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

BY

NOORAKMAR BINTI JUSOH

A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy (Biosciences)

Kulliyyah of Science
International Islamic University Malaysia

JUNE 2020

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 (Relenza^{TM}), oseltamivir (Tamiflu^{TM}), 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, ^{1}H NMR, ^{13}C 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 IC_{50} value of 44.13 µM.

خلاصة البحث

أظهرت حالات التفشي الحالية لسلالات الإنفلونزا الممرضة بشدة أن العقاقير الجديدة المضادة للأنفلونزا بحاجة إلى تطوير. إلى الآن تمت الموافقة على أربعة عوامل مضادة للفيروسات لعلاج عدوى الأنفلونزا، وهي الزانواميفير (Relenza™)، والأوسيلتاميفير (Tamiflu™)، 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 المدبب. لاستكشاف إمكانات الكارفون كمثبط للنيورامينيداز (NA) تم بنجاح استحداث سلسلة من أربعة عشر مركبا مشتقا من الكارفون باستخدام العديد من الاستراتيجيات منها الإبوأكسدة، وفتح حلقة الإيبوكسيد، والتحلل الأميني، إضافة الأمين الاختزالية، تفاعل التكثيف. تم عرض جميع المركبات المستحدثة باستخدام FT-IR، و ^{1}H NMR، و ^{12}C 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-الربيطة الرابط مع بروتين الـ NA. أظهرت دراسة المحاكاة أن مركب 3e-NA كان مستقرا مثل مركب OTV-NA خلال محاكاة الـ MD البالغة 50 ns. تم إجراء التقييم الإضافي للمركبات ذات الذوبان الجيد في محلول 2.5٪ DMSO لاختبار تثبيط الـ 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 µM.

APPROVAL PAGE

The thesis of Noorakmar Binti Jusoh has been approved by the following:

_____
Shafida Abd Hamid
Supervisor

_____
Noraslinda Muhamad Bunnori
Co-supervisor

_____
Khairul Bariyyah Abd Halim
Co-supervisor

_____
Deny Susanti Darnis
Internal Examiner

_____
Melati Khairuddean
External Examiner

_____
Roswanira Ab. Wahab
External Examiner

_____
Noriah Mohd Noor
Chairman

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Noorakmar Jusoh

Signature…….. Date ….....

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

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

I declare that the copyright holders of this thesis are jointly owned by the student and IIUM.

Copyright © 2019 Noorakmar Jusoh and International Islamic University Malaysia. All rights reserved.

No part of this unpublished research may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without prior written permission of the copyright holder except as provided below.

1. Any material contained in or derived from this unpublished research may be used by others in their writing with due acknowledgement.

2. IIUM or its library will have the right to make and transmit copies (print or electronic) for institutional and academic purposes.

3. The IIUM library will have the right to make, store in a retrieval system and supply copies of this unpublished research if requested by other universities and research libraries.

By signing this form, I acknowledge that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Noorakmar Jusoh

…… …….

Signature Date

ACKNOWLEDGEMENTS

First and foremost, praise be to ALLAH S.W.T. the Great and Almighty surrounded me under His auspices during my PhD journey. Alhamdulillah after almost four years and half of hard work and struggling, ALLAH S.W.T gave strength and blessing for me to complete my study.

I would like to express my sincere gratitude to my beloved supervisor, Assoc. Prof. Dr. Shafida Abd Hamid for her professional and amicable guidance, comments, support, constructive ideas, and encouragement during experimental work and thesis writing. Her integrity and moral support always encouraged me to complete my study and do my best. I would also like to thanks to Dr. Noraslinda Muhammad Bunnori, my co-supervisor, for her kind assistance in the computational part as well as whenever I needed it. Thanks also go out to my second co-supervisor, Dr. Khairul Bariyyah Abd Halim, for her supervision in molecular dynamics (MD) simulation analysis.

Special thanks are extended to my labmates Br Yousaf Ali, Br Ayisy Afti, Br Wafiy, Sr Najihah, Sr Amalina, and Sr Ishasanah for their help and support throughout my study. I also sincerely thank the persons who helped me with technical and administrative problems; Sr Norsa'adah, Sr Hafizah (UMP), Br Taufik, Br Muzzammil, Br Mizan, Sr Ain, Sr Hidayah, Sr Suhaila, Sr Hafiah, Sr Farah, Sr Muizzah and Sr Aisyah. I owe my deepest gratitude to my all my old friends in USM (As'malia, Haidar, Zaimas, Mardani, Juliani, Najihah, Munirah), friends in UIA (Ina, Mariam, Kema, Anis), the people with whom I shared the moments of excitements and disappointments.

I would like to express my thanks to MyBrain15 scholarship and FRGS for the financial scholarship support and founded this work.

Finally great thanks to my parents, Jusoh Awang Pa'su and Nik Rohati Raja Adam, parents in law, Abd Kadir@Mahmud and my late mother in law, Siti Rahmah and all my family members for their love and support in everything I do. To my beloved husband, Md Roidir Abd Kadir@Mahmud, I thank you for your unconditional love and support throughout my life and my studies. I could never have done this without you. This thesis is dedicated to him and my precious daughters, Qisya, Ainul, and Aafiyah.

Once again, we glorify Allah for His endless mercy on us, one of which is enabling us to successfully round off the efforts of writing this thesis. Alhamdulillah.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

COSY	Correlation Spectroscopy
d	Doublet
dd	Double doublet
dt	Double triplet
DCM	Dichloromethane
DMSO	Dimetyl sulphoxide
ESI-MS	Electronspray Onization-Mass Spectroscopy
HMQC	Heteronuclear Multiple Quantum Coherence
HRMS	High Resolution Mass Spectrometer
Hz	Hertz
H	Hour
^{1}H-^{1}H COSY	Correlated Spectroscopy
J	Coupling Constant
K*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
Δbind	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 Relenza^{TM}) 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 Tamiflu^{TM}) 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).

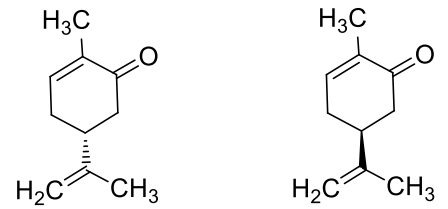

Figure 1.1 Enantiomers of Carvone

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 *Orthomyxiviridae*; *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).

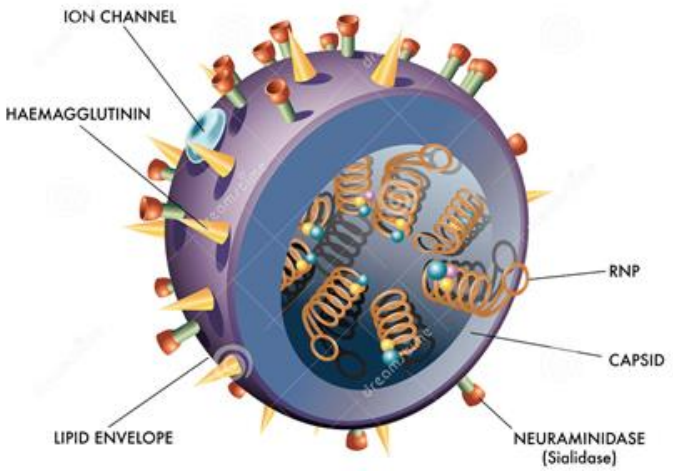

Figure 2.1 The Influenza Viral Lipid Envelope With a Nucleocapsid Containing Three Surface Proteins; Haemagglutinin (HA), Neuraminidase (NA), and Ion Channel Protein (Betakova, 2007)

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