internalisation process of the virus by fusing the viral envelope with the host cell (von Itzstein, 2007; Skehel & Wiley, 2002).

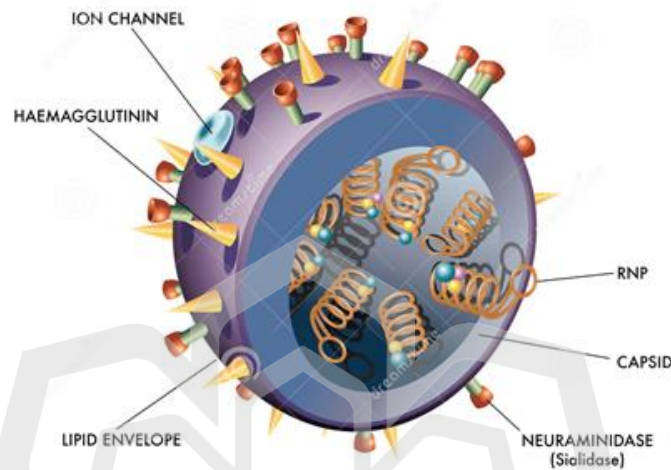


Figure 2.1 The Influenza Viral Lipid Envelope With a Nucleocapsid Containing Three Surface Proteins; Haemagglutinin (HA), Neuraminidase (NA), and Ion Channel Protein (Betakova, 2007)

Antigenic properties of influenza A virus are classified based on hemagglutinin (HA) and NA glycoproteins on the virus particles surface (Fouchier et al., 2005; Memorandum, 1980). To date, there are 18 distinct subtypes of hemagglutinin and 11 different subtypes of NA; H1 through H18 and N1 through N11, respectively (CDC 2017 website). Current influenza A subtypes reported in human are H1N1 and H3N2 (Glezen, 2013). Viruses of other subtypes such as H5N1, H7N7, and H9N2 have sporadically infected humans, but did not caused widespread outbreaks because of their limited ability to spread among humans (Walker, 2009).

The life cycle of influenza virus is essential for its virulence, replication and transmission that can be divided into six steps as described in Figure 2.2; (i) virus attachment; (ii) endocytosis and fusion; (iii) uncoating; (iv) RNA transcription and