



**UNIVERSITI PUTRA MALAYSIA**

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

**AZIRA BINTI MUHAMAD**

**IB 2014 10**



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**DOCTOR OF PHILOSOPHY  
UNIVERSITI PUTRA MALAYSIA**

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WITH PEPTIDES**

By

**AZIRA BINTI MUHAMAD**

**Thesis Submitted to the School of Graduate Studies,  
Universiti Putra Malaysia, in Fulfilment of the  
Requirements for the Degree of Doctor of Philosophy**

**December 2014**

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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

By

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**December 2014**

**Chair: Tan Wen Siang, PhD**  
**Institute: Bioscience**

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  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$  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  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{15}\text{N}$  NMR spectroscopy. Peptide **preS** has several structured region of  $\beta$ -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**

Oleh

**AZIRA BINTI MUHAMAD**

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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 berkisar 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 berkisar menggunakan kaedah pemilihan afiniti terhadap HBsAg. Peptida berkisar ini mengikat ketat pada rantau immunodominan HBsAg. Oleh yang demikian, kajian ini bertujuan untuk mengenalpasti struktur tiga dimensi peptida berkisar ini dan interaksi peptida tersebut dengan HBsAg secara *in silico*. Struktur tiga dimensi peptida berkisar dalam cecair ini telah dikenalpasti dengan spektroskopi NMR  $^1\text{H}$ ,  $^{13}\text{C}$ , dan  $^{15}\text{N}$  bersama simulasi dinamik molekul dengan jarak sekatan dan sudut regangan yang diperolehi dari eksperimen NMR. Peptida berkisar 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 berkisar 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  $^1\text{H}$ ,  $^{13}\text{C}$ , dan  $^{15}\text{N}$ . Peptida **preS** mempunyai beberapa kawasan berstruktur  $\beta$ -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  $\beta$ -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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I certify that a Thesis Examination Committee has met on 12 December 2014 to conduct the final examination of Azira Binti Muhamad on her thesis entitled “Nuclear Magnetic Resonance Spectroscopic Studies of the Structure and Interactions Between Hepatitis B Virus Core and Surface Antigens with Peptides” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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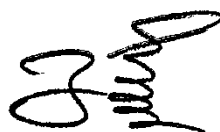
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## TABLE OF CONTENTS

	<b>Page</b>
<b>ABSTRACT</b>	i
<b>ABSTRAK</b>	iii
<b>ACKNOWLEDGEMENTS</b>	v
<b>APPROVAL</b>	vi
<b>DECLARATION</b>	viii
<b>LIST OF TABLES</b>	xiii
<b>LIST OF FIGURES</b>	xiv
<b>LIST OF APPENDICES</b>	xvi
<b>LIST OF ABBREVIATIONS</b>	xvii
<b>AMINO ACIDS ABBREVIATIONS</b>	xviii
<b>CHAPTER</b>	
<b>1 INTRODUCTION</b>	<b>1</b>
<b>2 LITERATURE REVIEW</b>	<b>3</b>
2.1 Hepatitis B Virus	3
2.1.1 Biology of hepatitis B virus	3
2.1.2 Antiviral approaches	8
2.2 Peptides	11
2.3 Structural study of biomolecules	12
2.3.1 NMR spectroscopy	12
2.3.1.1 Chemical shift	14
2.3.1.2 Coupling constants	14
2.3.1.3 The nuclear Overhauser effect (NOE)	15
2.3.1.4 Multidimensional NMR experiments for protein and peptide	16
2.3.1.5 Protein-ligand interaction	20
2.3.1.6 Applications of NMR in structural and interaction studies	21
2.3.2 Circular dichroism	22
2.3.3 Molecular modeling	23
2.4 Concluding remarks	25
<b>3 SOLUTION STRUCTURE AND <i>IN SILICO</i> BINDING OF A CYCLIC PEPTIDE WITH HEPATITIS B SURFACE ANTIGEN</b>	<b>26</b>
3.1 Introduction	26
3.2 Materials and Methods	27
3.2.1 Peptide synthesis	27
3.2.2 Circular Dichroism spectroscopy	27
3.2.3 NMR spectroscopy	28
3.2.4 Spectral assignment	28
3.2.5 Structure calculations	29

3.2.6	Molecular modelling	29
3.3	Results	30
3.3.1	Secondary structure determination by circular dichroism spectroscopy	30
3.3.2	Chemical shifts assignments and identification of secondary structure elements	31
3.3.3	Structure determination using restrained molecular dynamics	40
3.3.4	Docking studies	44
3.4	Discussion and Conclusion	47
<b>4</b>	<b>CLONING, EXPRESSION AND PURIFICATION OF PEPTIDES DERIVED FROM HEPATITIS B SURFACE ANTIGEN</b>	<b>49</b>
4.1	Introduction	49
4.2	Materials and Methods	51
4.2.1.	General protocols	51
4.2.1.1	Extraction of DNA by alkaline lysis	51
4.2.1.2	DNA quantification	51
4.2.1.3	Agarose gel electrophoresis	51
4.2.1.4	Purification of DNA fragments from agarose gel	52
4.2.1.5	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	52
4.2.1.6	Silver Staining	54
4.2.1.7	Western Blotting	54
4.2.1.8	The Bradford Assay	55
4.2.2	Cloning of HBsAg peptides	55
4.2.3	Expression and purification of the recombinant proteins	57
4.3	Results	58
4.3.1	Cloning of HBsAg peptides	58
4.3.2	Expression and purification of the recombinant peptides	58
4.4	Discussion and Conclusion	62
<b>5</b>	<b>STRUCTURAL AND INTERACTION STUDIES OF HEPATITIS B CORE ANTIGEN WITH PEPTIDES DERIVED FROM HEPATITIS B SURFACE ANTIGEN</b>	<b>64</b>
5.1	Introduction	64
5.2	Materials and Methods	65
5.2.1	Purification of Hepatitis B Virus Core Protein	65
5.2.1.1	Preparation of crude lysate	65
5.2.1.2	Ammonium sulfate precipitation	66
5.2.1.3	Size exclusion chromatography	66
5.2.2	Peptide synthesis	66

5.2.3	NMR spectroscopy	67
5.2.4	Spectral assignment	67
5.2.5	Structure calculations	68
5.2.6	Molecular docking	68
5.2.7	Saturation Transfer Difference Experiments	69
5.3	Results	69
5.3.1	Purification of hepatitis B core antigen	69
5.3.2	Chemical shifts assignments and identification of secondary structure elements	70
5.3.3	Structure determination using restrained molecular dynamics	75
5.3.4	Docking studies	77
5.3.5	STD NMR	81
5.4	Discussion and Conclusion	82
<b>6</b>	<b>SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH</b>	<b>87</b>
6.1	Summary	87
6.2	Conclusion	88
6.3	Recommendations for future research	88
	<b>REFERENCES</b>	<b>90</b>
	<b>APPENDICES</b>	<b>104</b>
	<b>BIODATA OF STUDENT</b>	<b>116</b>
	<b>LIST OF PUBLICATIONS</b>	<b>117</b>



## LIST OF TABLES

Table		Page
3.1	CP <sup>cis</sup> chemical shifts	36
3.2	CP <sup>trans</sup> chemical shifts	37
3.3	<sup>3</sup> J(H <sup>N</sup> -H <sup>α</sup> ) coupling constants and calculated phi angle	39
3.4	φ and ψ angles of representative structures of CP <sup>cis</sup>	41
3.5	φ and ψ angles of representative structures of CP <sup>trans</sup>	42
3.6	Structural statistics of the simulated cyclo(1,9)-CETGAKPHC peptide ensembles	44
4.1	6x Sample Buffer	53
4.2	Resolving Gel Solution	53
4.3	Stacking Gel Solution (12-15% Acrylamide)	53
4.4	1x Running Buffer	53
4.5	Silver Stain Protocol	54
4.6	Primers designed	56
4.7	PCR reagents	57
4.8	PCR protocol	57
5.1	Peptide sequences	67
5.2	<sup>3</sup> J(H <sup>N</sup> -H <sup>α</sup> ) coupling constant of peptides <b>preS</b> and <b>S</b>	73
5.3	Structural statistics of the simulated <b>preS</b> and <b>S</b> peptide ensembles	75

## LIST OF FIGURES

Figure		Page
2.1	A schematic representation of HBV virion structure	4
2.2	Biological assembly image of T4 capsid of human hepatitis B virus strain adyw, PDB code 2G33.	5
2.3	HBcAg dimer crystal structure, PDB code: 2QIJ	6
2.4	Schematic of the HBsAg gene	7
2.5	Mixed topology of L at the endoplasmic reticulum (ER) membrane	8
2.6	The life cycle of HBV	9
2.7	The spinning nucleus with a charge precessing in a magnetic field	13
2.8	Karplus curve for a peptide group	15
2.9	The pulse sequence of COSY	16
2.10	The pulse sequence of TOCSY	17
2.11	The pulse sequence of NOESY	17
2.12	Flowchart of assignment/structure determination process in peptide NMR	19
2.13	Illustration of ligand saturation process in an STD NMR experiment	21
2.14	Backbone CD spectra for different protein secondary structural motifs	24
3.1	CD spectrum of S <sup>1</sup> ,S <sup>9</sup> -cyclo-CETGAKPHC by CD spectroscopy	31
3.2	1D <sup>1</sup> H -NMR spectrum of the amide region of S <sup>1</sup> ,S <sup>9</sup> -cyclo-CETGAKPHC	32
3.3	The 1D <sup>1</sup> H -NMR spectra of the S <sup>1</sup> ,S <sup>9</sup> -cyclo-CETGAKPHC at different temperatures	33
3.4	Homonuclear NMR spectra	34
3.5	2D <sup>1</sup> H- <sup>13</sup> C HSQC-TOCSY spectrum of S <sup>1</sup> ,S <sup>9</sup> -cyclo-CETGAKPHC	35
3.6	Proton chemical shift index (CSI) for the (A) CP <sup>cis</sup> and (B) CP <sup>trans</sup> and a summary of the inter-residue NOEs, for (C) CP <sup>cis</sup> and (D) CP <sup>trans</sup> , respectively	38
3.7	Overlay of ten NMR restrained MD simulation structures of cyclic peptide	43
3.8	Docking of the cyclic peptides to HBsAg	46
4.1	Agarose gel electrophoresis of PCR analysis of positive clones carrying the coding sequences of the peptides	59
4.2	SDS-PAGE (A) and Western blotting (B) of the expression of fusion peptides, <b>preS</b> and <b>S</b>	60
4.3	SDS-PAGE gels of fusion peptide <b>preS</b> (A) and peptide <b>S</b> (B) purified using GSTrap FF column	61
4.4	Silver stain gels of peptides <b>preS</b> (A) and <b>S</b> (B)	62

5.1	Purification of HBcAg by SEC	70
5.2	2D $^1\text{H}$ - $^1\text{H}$ TOCSY spectra of <b>preS</b> and <b>S</b> peptides	71
5.3	2D $^1\text{H}$ - $^{15}\text{N}$ -HSQC spectra of peptide <b>S</b>	72
5.4	A summary of the inter-residue NOEs and proton chemical shift index (CSI)	74
5.5	Overlay of ten NMR restrained MD simulation structures and the representative structures of both peptides	76
5.6	<b>preS</b> and <b>S</b> peptides docked to HBcAg dimer by HADDOCK	78
5.7	<b>preS</b> peptide in complex with HBcAg	79
5.8	<b>S</b> peptide in complex with HBcAg	80
5.9	STD spectra of peptide <b>preS</b> -HBcAg complex	83
5.10	STD spectra of peptide <b>S</b> -HBcAg complex	84

## LIST OF APPENDICES

Appendix		Page
A1	NOESY spectra of the S <sup>1</sup> ,S <sup>9</sup> -cyclo-CETGAKPHC at different concentrations	104
A2	Ramachandran plot of CP <sup>cis</sup> ensemble	105
A3	Ramachandran plot of CP <sup>trans</sup> ensemble	106
B1	Vector map of pGEX-2T	107
B2	<b>preS</b> peptide DNA sequencing results	108
B3	<b>S</b> peptide DNA sequencing results	109
C1	<b>preS</b> peptide 1D <sup>1</sup> H spectrum	110
C2	<b>S</b> peptide 1D <sup>1</sup> H spectrum	111
C3	<b>preS</b> peptide chemical shifts at pH 4	112
C4	<b>S</b> peptide chemical shifts at pH 4	113
C5	Ramachandran plot of peptide <b>preS</b> ensemble	114
C6	Ramachandran plot of peptide <b>S</b> ensemble	115

## LIST OF ABBREVIATIONS

1D	one dimensional
2D	two dimensional
3D	three dimensional
$\alpha$	alpha
aa	amino acid
Å	Ångström
$\beta$	beta
bp	base pair
CD	circular dichroism
$\delta$	delta
D <sub>2</sub> O	deuterium oxide
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
FID	free induction decay
$\gamma$	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 $\beta$ -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
$\phi$	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
$\mu\text{g}$	microgram
$\mu\text{l}$	microliter
U	unit
v	volume
w	weight



## AMINO ACIDS ABBREVIATIONS

<b>Amino acid</b>	<b>One letter code</b>	<b>Three letter code</b>
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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