UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL ANTI-VIRAL PEPTIDE AGAINST AVIAN INFLUENZA VIRUS H9N2

MOHAMED RAJIK

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By

MOHAMED RAJIK

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Chairman: **Professor Datin Paduka Khatijah Mohd. Yusoff, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

Avian influenza viruses (AIV) are major cause of morbidity and mortality in the world. It is the causative agent of the most dangerous disease, called bird flu in common terms, among poultries. This virus belongs to the family of *Orthomyxoviridae* which contains two genera, influenza A & B and influenza C. Although avian influenza A viruses do not usually infect humans, several instances of human infections and outbreaks have been reported. Spanish flu, the well known influenza pandemic of 1918, is thought to have killed more than 50 million people worldwide. Although vaccination is the primary strategy for the control of infection, the antiviral drugs play an important role in the control of illness and transmission. Two classes of antiviral drugs are recommended for the treatment of influenza infection. They are adamantane derivatives (amantadine and rimantadine) and neuraminidase inhibitors (NAIs; zanamivir and oseltamivir). The rate of adamantane resistance has increased significantly from below 2% to an alarming 92.3% in recent years. Few viruses with altered susceptibility to NAIs have also been

isolated from the infected people. Several recent H5N1 influenza isolates recovered from the patients showed resistance to both classes of antiviral drugs. The increasing appearance of resistant strains of influenza virus and side effects of the currently available chemotherapeutic agents emphasises our need to identify new antiviral drugs.

In order to identify novel antiviral drugs, phage display library was utilised in this study. They were biopanned against the purified avian influenza virus H9N2. After four rounds of biopanning, four unique recombinant fusion phages bearing different peptide sequences were isolated. Their binding specificity was confirmed by modified phage-ELISA method. Among the four peptides, the peptide denoting the sequence NDFRSKT (P1) was taken into account for further analysis as it showed a high percentage of presence after the fourth round of panning and good inhibitory properties.

The *in vitro* inhibitory properties of the fusion phages were proved by the ability of the phage molecules to stop the multiplication of the virus in MDCK cell lines whereas, the *in ovo* inhibition ability of both peptides and fusion phages were assessed using the 9 days old embryonated chicken eggs. The antiviral molecule's ability to inhibit the hemagglutination activity and neuraminidase (NA) activity were also investigated using conventional hemagglutination inhibition test and neuraminidase inhibition test respectively. They were able to inhibit the HA activity but failed to inhibit the neuraminidase activity completely. The antibody-phage competition assay showed that the peptide molecule share some common epitopes of the viral surface proteins for their binding site. In order to investigate the *in vivo* binding ability of the molecules inside a

cellular environment and to identify the binding domain on the viral proteins, the peptide and virus surface glycoproteins interaction were analysed by the yeast two-hybrid system. The results showed that the C-terminal region of the HA protein was responsible for the interaction with the peptide. This was further confirmed by the co-immunoprecipitation experiment.

To understand the mechanism of the drug action, the effect of drug on the viral attachment and the viral entry to the host cell (MDCK cells) was studied by fluorescence microscopy and flow cytometry. The study revealed that the peptides prevent the attachment of the virus to the host cells, confirming the result of earlier haemagglutination inhibition experiment. Besides, it was also found that the peptides inhibit the early gene expression. But the peptides do not have any effect on preventing the entry of the virus molecules.

In summary, the current study had identified a novel antiviral peptide which inhibits the AIV H9N2 multiplication *in ovo* and *in vitro*, by binding the HA. This peptide prevents the attachment of the virus to the host cells thereby preventing its internalisation and early protein expressions. These new antiviral molecules may have the potential to control and treat the avian influenza virus infected individuals.

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

PENGENALPASTIAN DAN PENCIRIAN PEPTIDA ANTIVIRUS TERHADAP VIRUS INFLUENZA BURUNG H9N2

Oleh

Mohamed Rajik

November 2008

Pengerusi : Professor Datin Paduka Khatijah Mohd. Yusoff, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Virus influenza burung (AIV) adalah antara penyebab utama penyakit dan kematian spesis burung di dunia. Ia merupakan agen penyebab kebanyakan penyakit berbahaya yang lazimnya dikenali sebagai selsema burung di kalangan ternakan burung. Virus ini adalah dari keluarga *Orthomyxoviridae* yang terdiri daripada dua genera, influenza A & B dan influenza C. Walaupun virus-virus influenza A burung biasanya tidak menjangkiti manusia, terdapat beberapa kes wabak dan jangkitan manusia telah dilaporkan. 'Spanish flu', suatu pandemik influenza pada tahun 1918 yang dimaklumi ramai, dikatakan telah mengorbankan lebih 50 juta orang. Sungguhpun langkah pengvaksinan merupakan strategi utama bagi pengawalan jangkitan, antivirus dalam bentuk dadah memainkan peranan penting dalam pengawalan penyakit dan penyebaran virus.

Dua kelas dadah antivirus telah disyorkan bagi merawat jangkitan influenza. Mereka adalah terbitan dadah kelas adamantine (amantadine dan rimantadine) dan perencat neuraminidase (NAIs; zanamivir dan oseltamivir). Kadar kerintangan terhadap adamantine di Amerika Syarikat sahaja telah meningkat dari bawah paras 2% kepada nilai amaran iaitu 92.3% beberapa tahun

kebelakangan ini. Sekelompok virus dengan kadar keberkesanan terhadap NAIs telah diperolehi. Beberapa pemencilan influenza baru daripada pesakit jangkitan influenza H5N1 didapati mempamerkan kerintangan terhadap kedua-dua kelas dadah antivirus.

Peningkatan kehadiran strain-strain virus influenza dengan daya kerintangan serta kesan sampingan yang ada menekankan keperluan kita untuk mengenalpasti dadah-dadah antivirus yang baru. Dalam usaha mengenalpasti dadah-dadah antivirus novel, 'phage display library' telah digunakan dalam kajian ini. Mereka telah di*'biopanned'* ke atas virus influenza burung H9N2 yang telah ditulenkan. Selepas empat kitaran 'biopanning', empat peptida telah dipencilkan. Ketepatan ikatan mereka telah dipastikan menggunakan kaedah 'phage-ELISA' yang telah diubahsuai. Antara empat peptida tersebut, peptida yang membawa jujukan NDFRSKTC (P1) telah dipilih untuk analisis seterusnya kerana ia menunjukkan kadar kehadiran yang tinggi selepas kitaran keempat *'panning'* di samping mempunyai ciri-ciri perencat yang baik.

Kebolehan merencat secara *in vitro* oleh peptida-peptida dan 'fusion phages' telah dianalisa berdasarkan keupayaan faj atau molekul peptida untuk menyekat pembiakan virus di dalam sel MDCK sementara keupayaan merencat peptida oleh 'fusion phage' secara *in ovo* adalah menggunakan telur ayam berembrio berusia 9 hari. Keupayaan molekul antivirus untuk merencat aktiviti hemaglutinasi dan 'neuraminidase' NA juga telah dikaji masing-masing menggunakan ujian perencatan hemaglutinasi dan ujian perencatan 'neuraminidase' konservatif. Ujian-ujian tersebut berjaya menunjukkan kesan perencatan activiti HA tetapi gagal untuk merencat aktiviti 'neuraminidase' secara keseluruhannya.

‘Antibody-phage competition assay’ menunjukkan bahawa molekul peptida berkongsi beberapa epitop lazim pada protein permukaan virus sebagai tapak pengikatannya. Bagi mengkaji keupayaan pengikatan secara *in vivo* molekul-molekul tersebut dalam persekitaran sel, interaksi antara peptida dan glikoprotein permukaan virus telah dianalisa menggunakan sistem ‘yeast two-hybrid’. Kawasan hujung C pada protein HA didapati berupaya mengikat peptida tersebut sementara protein NA menunjukkan interaksi yang lemah terhadap peptida. Ini telah dipastikan lagi melalui eksperimen ‘co-immunoprecipitation’.

Untuk memahami mekanisme dadah dengan lebih mendalan, efek dadah tersebut terhadap pelekatan dan penembusan virus kedalam sel his telah dikaji mnggunakan kaedah mikroskop fluoresen dan “cytometry”. Kajian ini menunjukkan bahawa peptide-peptida ini mengelakkan pelekatan virus kepada sel hos, sekaligus mengesahkan hasil kajian eksperimen perencatan hemaglutinasi. Lagipun, peptide-peptida tersebut telah didapati boleh merencat ekspresi gen awal.

Namun demikian, mereka tak mempunyai sebarang efek bagi mengelakkkan kemasukkan molekul-molekul virus. Kesimpulannya, kajian ini telah mengenalpasti satu peptida antivirus novel yang berupaya merencat pembiakan AIV H9N2 secara *in ovo* dan *in vitro*, melalui pengikatan tapak hujung C pada protein HA. Peptida ini mengelakkan pelekatan virus pada sel hos, sekaligus mencegah penembusan serta ekspresi protein awal virus. Molekul-molekul antivirus baru ini mungkin berpotensi untuk mengawal dan merawat pesakit jangkitan virus influenza burung.

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I certify that a Thesis Examination Committee has met on 14 July 2009 to conduct the final examination of Mohamed Rajik on his thesis entitled "Identification and Characterization of a Novel Anti-Viral Peptide Against Avian Influenza Virus H9N2" 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.

Members of the Examination Committee were as follows:

Norhani Abdullah, PhD
Professor
Faculty of Biotechnology and Biomolecular sciences
Universiti Putra Malaysia
(Chairman)

Raha Abdul Rahim, PhD
Professor
Faculty of Biotechnology and Biomolecular sciences
Universiti Putra Malaysia
(Internal Examiner)

Raja Noor Zaliha Raja Abdul Rahman, PhD
Professor
Faculty of Biotechnology and Biomolecular sciences
Universiti Putra Malaysia
(Internal Examiner)

Ruben Omar Donis, PhD
Lecturer
Molecular Virology and Vaccines Branch Influenza Division
CCID Centers for Disease Control and Prevention
United States of America
(External Examiner)

BUJANG KIM HUAT, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 15 July 2009

The thesis 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:

Datin Paduka Khatijah Mohd. Yusoff, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Abdul Rahman Omar, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Sharifah Syed Hassan, PhD
Associate Professor
School of Medical and Health Sciences
Monash University Malaysia
(Member)

_____
HASANAH MOHD. GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 17 July 2009

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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MOHAMED RAJIK

Date:

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AD	activation domain
AIV	avian influenza virus
BCP	1-bromo-3-chloro-propane
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BD	binding domain
β-gal	β-galactosidase
bp	base pair
cDNA	complementary deoxyribonucleic acid
ci	curi
CITE	cap-independent translation enhancer
C-terminus	carboxy terminus
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides
DTT	1,4-dithiothritol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GST	glutathione S-transferase
h	hour
HA	Haemagglutinin activity
His	Histidine

kb	kilo base
kDa	kilo Dalton
LB	Luria Bertani
µg	microgram
µl	microlitre
µM	micromolar
mA	milliampere
mRNA	messenger RNA
NBT	nitro blue tetrazolium
NP	nucleocapsid protein
NP40	Nonidet P40
nt	nucleotide
N-terminus	amino terminus
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
pH	*puissance hydrogene*
PCR	polymerase chain reaction
RBC	red blood cell
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction

s	second
SDS	sodium dodecyl sulphate
SV5	simian virus 5
TAE	Tris-acetate – EDTA buffer
TBA	Tris-buffered saline
Taq	*Thermus aquaticus*
TEMED	tetramethyl ethylenediamine
U	unit
uv	ultraviolet
Vol	volume
w/v	weight/volume
YC	minimal medium for yeast

CHAPTER 1

INTRODUCTION

Avian influenza (AI) is caused by avian influenza virus (AIV) which belongs to the family *Orthomyxoviridae*, genus *Influenza* A. It rapidly infects poultry as well as many species of birds. The viruses are classified into subtypes on the basis of antigenic differences in their surface glycoprotein hemagglutinin (HA/H) and neuraminidase (NA/N). To date, 16 HA subtypes (H1-H16) and 9 NA subtypes (N1-N9) have been recognised (Webster *et al.,* 1992). AIV can also be categorised as high pathogenicity (HP) and low pathogenicity (LP) on the basis of the severity of clinical signs and mortality rates in experimentally inoculated chickens (Swayne *et al.,* 2000). HPAI is in list A disease of the Office International des Epizooties (OIE; http:www.oie.int, Septermber 2008).

AIVs occur naturally among birds. Wild birds worldwide carry the viruses in their intestines, but usually do not get sick from them. Domesticated birds may become infected with AIV through direct contact with infected waterfowl or other infected poultry, or through contact with contaminated surfaces (such as dirt or cages) or materials (such as water or feed) (Goldrick and Goetz, 2007).

Infection with AIVs in domestic poultry causes two main forms of disease that are distinguished by low and high extremes of virulence. The “low pathogenic” form may