UNIVERSITI PUTRA MALAYSIA

DESIGN, SYNTHESIS, AND BIOCHEMICAL ASSAY OF PEPTIDE INHIBITORS DERIVED FROM α-ENOLASE FOR USE AGAINST PEPTIDYLARGININE DEIMINASE TYPE 4

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By

IZZUDDIN BIN AHMAD NADZIRIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia
in fulfilment of the requirement for the degree of Master of Science

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By

IZZUDDIN BIN AHMAD NADZIRIN

October 2013

Chair: Bimo Ario Tejo, PhD

Faculty: Science

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease affecting about 0.5–1.0% of the world population. Peptidylarginine deiminase type 4 (PAD4) is a responsible protein for the occurrence of RA by catalyzing citrullination of proteins. The citrullinated proteins act as autoantigens by stimulating an immune response. Citrullinated α-enolase was identified as one of autoantigens for RA. Hence, α-enolase serves as a good template for design of potential peptide inhibitors against PAD4. The binding affinity of α-enolase-derived peptides and PAD4 was virtually determined using PatchDock and HADDOCK docking programs. Synthesis of the designed peptides was performed using a solid phase peptide synthesis (SPPS) method. The inhibitory potential of each peptide was determined experimentally by PAD4 inhibition assay and IC_{50} measurement. Based on the virtual experiment, binding energies given by molecular docking data suggested that N-P1 and NP-2 peptides were the most active substrates of PAD4. The synthesis of these peptides using SPPS yielded peptides with purity of more than 60%. PAD4 activity assay data

showed that the N-P2 peptide was the most favourable substrate among all peptides. Further modification of N-P2 by changing the Arg residue to canavanine (P2 (Cav)) rendered it an inhibitor against PAD4 by reducing the PAD4 activity to 35% with IC_{50} 1.39 ± 0.3 mM. The binding of P2 (Cav) and PAD4 changed the secondary structure of the peptide as indicated by circular dichroism spectroscopic data. As a conclusion, P2 (Cav) is a potential inhibitor against PAD4 and can serve as a starting point for the development of even more potent inhibitors.

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk ijazah Master Sains

REKA BENTUK, SINTESIS DAN ASAI BIOKIMIA PERENCAT PEPTIDA YANG BERASASKAN α-ENOLASE UNTUK KEGUNAAN MERENCAT PEPTIDILARGININA DEIMINASE JENIS 4

Oleh

IZZUDDIN BIN AHMAD NADZIRIN

Oktober 2013

Pengerusi: Bimo Ario Tejo, PhD

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Artritis Reumatoid (AR) adalah sejenis penyakit autoimun yang melibatkan 0.5-1.0% populasi dunia pada masa kini. Peptidilarginina deiminase jenis 4 (PAD4) telah dikenal pasti sebagai protein yang bertanggungjawab menyebabkan berlakunya AR dengan memangkin proses sitrulinasi protein yang lain. Protein yang telah melalui proses sitrulinasi ini kemudiannya akan bertindak sebagai autoantigen yang merangsang tidak balas sistem pertahanan badan. α-enolase yang telah melalui proses sitrulinasi telah dikenal pasti sebagai salah satu autoantigen penyebab AR. Oleh yang demikian, α-enolase adalah satu templat yang baik dalam usaha mereka bentuk peptida yang berpotensi merencat aktiviti pemangkinan PAD4. Daya penambatan peptida yang direka bentuk daripada α-enolase pada PAD4 telah ditentukan secara maya menggunakan dua perisian "*docking"* iaitu PatchDock dan HADDOCK. Sintesis peptida yang telah direka bentuk dilakukan menggunakan teknik sintesis peptida fasa pepejal (SPPS). Potensi perencatan dinilai bedasarkan asai perencatan PAD4 dan penentuan IC_{50}**.** Berdasarkan eksperimen maya, daya

penambatan yang didapati oleh peirisian "*docking"* menunjukkan bahawa peptida N-P1 dan N-P2 ialah substrat yang paling aktif untuk PAD4. Sintesis peptida yang menggunakan teknik SPPS menghasilkan peptida yang mempunyai peratusan ketulenan melebihi 60%. Asai aktiviti PAD4 pula menunjukkan peptida N-P2 sebagai substrat yang paling sesuai dengan PAD4. Kemudian, N-P2 telah dimodifikasi dengan menukarkan asid amino Arg dalam jujukan asid aminonya kepada kanavanina (P2 (Cav)), sekali gus mentransformasikan sifat awalnya iaitu sebagai substrat kepada sifat merencat. P2 (Cav) telah didapati mengurangkan aktiviti PAD4 kepada 35% dan IC_{50} yang dicatatkan adalah 1.39 ± 0.3 mM. Penambatan antara P2 (Cav) dan PAD4 juga didapati telah mengubah struktur sekunder peptida tersebut seperti yang ditunjukkan data spektroskopi dikroisme bulat. Secara rumusannya, P2 (Cav) bertindak sebagai perencat yang berpotensi merencat aktiviti PAD4 dan boleh dijadikan batu loncatan untuk menghasilkan perencat yang lebih berpotensi.

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I certify that a Thesis Examination Committee has met on 11th October 2013 to conduct the final examination of Izzuddin Bin Ahmad Nadzirin on his thesis entitled "Design, Synthesis, and Biochemical Assay of Peptide Inhibitors Derived from α-Enolase for Use against Peptidylarginine Deiminase Type 4" 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 Master of Science.
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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 Master of Science. The members of the Supervisory Committee were as follows:

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Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

IZZUDDIN BIN AHMAD NADZIRIN

Date: 11 October 2013

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LIST OF ABBREVIATIONS

3D	three-dimensional
ACN	acetonitrile
ACPA	anticitrullinated protein antibodies
ACR	American College of Rheumatology
AKA	antikeratin antibody
Ala/A	alanine
APF	antiperinuclear factor
Arg/R	arginine
$ArgNO_2$	nitroarginine
Asp/D	aspartic acid
BAA	N-α-benzoyl arginine amide
BAEE	N-α-benzoyl-L-arginine ethyl ester
BB-RMSD	root mean square deviation of backbone
BMO	2,3-Butanedione Monoxime
BSA	bovine serum albumin
Bz-L-Arg	N-α-benzoyl-L-arginine
bp	base pair
Cav	canavanine
CD	circular dichroism
Cit	citrulline
Cys/C	cysteine
DCM	dichloromethane
ddH_2O	deionized distilled water

DIC	diisopropylcarbodiimides
DIEA	N,N-Diisopropylethylamine
DMARD	disease modifying anti-rheumatic drug
DMF	N,N-Dimethylformamide
DTT	1,4-Dithiothreitol
EULAR	European League Against Rheumatism
Fmoc	9-fluorenylmethyloxycarbonyl
GDH	glutamate-dehydrogenase
GIT	gastrointestinal tract
Gln/Q	glutamine
Glu/E	glutamic acid
Gly/G	glycine
HCTU	2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
His	histidine
HMM	Hidden Markov Model
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxy-1H-benzotriazole
HPLC	High Performance Liquid Chromatography
HT	high tension voltage
IC_{50}	half maximal inhibitory concentration
IL-15	interleukin-15
IL-17	interleukin-17
IL-22	interleukin-22
Ile/I	isoleucine

KCN	potassium cyanide
LC/MS	liquid chromatography/mass spectroscopy
Leu/L	leucine
Lys/K	lysine
Met	methionine
MHC	major histocompatibility complex
MT-MMP	membrane-type matrix metalloproteinases
MT1-MMP	membrane-type 1 matrix metalloproteinases
MTX	methotrexate
MW	molecular weight
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NMP	1-methyl-2-pyrrolidinone
NMR	nuclear magnetic resonance
NSAID	non-steroidal anti-inflammatory drug
Orn	ornithine
PAD	peptidylarginine deiminase
PAD4	peptidylarginine deiminase type 4
Pbf	pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl
PDB	Protein Database
Pro/P	proline
RA	rheumatoid arthritis
RF	Rheumatoid Factor
RMSD	root mean square deviation
SA	structural alphabet

SE	shared epitope
Ser/S	serine
SFB	segmented filamentous bacterium
sOPEP	coarse-grained energy score
SPPS	solid phase peptide synthesis
TFA	trifluoroacetic acid
Thr/T	threonine
TIS	triisopropylsilane
Trp/W	tryptophan
Tyr/Y	tyrosine
Val/V	valine

CHAPTER 1

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by pain at lower and upper joints. The term autoimmune can be defined into two separate words; auto and immune. Auto means self and immune refers to immune system. Thus, autoimmune disease can be inferred as a disease caused by self immune system response against one self's own cells or tissues. The development of RA is caused by the immune system stimulation upon detection of autoantigen in serum. The stimulation is followed up with the attack of self cells, particularly joint cells, of an affected person. After certain period of time, RA patients' joints will be swollen and they will feel extreme pain because of chronic inflammation in synovial space surrounding upper and lower limb. Eventually, the affected joints will be totally destroyed which results in disability and lower quality of life (Vossenaar & van Venrooij, 2004).

RA has become more common among people and recent estimation showed that about 0.5-1.0% of world population to be affected by the disease (Gabriel, 2001). It was estimated approximately three million United States people are afflicted with RA and the same number of cases was reported in Europe (Helmick *et al.*, 2008). Statistics proved that more than 50% of RA patients have lost their capabilities of doing normal jobs after six years of first diagnosis (Lawrence *et al.*, 2008). The situation is worse in Malaysia because most of RA patients seeking for their first treatment are already in bad health condition and are jobless. Worst still, most of

them are in their 20's to 40's who should be productive in their careers (Fong, 2010). Such situation indirectly gives bad consequence to the country because it can reduce the country's human capital and productivity. Apart from that, RA treatment also involves a big amount of money for prolonged period of time and thus, it can affect the status of economy of a country as well. Hence, RA is indeed a dangerous affliction to the country in general and people particularly.

All autoimmune diseases involve recognition of autoantigen. Autoantigen may originate from external pathogen or internal substances. An affected person has autoantigen in his/her serum which is later detected by the immune system. The system then reacts and produces antibodies against the autoantigen. The same occurrence applies in RA patients. Various autoantigens have been identified as probable autoantigens for RA, but none is confirmed to be the main culprit. Despite the fact, researchers have been studying those autoantigens for manipulating it into a serological marker to diagnose the disease at early stage. A few markers have been identified so far which includes the long identified Rheumatoid Factor (RF), yet the specificity of RF towards RA is limited where only 75% of RA patients exhibit RF and 5% of healthy patients also exhibit RF in their sera (Kinloch *et al.*, 2005).

There were other markers reported present in the sera of RA which include antiperinuclear factor (APF) and antikeratin antibody (AKA) (Serre, 2001). Until recently, a new marker has been identified as a great specific marker for RA which is anticitrullinated protein antibodies (ACPA) (Anzilotti *et al.*, 2010). Its diagnostic value has been proven in several studies (Kang *et al.*, 2006; Steiner & Smolen, 2002;

van Boekel *et al.*, 2002). The promising news is that the detection of ACPA is possible even before the onset of RA symptoms.

ACPA recognizes and attacks citrullinated proteins, i.e. proteins having arginine (Arg) undergo citrullination process which alters it into citrulline (Cit), as shown in Figure 1.1. The citrullination process is catalyzed by a protein called peptidylarginine deiminase type 4 (PAD4). There are several types of ACPAs identified in RA patients which include anticitrullinated filaggrin, anti-Sa and anti-α-enolase antibodies; depending on types of citrullinated proteins it attacks. Anti-α-enolase antibodies have been recently identified as candidate autoantibodies that play important role in the development of RA (Kinloch *et al.*, 2005).

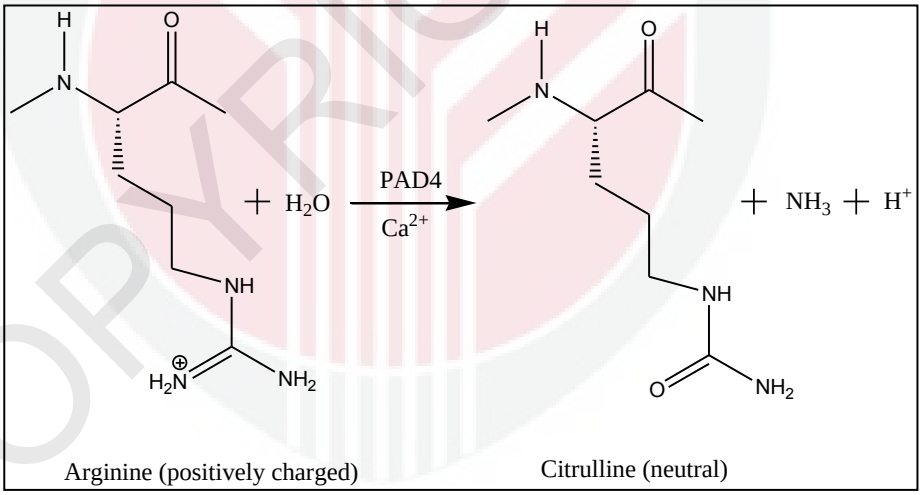

Figure 1.1. Citrullination process by PAD4. Arg is converted into citrulline by PAD4 in the presence of Ca^{2+}. The alteration also includes the positive charged carried by Arg being deprotonated into neutral citrulline (Nijenhuis *et al.*, 2004).

Scientists all over the world have done extensive researches on this disease, yet the underlying causes and pathogenesis of RA still remain blurry. There is no single explanation or hypothesis that is agreed by all scientists to describe its aetiology and pathogenesis. Despite incomplete knowledge about the disease, scientists have

developed several drugs to overcome the disease. However, current medications are only for relieving RA symptoms, leaving the RA causative agent unattended. This subsequently leads to deterioration of the joints of RA patients (Knuckley *et al.*, 2008). At present, there are three classes of drugs available in the market to alleviate the symptoms of RA which are disease modifying anti-rheumatic drug (DMARD), non-steroidal anti-inflammatory drug (NSAID), and corticosteroid (Smolen & Steiner, 2003).

The most commonly used class of drugs among RA patients is DMARD. Even though it has been long known as having unspecific target and producing multiple side effects, DMARD is still regarded as the best medication for RA patients. Example of a DMARD drug is Methotrexate (MTX). Long term use of MTX aggravates the probability of developing diverse side effects on pulmonary, central nervous, hepatic, hematologic, and gastrointestinal systems (Smolen *et al.*, 2005). Its consumption by RA patients is proven to be effective against RA with the best efficacy-toxicity ratio. Nevertheless, discontinuation of the drug among 30% of its users was observed after one year of therapy. The reason of discontinuation is toxicities rather than lack of efficacy (van Ede *et al.*, 1998).

Since toxicity is the major concern of MTX, the direction of drug design approach has been re-routed to peptide-based drug design. Peptide-based drug is a drug made of peptide, i.e. polymer of amino acids linked together via peptide/amide bond in a straight or cyclic chain. Amino acids and peptides are readily present in human body and thus, are natural to human body. Therefore scientists believe that peptide-based drugs can overcome the toxicity problem while conserving the potency of a drug

(Otvos, 2008). With the discovery of PAD4 as the responsible protein for catalyzing citrullination of proteins and α-enolase as protein targeted by PAD4, development of peptide-based drug is possible.

Therefore, the present research aimed to synthesize a novel peptide-based drug to treat RA. The general idea that gives rise to this research is that RA is caused by the activation of PAD4 that citrullinates a few types of proteins including the protein of interest which is α-enolase. Citrullinated α-enolase becomes an autoantigen which stimulates the immune system to attack the citrullinated α-enolase. Hence, this study aimed to design and synthesize peptide inhibitors against PAD4 based on the sequence of α-enolase. The design was primarily based on α-enolase sequence and the designed peptides were docked with PAD4 virtually. Subsequently, the peptides were synthesized and assayed *in vitro* to determine the binding likeliness with PAD4 before being modified to become potential inhibitors. The modified peptides were then synthesized and assayed to observe its inhibition on PAD4 activity. The most potent inhibitor was chosen to determine its half maximal inhibitory concentration (IC_{50}). The secondary structures of particular peptides were later determined using circular dichroism (CD).

The objectives of this study are:

1. To design peptides and determine binding energy of the designed peptides with PAD4 *in silico*.
2. To synthesize the designed peptides, observe inhibitory properties of the modified peptides *in vitro* and determine IC_{50} of the best-inhibiting peptide.
3. To study the secondary structure of peptides using circular dichroism.

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