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MODELLING THE INFLUENCE OF LIGAND BINDING TOWARDS THE STRUCTURE AND DYNAMICS OF PROTEIN ARGININE DEIMINASE IV

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By

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May 2017

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An enzyme called Protein Arginine Deiminase IV (PAD4) has gained tremendous attention due to its role in rheumatoid arthritis (RA). It catalyses the citrullination reaction, whose products was reported to be dysregulated in the RA patients. Therefore, modulation of PAD4 activities is considered as an alternative therapeutic strategy against the disease. The early view of protein as a rigid body has been replaced by dynamics model. The use of dynamics model allows exploration of unique conformational changes that are varied depending on the protein environment. Although the structure of PAD4 has been solved experimentally, it does not reflect the dynamical changes of the protein. The present thesis employed molecular dynamics (MD) simulations to enhance understanding on PAD4 behaviour, and on how a ligand can influence the protein structure, dynamics and catalytic pocket.

The MD simulations were performed in the ligand-bound and the unbound form of the enzyme. For ligand-bound systems, two known ligands were used: *o*-F-amidine and GSK147, which binds at two different binding regions. For the PAD4-GSK147 systems, the simulations were performed in the presence of five and two calcium ions conditions. This provided a platform for inspection and direct comparison of the structural and dynamics changes at the atomic level.

The binding of either ligand showed a significant reduction in the local fluctuation profiles up to 30% at regions that are distal from the catalytic pocket, particularly residues in Subdomain I and 387-407, in comparison to that in the unbound-PAD4-$5Ca^{2+}$ system. Comparison on the collective motion from the MD simulations revealed that the key of PAD4 inhibition by these ligands were through constraining

the movement of Subdomain I in relative to Subdomain II, with two different hinge points. A reduction in the number of calcium ions in the PAD4-GSK147 simulation was also observed to greatly affect not only PAD4 structure and dynamics, but also protein-ligand interaction. This observation suggests that residues at the calcium binding sites can be utilised for modulating the enzyme.

A clustering analysis on the PAD4 trajectories revealed the dynamics behaviour of H640 side chain, which is located in between the two door of PAD4 catalytic pocket. The upward movement of the H640 side chain changes the topography of the two-door catalytic pocket, which resembles an open-cleft event. This new insight on the H640 side chain has assisted in the identification of 47 drug-like compounds that exerts mean binding affinity in the range of -9.12 to -7.33 kcal/mol towards PAD4 and binds at either front door, back door or in between the two-door catalytic pocket. Binding interaction analyses of the top PAD4-binder complexes showed that the top binders were interacting to one or more key residues lining either the PAD4 front door or the back door; thus may be blocking the citrullination reaction. With additional optimisation, these binders may serve as lead compounds in future drug and development against PAD4. Overall, this work provides new and promising insights into the PAD4 dynamics and catalytic pocket.

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MEMODEL KESAN PENGIKATAN LIGAN TERHADAP STRUKTUR DAN DINAMIK PROTIN ARGININA DEIMINASI IV

Oleh

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Enzim yang dipanggil Protin Arginina Deiminasi IV (PAD4) telah mendapat perhatian besar kerana peranannya di dalam artritis reumatoid (RA). Ia menjadi pemangkin tindak balas sitrulinasi, yang tidak seimbang di dalam pesakit RA. Oleh itu, modulasi aktiviti PAD4 dianggap sebagai strategi terapeutik terhadap penyakit tersebut. Pandangan awal protin sebagai sebuah badan tegar telah digantikan dengan model dinamik. Penggunaan model dinamik membolehkan eksplorasi terhadap perubahan konformasi unik protin yang berubah mengikut persekitaran protein. Walaupun struktur PAD4 telah diselesaikan melalui eksperimen, ianya tidak mencerminkan perubahan dinamik protein itu. Tesis ini mengaplikasikan simulasi dinamik molekul (MD) untuk meningkatkan pemahaman tentang dinamik PAD4, dan bagaimana ligan boleh mempengaruhi dinamik protin ini. Simulasi PAD4 dilaksanakan menggunakan dua ligan, *o*-F-amidine dan GSK147, selama 50 ns.

Analisis struktur terhadap simulasi PAD4-ligan menunjukkan bahawa struktur PAD4 mengalami kesan yang minima sekitar 3% apabila ligan mengikat. Profil fleksibiliti daripada simulasi PAD4 bersama ligan menunjukkan pengurangan yang ketara sehingga 30% di kawasan-kawasan yang jauh dari poket pemangkin, terutamanya di asid amino di dalam Subdomain I dan 387-407. Analis domain menunjukkan bahawa pergerakan PAD4 sangat bergantung kepada pergerakan Subdomain I. Perbandingan kolektif simulasi PAD4 terikat bersama *o*-F-amidine dan PAD4 bersama GSK147 menunjukkan bahawa kunci perencatan oleh ligan-ligan ini ke atas PAD4 adalah dengan mengekang pergerakan Subdomain I kepada Subdomain II, dengan dua titik engsel yang berbeza. Pengurangan jumlah ion kalsium di dalam simulasi PAD4-GSK147 bukan sahaja diperhatikan mempengaruhi struktur dan dinamik protin, malah interaksi di antara protin dan ligand.

Satu analisis kelompok kepada trajektori PAD4 memdedahkan tingkah laku dinamik rantaian sampingan H640, yang terletak di antara poket pemangkinan dua pintu PAD4. Pergerakan menaik rantaian sampingan H640 mengubah topografi poket pemangkinan dua pintu menjadi satu poket yang luas, seakan satu rekahan terbuka. Pendekatan baru tentang rantaian sampingan H640 ini digunakan dalam pencarian maya perencat kepada PAD4. Sebanyak 47 sebatian yang dijangka mengikat dengan min tarikan pengikatan di antara -9.12 hingga -7.33 kcal/mol kepada PAD4 telah dikenalpasti. Analis interaksi pengikatan ke atas kompleks PAD4-pengikat teratas menunjukkan bahawa pengikat teratas berinteraksi dengan satu atau lebih asid amino penting samada di pintu depan atau pintu belakang PAD4. Ini dijangka dapat mengawal tindak balas sitrulinasi. Penggunaan struktur poket pemangkinan PAD4 dengan rantaian sampingan H640 yang menaik membantu dalam mengenalpasti perencat yang berpotensi untuk mengikat di pintu belakang poket pemangkinan. Dengan pengubahsuaian tambahan, sebatian-sebatian ini dipercayai boleh menjadi dasar di dalam reka bentuk dan pembangunan perencat terhadap PAD4. Secara keseluruhannya, hasil dari penyelidikan ini memberikan pendekatan baharu ke atas dinamik PAD4 dan poket pemangkinan.

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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LIST OF ABBREVIATIONS

ACPA	Anti-Citrullinated Peptide Antibody
AM1-BCC	AM1 atomic charge with Bond Charge Correction
AMBER	Assisted Model Building with Energy Refinement
BAA	Benzoyl-Arginine Amide
BPTI	Bovine Pancreatic Trypsin Inhibitor
DMARD	Disease-Modifying Anti-Rheumatic Drug
GROMACS	GROningen MAchine for Chemistry Simulation
IC50	Concentration of an inhibitor causing 50% inhibition of the desired activity
LJ	Lennard-Jones
MD	Molecular Dynamics
MM	Molecular Mechanics
MTX	Methotraxate
NCIDS	National Cancer Institute Diversity Set
NMR	Nuclear Magnetic Resonance
NPT	Number of atoms, Pressure and Temperature
ns	nanosecond
NSAID	Non-Steroidal Anti-Inflammatory Drug
NVT	Number of atoms, Volume and Temperature
PAD	Protein Arginine Deiminase
PAD4	Protein Arginine Deiminase type 4
PAD4-YFF-5Ca^{2+}	PAD4-*o*-F-amidine complex with 5 calcium ions
PAD4-GSK147-5Ca^{2+}	PAD4-GSK147 complex with 5 calcium ions
PAD4-GSK147-2Ca^{2+}	PAD4-GSK147 complex with 2 calcium ions
PBC	Periodic Boundary Condition
ps	picosecond
QM	Quantum Mechanics
RA	Rheumatoid Arthritis
RCS	Relaxed Complex Scheme
RMSD	Root-Mean Square Deviation
RMSF	Root-Mean Square Fluctuation
R_g	Radius of Gyration
SBVS	Structure-Based Virtual Screening
Unbound-PAD4-5Ca^{2+}	Unbound PAD4 with 5 calcium ions
VMD	Visual Molecular Dynamics
VS	Virtual Screening
YFF	*o*-F-amidine

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Proteins are macromolecules that are vital in human body and biochemical process. Structurally, a protein is made up of simple building blocks based on the 20 naturally produced amino acids which differ at their side chain. Since each amino acid has a unique side chain, the chemistry is different too. Some are hydrophobic, and several others are polar and nonpolar. This chemistry of amino acid is vital, as it will determine the shape of the protein. Because of the amino acid backbone and side chain interactions, the sequence bends and folds into a three-dimensional (3D) structure. The newly formed structure is closely packed; with some atoms remain capable to make small movements (Fersht, 1999).

Proteins carry several roles in human body. They could act as enzyme, transporter, or carrier. For a protein to have such specialized function, it has to undergo modification known as post-translational modification (PTM). The modifications include glycosylation, acetylation, phosphorylation, and citrullination. Citrullination converts peptidyl-arginine into peptidyl-citrulline, which is catalysed by an enzyme called protein arginine deiminase (PAD) (van Venrooij & Pruijn, 2000). Since the conversion involves the removal of an imine group to a carbonyl group, the chemical reaction is also referred to as deimination. Although citrullination was first reported early in 1958 (Rogers & Simmonds, 1958), appreciation towards the modification only started 30 years later when the peptidyl-citrulline was found to be associated with a disease called rheumatoid arthritis (RA) (Schellekens *et al.*, 1998).

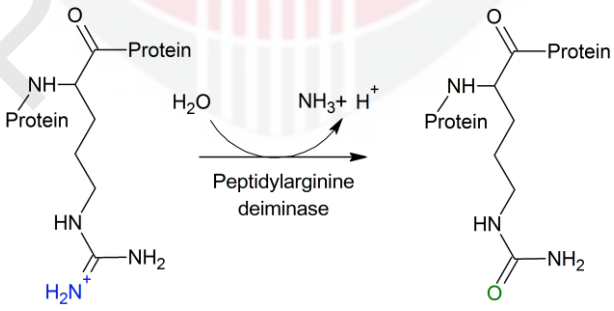

Figure 1.1 : Enzymatic conversion of peptidylarginine into peptidylcitrulline.
Note: PAD catalyzes the hydrolytic deimination of peptidylarginine to produce peptidylcitrulline and ammonia.

RA is a chronic inflammatory disease that is characterized by symmetrical pain in one or more joints (Landre-Beauvais, 2001). The symptoms usually start with stiffness in the morning, followed by joint tenderness and swelling. The number of affected joints varies, but most of the disease development involves five or more joints, including both small and big joints. Since its identification, RA has been the cause of joint inflammations in 0.5 to 1% of the world's population (Gabriel, 2001). The number of people diagnosed with RA in year 2008 has been estimated to be nearly 1.3 million in the United States (Helmick *et al.*, 2008; Ong *et al.*, 2013). Majority of the affected patients were reported to have a low health-related quality of life due to physical damages induced by the disease (Salaffi *et al.*, 2009). In Malaysia, it is estimated that approximately 140000 people are suffering from the disease (Malaysian Society of Rheumatology, 2011). However, the scenario in Malaysia is poorer as some of the patients had delayed treatment, due to the lack of awareness. This caused difficulties for the patients to perform their routine activities, and for some, it resulted in losing their jobs. With the assumption that human life span increases every year, it is expected that the frequency of correctly diagnosed patients with RA will increase in the future.

RA is regarded as an autoimmune disease ('auto' means self, while 'immune' means immune system) (Davidson & Diamond, 2001). In general, an autoimmune disease can be understood as a disease that is triggered by the abnormal response of the immune system against one's substance or tissue that is naturally presented in the body. In the case of RA, the target is the joint and the tissues that surround it. The onset of RA is caused by the stimulation of the immune system that is triggered by foreign substrates that mimic the joint cells (Davidson & Diamond, 2001). Upon the trigger, the immune components start to attack the invaders and coincidentally, the joint cells too. This in turn, causes the swelling of the synovial membrane (see Figure 1.2). In comparison to a healthy joint, a joint affected by RA has almost no gaps in between because the gap was filled with the swelling of synovial membrane. This consequently limits the range of motion of the joint and may gradually disrupt other joint components, for example, the joint cartilage.

Over the past 30 years, several drugs have been prescribed to treat RA. The main aim is to minimize RA joint inflammations, but the drugs do not treating the underlying causes. At present, three classes of drugs are commercially available, namely corticosteroids, non-steroidal anti-inflammatory drug (NSAID) and disease-modifying anti-rheumatic drug (DMARD) (O'dell, 2004). Depending on the disease severity, an appropriate drug is prescribed. DMARD is the most commonly prescribed medication for RA patients. An example of a drug from this class is methotrexate (MTX) (Aletaha *et al.*, 2010). Although it is effective in relieving RA symptoms, the mechanism of action of MTX in treating RA remains unclear. A major drawback of MTX is that it can cause multiple side effects on the central nervous, hepatic, pulmonary, hematologic and gastrointestinal systems in long-term usage (Smolen *et al.*, 2005). This drug also has a toxicity issue, which is why MTX was discontinued in certain, more susceptible RA patients (Smolen *et al.*, 2010).

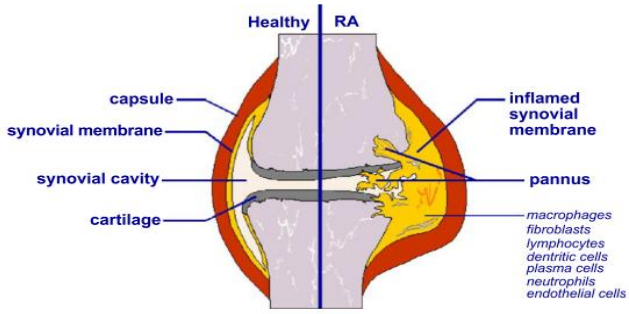

Figure 1.2: Schematic diagram of a healthy joint versus RA joint.

Note: RA is characterised by chronic inflammation of the joints, caused by massive infiltration of inflammatory cells and proliferation of the synovial lining cells. As the disease progresses, the synovium will expand and develop pannus tissue. During the disease process, the pannus tissue attacks the articular cartilage and the underlying bone, which can lead to progressive joint destruction. (Adopted from Nijenhuis *et al.*, 2004)

RA is not a fatal disease, however, severe and unmanageable conditions may arise that gradually disrupts overall human function, and thus, the quality of life (Salaffi *et al.*, 2009). Long-standing RA patients have an increased risk of suffering multiple health problems. Therefore, it is crucial to diagnose RA at an earlier stage of the disease; so that treatment can be administered sooner and major damage of joint tissues can be prevented. Considering the close relation between RA and citrullination pathway, suppression of the pathway has been hypothesized to be a strategic way to slow down the disease (Schellekens *et al.*, 1998, 2000; Wegner *et al.*, 2009). An alternative is by controlling the activity of the enzyme that catalyses the pathway, PAD type 4 (PAD4). On-going researches on PAD4 include studies on protein structure and function, as well as inhibitor design and development.

1.2 Problem Statement

PAD4 catalyses citrullination reaction and is considered to be a promising target to treat RA. Based on the crystal structures available, known PAD4 ligand can be categorised into two classes: the front door ligands, which mimic natural PAD4 substrate, benzoyl-arginine amide and bind covalently to the front door of PAD4 catalytic pocket (Arita *et al.*, 2004; Causey *et al.*, 2011; Jones *et al.*, 2012; Luo *et al.*, 2007, 2006) and the back door ligands that binds non-covalently at the water channel or the back door of the catalytic pocket (Lewis *et al.*, 2015). The front door ligands were proposed to follow competitive inhibition mechanism, which inactivates PAD4 by interacting with the catalytic residues (Kearney *et al.*, 2005). Meanwhile, the back door ligand binding strongly dependent on the calcium ions environment and it works best when only two out of five calcium-binding sites were occupied.

The crystallographic studies on PAD4-ligand complexes have provided insightful

observations on the structural features of PAD4 and the ligand binding mechanism. In addition, studies on the reaction mechanism of PAD4-ligand complexes have also been performed (Ke *et al.*, 2009; Li *et al.*, 2015). However, there is no work reporting on the dynamics of PAD4 in the presence and absence of ligand. It is indeed difficult to gain the dynamics information based on the crystal structure. In order to elucidate the ligand binding impact, further studies on the dynamical behaviour of PAD4 in its unbound and ligand-bound states at atomic level need to be acquired. This would provide understanding on the dynamics of PAD4 and PAD4-ligand complexes, together with the catalytic pocket dynamics and interaction between PAD4 and the ligand. This is essential particularly in discovering or designing suitable ligands to occupy the PAD4 catalytic pocket.

Computational technique such as molecular dynamics (MD) simulations is proved to be a very useful and reliable way to study dynamics of biomacromolecular and is a complementary means to experimental study. The influence of ligand binding towards PAD4 dynamics can be investigated by simulating the PAD4-ligand interactions. By relating experimental evidences and known crystal structures with the atomistic observation provided by MD simulations, the mechanistic and dynamics properties of PAD4 and its relation with ligand can be fully elucidated. In addition, several predictions on the type of ligands that are suitable to occupy the front door, back door and both door based on the dynamics of the PAD4 catalytic pocket can be deduced for future successful drug design efforts against this enzyme.

1.3 Scope of Research

In order to obtain the relation between ligand binding and PAD4, the atomistic features of unbound PAD4 should be first described. Concurrently, the investigation on the ligand binding impact on PAD4 was performed in the presence of two classes of PAD4 ligands: *o*-F-amidine (YFF) representing the front door ligand, and GSK147 as the back door ligand. Analyses related to structural and dynamics properties were performed on PAD4 and its catalytic pocket. In order to further investigate the type of ligand suitable to bind at the PAD4 catalytic pocket, a virtual screening using ensemble-based approach was performed. The ensemble-based approach was utilised to integrate the dynamics information of the PAD4 catalytic pocket obtained from the MD simulations. The compound library used during the screening was limited to the compounds from National Cancer Institute Diversity Set 3 (NCIDS3). The identified ligands were discussed based on their predicted mean binding affinities, drug-likeness properties and relevant enzyme-ligand interactions.

1.4 Objectives

The present research was aimed to elucidate the influence of ligands binding towards PAD4 stability and dynamics and to identify putative PAD4 binders using computational approaches. Therefore, these objectives were pursued:

i. To study the PAD4 stability and dynamics
ii. To investigate the effect of PAD4 ligands towards PAD4 stability and dynamics
iii. To analyse the PAD4 catalytic pocket conformational ensemble
iv. To study the type of potential ligands that bind to the PAD4 catalytic pocket ensemble

In the next chapter, the literature related to the project is discussed. The theoretical background and methodologies that were used throughout the project are presented in Chapter 3. Chapter 4 presented the findings including the impact of ligand binding towards the stability and dynamics of PAD4 and its catalytic pocket. The type ligands that would bind to the PAD4 catalytic pocket ensemble are also discussed. The results are further correlated to the currently available findings whenever applicable. Overall conclusion from this study and some recommendations to improvise the study are presented in the last chapter.

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