UNIVERSITI PUTRA MALAYSIA

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPIC STUDIES OF THE STRUCTURE AND INTERACTIONS BETWEEN HEPATITIS B VIRUS CORE AND SURFACE ANTIGENS WITH PEPTIDES

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By

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December 2014

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Hepatitis B virus (HBV) infection remains a health problem globally despite the availability of effective vaccines. There are several approaches in designing an antiviral drug based on the virus life cycle. This study focuses on two antiviral approaches. The first approach is to discover a peptide that can block the virus from entering hepatocyte cells. Previous studies have implicated HBV surface antigen (HBsAg) to be involved in the virus entry into the host cells. Thus, a specific ligand targeting the immunodominant region of HBsAg is desired in neutralizing the infectivity of the virus. In a previous study, a disulfide constrained cyclic peptide cyclo S1,S9 Cys-Glu-Thr-Gly-Ala-Lys-Pro-His-Cys (S1, S9-cyclo-CETGAKPHC) was isolated from a phage displayed cyclic peptide library using an affinity selection method against HBsAg. The cyclic peptide binds tightly to the immunodominant region on HBsAg. Consequently, this study was aimed to elucidate the three-dimensional structure of the cyclic peptide and its interaction with HBsAg *in silico*. The solution structure of this cyclic peptide was solved using ^{1}H, ^{13}C, and ^{15}N NMR spectroscopy and molecular dynamics simulations with NMR-derived distance and torsion angle restraints. The cyclic peptide adopted two distinct conformations due to the isomerization of the Pro residue with one structured region in the ETGA sequence. Docking studies of the peptide ensemble with a model structure of HBSAg revealed that the cyclic peptide can potentially be developed as a therapeutic drug that inhibits the virus–host interactions. The second approach is to design a peptide based on the viral surface antigen as an inhibitor to block the viral assembly. This strategy involves interaction studies between HBV core antigen (HBcAg) and HBsAg. It is of important to understand the interactions between the two viral proteins because of their involvement in the assembly of the virus. In this study, two peptides of 25 residues long were chosen from different regions of HBsAg. The peptides, designated **preS** and **S** were ligated into pGEX-2T

vector and expressed in *Escherichia coli*. The peptides were purified using the GSTrap FF column and the recombinant peptides were chopped off from the GST tag using thrombin. The peptides each at 2.9 kDa were detected by silver staining. However, the yield obtained after cleavage was too little to carry out further studies. As such, these peptides were synthesized chemically using Fmoc chemistry and were analyzed using NMR. The solution structures of both peptides were solved using ^{1}H, ^{13}C, and ^{15}N NMR spectroscopy. Peptide **preS** has several structured region of β-turns at Ser7-Pro8-Pro9, Arg11-Thr12-Thr13 and Ser22-Thr23-Thr24 sequences whereas peptide **S** has only one structured region observed at Ser3-Asn4-His5. Both peptides contain bend-like structures surrounding the turn structures. Saturation Transfer Difference (STD) NMR experiments were performed to study the interaction of the **preS** and **S** to HBcAg. Several aromatic residues of **preS** and **S** were involved in the interaction with HBcAg, indicating their potential as antiviral agents that inhibit the virus morphogenesis. The experiments carried out on the peptides were fundamental methods in designing new antiviral agents for HBV. Promising results were obtained for each peptide, paving the way for future studies on the potential HBV peptide inhibitors.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KAJIAN SPECTROSKOPI MAGNETIK RESONANS NUKLEAR PADA STRUKTUR DAN INTERAKSI DI ANTARA VIRUS HEPATITIS B ANTIGEN TERAS DAN PERMUKAAN BERSAMA PEPTIDA

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Hepatitis B virus (HBV) masih merupakan masalah kesihatan di seluruh dunia walaupun terdapat vaksin yang berkesan. Terdapat beberapa pendekatan dalam mereka-bentuk ubat antiviral berdasarkan kitaran hidup virus. Kajian ini memberi tumpuan kepada dua pendekatan antivirus. Pendekatan pertama adalah untuk menemui peptida yang boleh menghalang virus daripada memasuki sel hati. Kajian sebelum ini melibatkan HBV antigen permukaan (HBsAg) semasa kemasukan virus ke dalam sel perumah. Oleh itu, ligan khusus yang disasarkan ke rantau immunodominan HBsAg ini dikehendaki untuk meneutralkan infektiviti virus. Dalam kajian sebelum ini, peptida berkitar di mana disulfidanya dikekang iaitu cyclo S1,S9 Cys-Glu-Thr-Gly-Ala-Lys-Pro-His-Cys (S1,S9-cyclo-CETGAKPHC) telah dipencilkan daripada faj yang dipaparkan dengan perpustakaan peptida berkitar menggunakan kaedah pemilihan afiniti terhadap HBsAg. Peptida berkitar ini mengikat ketat pada rantau immunodominan HBsAg. Oleh yang demikian, kajian ini bertujuan untuk mengenalpasti struktur tiga dimensi peptida berkitar ini dan interaksi peptida tersebut dengan HBsAg secara *in silico*. Struktur tiga dimensi peptida berkitar dalam cecair ini telah dikenalpasti dengan spektroskopi NMR ^{1}H, ^{13}C, dan ^{15}N bersama simulasi dinamik molekul dengan jarak sekatan dan sudut regangan yang diperolehi dari eksperimen NMR. Peptida berkitar mempunyai dua bentuk berbeza kerana pengisomeran Proline dengan satu kawasan berstruktur di urutan ETGA. Kajian 'docking' menggunakan struktur 3D peptida dengan struktur model HBsAg menunjukkan bahawa peptida berkitar adalah berpotensi dibangunkan sebagai ubat terapeutik yang menghalang interaksi virus perumah. Pendekatan kedua adalah untuk mereka-bentuk peptida berdasarkan antigen permukaan virus sebagai perencat untuk menyekat pergabungan virus. Strategi ini melibatkan kajian interaksi antara HBV antigen teras (HBcAg) dan HBsAg. Adalah penting untuk memahami interaksi antara

kedua-dua protein virus ini kerana mereka terlibat dalam pergabungan virus. Dalam kajian ini, dua peptida yang mempunyai 25 asid amino telah dipilih dari urutan HBsAg yang berada di kawasan-kawasan yang berlainan. Peptida yang dinamakan **preS** dan **S** telah digabungkan dengan vektor pGEX-2T dan diekspreskan ke dalam *Escherichia coli*. Penulenan peptida ini dilakukan dengan menggunakan turus GSTrap FF dan dipotong dari tag GST menggunakan trombin. Setiap peptida ini dikesan dengan pewarnaan perak dengan berat molekul 2.9 kDa. Walau bagaimanapun, peptida tulen hasil daripada belahan dari GST terlalu sedikit untuk dijalankan kajian selanjutnya. Oleh yang demikian, kedua-dua peptida ini telah disintesis dengan kaedah kimia Fmoc dan dianalisis selanjutnya menggunakan NMR. Struktur di dalam cecair bagi kedua-dua peptida telah diselesaikan menggunakan spektroskopi NMR ^{1}H, ^{13}C, dan ^{15}N. Peptida **preS** mempunyai beberapa kawasan berstruktur β-belok di Ser7-Pro8-Pro9, Arg11-Thr12-Thr13 dan urutan Ser22-Thr23-Thr24 manakala peptida **S** mempunyai hanya satu kawasan berstruktur pada Ser3-Asn4-His5. Kedua-dua peptida mengandungi struktur bengkok di sekitar struktur β-belok. Ujikaji Pemindahan Perbezaan Ketepuan (STD NMR) telah dijalankan untuk mengkaji interaksi di antara **preS** dan **S** bersama HBcAg. Beberapa asid amino aromatik **preS** dan **S** telah terlibat dalam interaksi dengan HBcAg. Ini menunjukkan peptide-peptida ini berpotensi sebagai agen antivirus yang menghalang morfogenesis HBV. Eksperimen yang telah dijalankan ke atas peptida-peptida ini adalah kaedah asas dalam mereka-bentuk agen antivirus baharu untuk HBV. Kejayaan awal telah diperolehi bagi setiap peptida ini membuka jalan untuk kajian masa hadapan mengenai potensi perencat peptida HBV.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

1D	one dimensional
2D	two dimensional
3D	three dimensional
α	alpha
aa	amino acid
Å	Ångström
β	beta
bp	base pair
CD	circular dichroism
δ	delta
D_2O	deuterium oxide
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
FID	free induction decay
γ	gamma
GST	glutathione-S-transferase
h	hour
HBcAg	hepatitis B core antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCl	hydrogen chloride
HSQC	Heteronuclear Single Quantum Coherence
Hz	hertz
IPTG	isopropyl β-D-1-thiogalactopyranoside
kD	dissociation constant
LB	Luria Bertani
M	Molar
MD	molecular dynamics
min	minute
mg	milligram
MgCl	magnesium chloride
ml	milliliter
ms	millisecond
MW	molecular weight
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
nm	nanometer
nM	nanoMolar
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
K	Kelvin
kDa	kiloDalton

OD	optical density
φ	phi
PDB	Protein Data Bank
ppm	parts per million
ps	picosecond
RMSD	root-mean-square-deviation
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
STD	saturation transfer difference
TOCSY	Total Correlation Spectroscopy
µg	microgram
µl	microliter
U	unit
v	volume
w	weight

AMINO ACIDS ABBREVIATIONS

Amino acid	One letter code	Three letter code
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamic acid	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

CHAPTER 1

INTRODUCTION

The existence of viral hepatitis has been documented many centuries ago, but its causes were only discovered in 1940s when a virus was suspected to be present in human blood of infected individuals (MacCallum, 1947). Hepatitis B virus (HBV) was eventually discovered in 1965 when the Australia antigen was found (later known to be HBsAg) in the blood of Australian aboriginal people (Alter and Blumberg, 1966). HBV is a major cause of acute and persistent liver diseases in humans. Currently, it is estimated that there are more than 360 million chronic HBV carriers worldwide (Shepard *et al*., 2006). Despite availability of effective vaccines, HBV infection remains a health problem globally. This is due to the increasing number of chronically infected people with the rising world population and the limited accessibility of the vaccine in developing countries. The virus is transmitted through direct contact with the blood or body fluid of an infected patient.

The currently approved antiviral drugs used to treat chronic HBV patients are expensive and only 20-30% of them show sustained virological responses after withdrawal of the substances. There are several approaches in designing an antiviral drug based upon the virus life cycle. Antivirals can be designed during the viral entry, viral synthesis, viral assembly and during the release phase. Most of the antivirals available for HBV are inhibitors designed to block the viral synthesis. These inhibitors are grouped as nucleotide/nucleoside analogs. However, the major problem of prolonged therapies with these inhibitors often result in the selection of drug resistant mutants. Thus new discoveries of potent inhibitors for HBV are still required.

Small molecules such as peptide inhibitors have been studied extensively in discovering new compounds for the treatment of HBV. The perfect therapeutic agent would be a small-molecular-mass chemical mimic of a receptor ligand, which would be cheap to manufacture and could get to the site of action after an oral administration, which makes a peptide as the perfect candidate to serve as an inhibitor. Other benefits of working with peptides are: they are small, easily optimized, and can be quickly investigated for therapeutic potential.

This study focuses on two antiviral approaches. The first approach is to discover a peptide that can block the virus from entering the cell. The hypothesis of this approach is based on previous studies whereby HBV surface antigen (HBsAg) has been implicated to be involved in the virus entry. A study carried out by Tan *et al* (2005) showed that a cyclic peptide CETGAKPHC derived from a phage-display library, interacts tightly with the HBsAg. Thus, further studies on this cyclic peptide are of interest as it can be a potential HBV inhibitor. Consequently, understanding the

interaction properties of a lead compound with HBsAg will open possibilities of novel therapeutic options addressing virus entry.

The second approach is to design a peptide based on the viral surface antigen as an inhibitor to block the viral assembly. Many studies have shown that the virus core antigen (HBcAg) and the large surface antigen (L-HBSAg) play important roles during the virus assembly. Therefore, interaction studies between HBcAg and the viral surface antigen are critical to understand due to their involvement in the virus morphogenesis. Regardless of the amount of studies that has been carried out on HBV, detailed structural insights into the arrangement of HBsAg in virions and its interaction with the HBcAg are still obscure.

This dissertation is divided into three research chapters. The first research chapter focuses on the cyclic peptide as a potential inhibitor during virus entry. Nuclear Magnetic Resonance (NMR) and molecular modeling approaches were applied in this chapter. The second research chapter concentrates on the designing of prospective peptides as inhibitors during virus morphogenesis. These peptides were produced using the *Escherichia coli* system. The third research chapter looks further into the potential of the peptides as inhibitors by using NMR as the main method in gaining structural as well as interaction information of the peptides with HBcAg.

Synthetic peptide inhibitors designed based on rational approach of structural information and interaction studies are the latest techniques used in discovering new potential drugs. Therefore, the objectives of this study were:

- To determine the three-dimensional structure of the phage display derived cyclic peptide CETGAKPHC
- To study the interaction of the cyclic peptide with HBsAg
- To synthesize peptides of HBsAg by using *Escherichia coli*
- To determine the three-dimensional structure of the HBsAg peptides
- To study the interactions of the peptides with hepatitis B core antigen (HBcAg)

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