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SCREENING, DESIGN, SYNTHESIS AND BIOACTIVITY OF INHIBITORS AGAINST PROTEIN ARGININE DEIMINASE TYPE IV

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SCREENING, DESIGN, SYNTHESIS AND BIOACTIVITY OF INHIBITORS AGAINST PROTEIN ARGININE DEIMINASE TYPE IV

By

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SCREENING, DESIGN, SYNTHESIS AND BIOACTIVITY OF INHIBITORS AGAINST PROTEIN ARGININE DEIMINASE TYPE IV (PAD4)

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Chair: Professor Mohd Basyaruddin Abdul Rahman, PhD

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Protein Arginine Deiminase IV (PAD4) is a new target for rheumatoid arthritis (RA) therapeutic. It catalyses the citrullination process in human body that produces citrullinated protein which is believed to be the root cause of RA. Inhibitor for the enzyme can be a drug for the treatment of RA. The main objective of this research is to search for potent inhibitors for PAD4. Human PAD4 has been successfully expressed in *Escherichia coli* BL21 (DE3). PAD4 expressed from pET32b vector with the use of auto-induction media introduced by Studier gave higher solubility and activity compared to conventional induction system. PAD4 was successfully purified using affinity chromatography technique with purity of 90%, approximately. Several compounds have been identified as potential inhibitors for PAD4 by structure-based virtual screening using Ligand Discovery at Edinburgh University (LIDAEUS) programme. Three compounds out from 22 top-ranked water-soluble compounds showed significant inhibition to PAD4 and their IC_{50} values were ranged from 1.49 ± 0.03 to 2.96 ± 0.01 mM. Binding affinities of the compounds to PAD4 obtained from molecular docking were -7.49, -7.27 and -6.00 kcal/mol. The structures of the three compounds showed no resemblance with previously discovered PAD4 inhibitors, nor with existing drugs for RA treatment. Ultrafast Shape Recognition (USR) was utilized in searching of compounds possess similar molecular shape with previous reported inhibitor, streptonigrin. Five compounds out of the selected 37 compounds inhibited enzymatic activity of PAD4 significantly, with more than 10% inhibition at 100 μM. The best compound, (2*E*)-*N*-{(2*R*)-1-[(furan-2-ylmethyl)(methyl)amino]-1-oxopropan-2-yl}-3-(4-methoxyphenyl) prop-2-enamide, is a moderate inhibitor for PAD4 with IC_{50} value of 362.67 ± 4.13 μM. The structure of the compound showed no resemblance with the parent compound. Interestingly, furan ring in USR discovered compound was able to enter the active site cleft of PAD4. Four peptide-based inhibitors incorporated with non-standard amino acid containing furan ring (X) with sequence based-on PAD4 natural substrate, nucleophosmin were designed and synthesized using solid phase technique. Circular dichroism spectroscopy showed that the structure of the designed peptide-based inhibitors was predominantly unordered. The IC_{50} value of the best peptide-based

inhibitors with sequence KSIXDTP is 243.2 ± 2.4 μM which is lower than compounds obtained from LIDAEUS and USR. Kinetic studies revealed that it inhibited PAD4 reversibly and competitively. Three dimensional structure of the best peptide-based inhibitor was further elucidated using nuclear magnetic resonance spectroscopy. Molecular docking of the peptide-based inhibitor suggested favourable interaction between the peptide with PAD4 with binding affinity of -5.4 kcal/mol. Inhibitors containing furan ring in the structure show high potential in inhibiting PAD4 and the structure of the inhibitors discovered in this research can be modified to be a better drug candidate for treatment of rheumatoid arthritis.

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia
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SARINGAN, REKA BENTUK, SINTESIS DAN BIOAKTIVITI PERENCAT TERHADAP PROTEIN ARGININA DEIMINASE JENIS 4

Oleh

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Protein Arginina Deiminase jenis IV (PAD4) adalah sasaran baru untuk teraputik artritis reumatoid (RA). Ia merupakan pemangkin proses pensitrulinaan yang menghasilkan protein mengandungi sitrulina yang dipercayai menjadi punca kepada RA. Perencat enzim ini boleh menjadi ubat untuk rawatan RA. Objektif utama kajian ini adalah mencari perencat untuk PAD4. PAD4 manusia telah berjaya dihasilkan dalam *Escherichia coli* BL21 (DE3). PAD4 yang dihasilkan dari vektor pET32b dengan penggunaan media auto-induksi yang diperkenalkan oleh Studier mempunyai kelarutan dan aktivity yang lebih tinggi berbanding dengan sistem induksi konvensional. PAD4 telah berjaya ditulenkan dengan menggunakan teknik kromatografi afiniti dengan 90% ketulenan. Beberapa sebatian telah dikenalpasti sebagai perencat berpotensi untuk PAD4 oleh program *Ligand Discovery at Edinburgh University* (LIDAEUS) berasaskan struktur PAD4. Dua puluh dua sebatian paling bagus yang larut air telah dipilih untuk pemeriksaan potensi perencatan mereka terhadap PAD4. Tiga sebatian menunjukkan perencatan yang ketara kepada PAD4 dan nilai-nilai IC_{50} mereka adalah di antara $1.49 \pm 0.03 – 2.96 \pm 0.01$ mM. Tenaga penambatan untuk sebatian tersebut dengan PAD4 yang diperolehi daripada *docking* molekul adalah -7.49, -7.27 dan -6.00 kcal/ mol. Struktur tiga sebatian itu tidak menunjukkan persamaan dengan perencat PAD4 yang ditemui sebelum ini atau dengan dadah yang sedia ada untuk rawatan RA. *Ultrafast Shape recognition* (USR) telah digunakan untuk mencari sebatian yang mempunyai bentuk molekul yang menyerupai satu perencat yang dilaporkan sebelum ini, iaitu *streptonigrin*. Lima sebatian daripada 37 sebatian terpilih merencat aktiviti enzim PAD4 dengan ketara, lebih daripada 10% perencatan pada 100 μM. Sebatian yang terbaik, (2E)-N-{(2R)-1-[(furan-2-ilmetil)(metil)amino]-1-oxopropan-2-il}-3-(4-metoksilfenil)prop-2-enamida, adalah perencat sederhana untuk PAD4 dengan nilai IC_{50} 362.67 ± 4.13. Struktur sebatian tidak menunjukkan persamaan dengan sebatian induknya. Gelang furan di sebatian yang ditemui dengan USR dapat memasuki rekahan tapak aktif PAD4. Empat perencat berasaskan peptida yang diperbadankan dengan asid amino tidak semula jadi yang mengandungi gelang furan (X) dengan urutan berasaskan substrat semula jadi PAD4 iaitu nucleophosmin telah direka dan

disintesis dengan menggunakan teknik fasa pepejal. Spektroskopi dikroisme bulatan menunjukkan bahawa struktur perencat berasaskan peptida yang direka adalah kebanyakannya tidak tertib. Nilai IC_{50} perencat berasaskan peptida yang terbaik dengan urutan KSIXDTP ialah 243.2 ± 2.4 μM iaitu lebih rendah daripada sebatian yang diperolehi daripada LIDAEUS dan USR. Kajian kinetik menunjukkan perencat tersebut merencat PAD4 secara berbalik dan kompetitif. Struktur tiga dimensi bagi perencat berasaskan peptida yang terbaik telah dijelaskan selanjutnya dengan menggunakan spektroskopi resonans magnet nukleus. *Docking* molekul untuk perencat berasaskan peptida mencadangkan kewujudan interaksi tersesuaikan antara peptida tersebut dengan PAD4 dengan tenaga penambatan -5.4 kcal/mol. Perencat yang mengandungi gelang furan dalam strukturnya menunjukkan potensi yang tinggi dalam merencat PAD4 dan struktur perencat yang ditemui dalam kajian ini boleh diubah suai untuk menjadi calon dadah yang lebih baik untuk rawatan artritis reumatoid.

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ABPP	Activity-Based Protein Profiling
ACN	Acetonitrile
ACPA	anticitrullinated protein autoantibody
AKA	anti-keratin antibodies
APF	anti-perinuclear factor
Ala/A	alanine
Arg/R	arginine
Asn/N	asparagine
Asp/D	aspartic acid
BAA	N-α-benzoyl arginine amide
BAEE	N-α-benzoyl-L-arginine ethyl esther
BMO	2, 3-Butanedione monoxime
BSA	Bovine serum albumin
BzADMA	benzoyl-N^G-asymmetrical dimethyl-Arg
bp	base pairs
CCP	cyclic citrullinated peptide
CD	circular dichroism
CSI	Chemical shift index
Cys/C	cystein
DCM	Dichloromethane
DIEA	N, N – diisopropylethylamine
DMARD	Disease Modifying Anti-Rheumatic Drug
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
Dab	L-2,4-diaminobutyric acid
Dap	L-2,3-diaminopropionic acid
Dfa	Dap-Fal
EDTA	Ethylenediaminetetraacetic acid disodium salt dihydrate
Fal	L-2-furylalanine
Fmoc	fluorenylmethyloxycarbonyl
Gln/Q	glutamine
Glu/E	glutamic acid
Gly/G	glycine
HCTU	2-(6-Chloro-1H-benzotriazole-1-yl)-1, 1, 3, 3-tetramethylaminium hexafluorophosphate
HTS	High-Throughput Screening
His/H	histidine
IC_{50}	half maximal inhibitory concentration
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Ile/I	isoleucine
LB	Luria-bertani
LIDAEUS	Ligand Discovery At Edinburgh University
LPPS	Liquid phase peptide synthesis
Lys/K	L-lysine
MTX	Methotrexate
Met/M	methionine
Mtt	Methyltrityl

NLS	nuclear localization signal
NMF	natural moisturizing factor
NMP	N-methyl-2-pyrrolidone
NSAID	Non-Steroidal Anti-Inflammatory Drug
Orn	L-orthinine
PAD	protein arginine deiminase
PAD4	protein arginine deiminase type IV
Pro/P	proline
RA	rheumatoid arthritis
RF	Rheumatoid factor
RFA	rhodamine conjugated F-amidine
SE	Shared epitope
SNP	single-nucleotide polymorphism
SPPS	solid phase peptide synthesis
Ser/S	serine
TEMED	N, N, N', N'-tetramethylethylenediamine
TFA	trifluoroacetic acid
TIS	Triisopropylsilane
TNF	Tumor Necrosis Factor
Thr/T	threonine
Trp/W	tryptophan
USR	Ultrafast Shape Recognition
vHTS	virtual High-Throughput Screening
Val/V	valine

CHAPTER 1

INTRODUCTION

The immune system protects us against diseases and infections. Substances which are foreign and harmful to our body are called antigens. Examples of antigens are viruses, cancer cells and blood or tissues from other person or species. When antigens enter our body, our immune system will produce antibodies to destroy the harmful substances. Autoimmune disease occurs when our immune system attacks body's own tissues by mistake. It cannot differentiate between healthy body's tissues and antigens. Rheumatoid arthritis (RA) is an autoimmune disease where the immune system attacks synovial membrane around joints. RA is a disease which lasts for a long period of time, and it may affect many tissues and organs, but principally attacks joints. The attacked joints will become swollen, stiff, red and painful. This will lead to joint destruction and functional disability.

About 0.5-1.0% of the adult population is affected by RA (Wegner *et al.*, 2010). It is the second most common type of arthritis that most often starts after 40 years of age and before 60 years of age (Knuckley *et al.*, 2008). Similar to other autoimmune diseases, such as multiple sclerosis and type-1 diabetes, the etiology of the disease is still unknown. The treatment of the disease is only to reduce the symptoms of the disease, but not to cure the disease. Most of the autoimmune diseases are still not curable.

As an autoimmune disease, there are many autoantibodies that react with various autoantigens are detectable in the sera of RA patients. RA associated autoantibodies are useful in diagnosis of the disease. Diagnosis at the early stage of the disease can prevent irreversible joint damage, reducing signs and symptoms of erosion and improving physical function (Conrad *et al.*, 2010). Among the autoantibodies, anticitrullinated protein autoantibody (ACPA) has been documented as a highly specific marker for RA and has the most valuable diagnosis and prognosis potential for RA (van Boekel *et al.*, 2002).

There are five highly related calcium-dependent protein arginine deiminases (PADs), PAD1 to PAD4 and PAD6, exist in humans. Among the isozymes, PAD4 has emerged as a potential therapeutic target for the treatment of RA (Jones *et al.*, 2009). By inhibiting the enzymatic activity of PAD4, it is believed that the development of RA can be suppressed and subsequently the disease can be cured.

Several drugs are available for treating RA. They can be generally divided into three groups: Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), corticosteroidals, and Disease Modifying Anti-Rheumatic Drugs (DMARDs) (Masumoto *et al.*, 1998). Although the existing drugs can treat the disease, they do bring many side effects to the patients. For examples, long term use of NSAIDs will lead to gastrointestinal disturbance such as ulcers and bleeding while corticosteroidals such as prednisone will cause weight gain, increase blood pressure and blood sugar (Masumoto *et al.*, 1998).

The drug that is now considered as the most commonly used drug for RA patients is methotrexate, an examples of DMARDs. The drug will also cause diarrhea, nausea and fatigue (Masumoto *et al.*, 1998). Several strong inactivators of PAD4 have been reported, such as o-F-amidine and Thr-Asp-F-amidine (TDFA) (Causey *et al*., 2011; Jones *et al.*, 2012). However they inactivate PAD4 irreversibly. Introduction of the inactivators in human body will cause PAD4 to lose its functions permanently and this may lead to other health problems.

Searching for effective drugs for a disease in a short period of time is crucial in curing the disease or even in saving the patients' lives. Yet drug discovery is a time consuming and complex process. The process from discovery to approval of a new drug may take more than 10 years and cost hundreds of millions of Dollars (Morgan *et al.*, 2011). However practice of bioinformatics techniques generally accelerates the drug discovery process and also reduces the overall risk and cost throughout the process (Katara, 2013). Bioinformatics helps in the gene identification for druggable targets and discovery of new targets for new drugs (Chen & Chen, 2008; Katara *et al.*, 2011). Bioinformatics approach supports rational drug discovery and subsequently encourages the application of computer-aided drug design (CADD) in drug development. The first drug approved with the aid of bioinformatics tools is Relenza, an inhibitor of neuraminidase for influenza treatment (MacConnachie, 1999). With the use of bioinformatics tools, more and more drugs were discovered later. For instances, with the use of structure-based drug design, Indinavir was discovered for human immunodeficiency virus (HIV) infection treatment (Askin *et al.*, 1994).

Peptide-based drugs attract attention from public for their attractive advantages such as high specificity and low toxicity in comparison to conventional small molecule drugs. As peptide is a natural substance, introduction of peptide-based drug to human body is believed to have less negative impact compared to small molecules. Strong binding between the peptide-based inhibitor to the receptor can be achieved by structure modification of the existing natural amino acids. Combination of the CADD techniques and peptide-based drug design can be a fascinating method in designing PAD4 inhibitors with strong binding and low toxicity properties.

Since current therapies for rheumatoid arthritis are not completely effective in treating the disease, new drugs with minimum side effects need to be discovered. PAD4 inhibitors were searched by different approaches in this research. The objectives of this study are:

1. To express and purify human PAD4 using recombinant DNA technology.
2. To screen and evaluate inhibitors of PAD4 by structure-based virtual screening.
3. To screen and evaluate inhibitors of PAD4 by ligand-based virtual screening.
4. To design and synthesize peptide-based inhibitors of PAD4.
5. To elucidate inhibition characteristics and structure of PAD4 inhibitor.

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