Study of the Structural Pathology Caused by CYP2C9 Polymorphisms towards Flurbiprofen Metabolism Using Molecular Dynamics Simulation

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Abstract. CYP2C9 is one of the major cytochrome P450 enzymes that play a crucial role in metabolic clearance of several drugs in the current clinical used. CYP2C9 has several allelic variant forms each of which arises from single amino acid substitution and could reduce/increase enzyme activities and affect drug metabolism. Mutant alleles may cause serious toxicity in some narrow therapeutic index drugs. CYP2C9*13, one of the CYP2C9 variant forms that is commonly found in Asian population, has a Leu90Pro amino acid substitution that leads to defective drug metabolism in individuals who carry this allele. It has been reported that metabolic activity of CYP2C9*13 was reduced towards some CYP2C9 substrates compared to wildtype. In this study, X-ray crystal structure of human cytochrome P450 2C9 complexed with flurbiprofen (PDB code: 1R9O) was represented to wildtype and the structure of CYP2C9*13 was constructed based on the X-ray crystal structure of CYP2C9-flurbiprofen complex. Herein, molecular docking of CYP2C9*1 and CYP2C9*13 with flurbiprofen was performed in search for flurbiprofen orientation that corresponds to its binding state before undergoing monooxygenation. Subsequently, molecular dynamics simulation was operated to compare binding of flurbiprofen in catalytic cavity of these 2 variants. Substrate access channel of CYP2C9*13 has a dramatic effect on an interaction between the drug and the enzyme. Consequently, this study can lead to an understanding of structural pathology caused by single amino acid change in CYP2C9*13 variant.

Keywords: Cytochrome P450 2C9, CYP2C9*13, Genetic polymorphisms, Flurbiprofen, Molecular dynamics simulation.

1 Introduction

Cytochrome P450s (P450s) are a diverse superfamily group of enzymes that have been found in all kingdoms of life [1]. P450s are heme-containing enzymes, which

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have a main function to catalyze the oxidation of organic substances, so called monooxygenation reaction. They are involved in biotransformation for a large number of endogenous compounds, drugs and xenobiotics [2-3]. Moreover, they are able to activate or metabolize chemical carcinogens, degrade several substances and synthesize important compounds such as steroidal hormones and soluble vitamin. More than 40% and 55% of primary amino acid sequence identities are shared among P450 families and subfamilies, respectively [4]. Despite their difference in amino acid similarity, three-dimensional structures of P450 enzymes are generally conserved [5].

Human P450s are membrane bound proteins that are found on the endoplasmic reticulum, few are identified on mitochondria. P450s anchor membrane by their N-terminal α-helix [6]. More than 57 cytochrome P450 enzymes [7] are encoded in the human genome. CYP2C9 is one of the four functional CYP2C genes (including CYP2C8, CYP2C18 and CYP2C19) that locate on the chromosome 10 [8]. One of the most important and abundant 2C subfamily P450 enzymes in the human liver is CYP2C9. It is responsible for numerous metabolic clearances of therapeutic agents approximately 15% in current clinical used [9] which include a wide range of narrow therapeutic drugs and many non-steroidal anti-inflammatory agents (NSAIDs). However, therapeutic treatments are varied among individuals because of lethal effects caused by polymorphic variants of P450 enzymes. CYP2C9 has several variant forms, which arise from single amino acid substitution, resulting in reduce/increase enzyme activities as well as drug metabolism [8-9]. The narrow therapeutic index drugs could cause serious toxicity to the people who carry the mutant CYP2C9 allele(s). A lot of studies on human CYP2C9 polymorphisms in vivo and in vitro have been conducted in order to elucidate the enzyme-drug interactions. In addition, these studies have made a great effort to clarify the influences of CYP2C9 polymorphisms that alter enzyme activities and drug metabolism [9-11].

CYP2C9*13 is a novel CYP2C9 variant form that is commonly found in Asian population [12, 13]. It emerges from a T269C transversion of the CYP2C9 gene, causing a substitution of residue 90 leucine to proline (L90P) [13]. This mutation is located in N-terminal loop that is closed to an entrance for substrate access path. Regarding to allele frequency analysis, the incident of this allele is approximately 1.02% in the Chinese population [12] and 0.6% in the Korea population [14]. Furthermore, numerous studies have measured the catalytic activities of CYP2C9 mutant in comparison to wildtype against various substrates [10, 15-16]. Owing to the important role of CYP2C9 polymorphisms in defective drug metabolism including the serious toxicity in the poor metabolizers carrying CYP2C9*13 allele, it is necessary to describe structural pathology of CYP2C9*13 variant for better understanding on how single amino acid substitution in this allele affects enzymatic activities and influences drugs metabolism.

In 2003, X-ray crystal structure of CYP2C9 both unliganded and complexed with the anti-coagulant drug warfarin (PDB code: 1OG2 and 1OG5) were determined [17]. Later, a crystal structure of CYP2C9 with flurbiprofen bound (PDB code: 1R9O) was investigated in 2004 [18]. With an advantage of these available X-ray crystal structures of CYP2C9, structural analyses of CYP2C9-drug interaction can be attained, especially the effect of single amino acid substitution in mutant alleles. Previously, Zhou et al. determined the structure of CYP2C9*13 and found out that the size of

substrate access channel was altered compared to CYP2C9*1, resulting in difficulty of substrates to enter into active site cavity [19].

In this study, the crystal structure of CYP2C9-flurbiprofen complex (PDB code: 1R9O) was designated as wild type structure while the structure of CYP2C9*13 was constructed based on the X-ray crystal structure of CYP2C9-flurbiprofen complex. The constructed CYP2C9*13 with bound flurbiprofen was implemented to investigate the structural pathology that is induced by single nucleotide polymorphism resulting in reduced metabolic activities. Molecular dynamics simulation can provide the information on a characteristic of specific single amino acid substitution in CYP2C9 polymorphism causing defective enzymatic activities and influences drug metabolism. It gives a more comprehensive on how the single amino acid substitution in CYP2C9*13 has an effect on drug-enzyme complex. The aim of this study is to introduce the molecular dynamics simulation as a tool to explore the underlying structural pathology of CYP2C9 polymorphisms towards ineffective flurbiprofen metabolism. In addition, interaction of enzyme-drug complex can be evaluated by means of pharmacophore model.

2 Methods

2.1 Structure Preparation

Crystal structure of human cytochrome P450 2C9 with flurbiprofen bound (PDB code 1R9O) [18] was obtained from the Brookhaven Protein Databank (http://www.pdb.org/). This structure was solved by X-ray crystallography at 2.0 Å resolution and has a number of missing residues. These missing residues (residues 38-42 and residues 214-220) were resolved by MODELLER9v6 [20]. The CYP2C9-flurbiprofen complex was employed to construct CYP2C9*13 which has mutation of residue 90 leucine to proline (L90P) by using SCWRL3.0 [21].

Additionally, initial atomic coordinates of flurbiprofen were obtained from ChemIDplus database (http://chem.sis.nlm.nih.gov/chemidplus/) in order to perform molecular docking and further molecular dynamics simulation. All computation was carried out on Linux high performance cluster AMD quad cores 2.3 GHz 64 GB memory available at BIOTEC, NSTDA, Thailand.

2.2 Molecular Docking

The molecular interaction between the CYP2C9-flurbiprofen complex was computed by using AutoDock4.0 program [22]. Partial charges of flurbiprofen and CYP2C9 were assigned using Gasteiger method with the aid of AutoDockTools [23].

Affinity maps were generated using AutoGrid program [23] and centered on heme. The maps were manually adjusted following substrate recognition sites of CYP2 family that were previously proposed by Gotoh [24]. Dimension of cubic box was set to be 60 x 60 x 60 grid points and 0.375Å spacing. AutoDock4.0 program was employed to dock ligands into catalytic cavity of CYP2C9 by using Lamarckian genetic algorithm (LGA) consisting 200 runs with 270,000 generations.

Estimated free binding energy of each substrate-protein complex was considered. Dock conformations were clustered and analyzed using AutoDockTools.

2.3 Molecular Dynamics Simulation

Simulations of CYP2C9-flurbiprofen complex both CYP2C9*1 and CYP2C9*13 were performed by using AMBER10.0 package [25]. The SANDER program of AMBER10.0 was used for minimization and MD simulations. The initial structures were first energy minimized for 2,000 steps (1,000 steepest descent [26] and 1,000 conjugate gradient) and then simulated at a temperature of 300 K. The Shake algorithm was applied to all bonds containing hydrogen atoms, and a time step of 2 fs was used. The method of Berendsen was used to couple the system to constant temperature and pressure. The carbon atoms were restrained for 20 ps and followed by an unrestrained simulation of 2.5 ns.

2.4 Pharmacophore Model

LigandScout2.03 [27], a software tool that automatically derives pharmacophores from protein-ligand complexes, was used to determine interaction patterns between CYP2C9 and flurbiprofen obtained from molecular dynamics simulation.

3 Results

3.1 Molecular Docking of CYP2C9*1

The crystal structure of CYP2C9-flurbiprofen complex (PDB code: 1R9O) was docked with flurbiprofen in order to find a suitable conformation of flurbiprofen accessing into interior cavity of CYP2C9*1. The initial structure of CYP2C9-flurbiprofen complex is illustrated in figure 1. Estimated free binding energy of this substrate-protein complex was -6.66 kcal/mol, which is favorable for the drug to bind in this position of the enzyme.

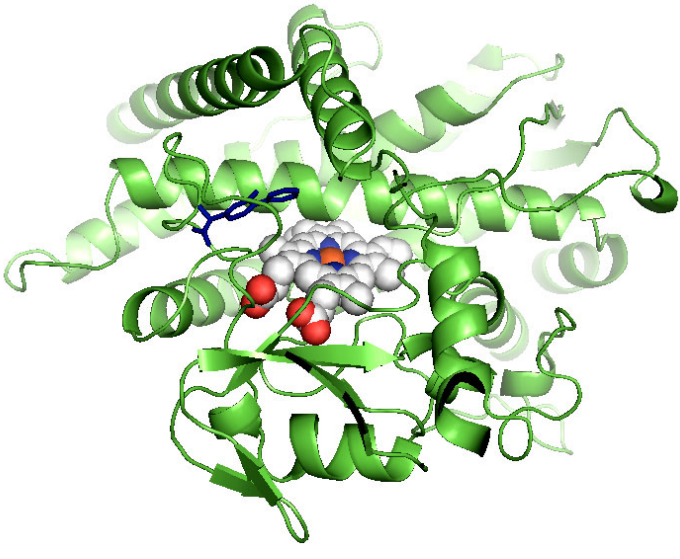

Fig. 1. Initial binding orientation of flurbiprofen obtained from molecular docking of CYP2C9*1. This conformation of flurbiprofen was also applied to CYP2C9*13. Flurbiprofen is represented by blue stick.

3.2 Molecular Dynamics Simulation of CYP2C9*1 and CYP2C9*13

Molecular dynamics simulation was performed to determine enzyme-drug interaction. In addition, it allows an observation of drug motions in substrate access channel of the protein molecule. The conformation of CYP2C9-flurbiprofen complex selected from the molecular docking study was used as a starting structure for simulations. Simulations of both CYP2C9*1 and CYP2C9*13 were performed by using AMBER10.0 package. BC loop of CPY2C9*13 was dramatically altered, and it led to different drug-enzyme interactions between CYP2C9*1 and CYP2C9*13. For CYP2C9*13, Arg108 bends upwards aromatic ring of flurbiprofen, while that of CYP2C9*1 points away from flurbiprofen as demonstrated in figure 2. This slight difference caused a dramatic effect on orientation of flubiprofen in that flurbiprofen is likely to rise up in CYP2C9*13 despite of lying horizontally as observed in CYP2C9*1.

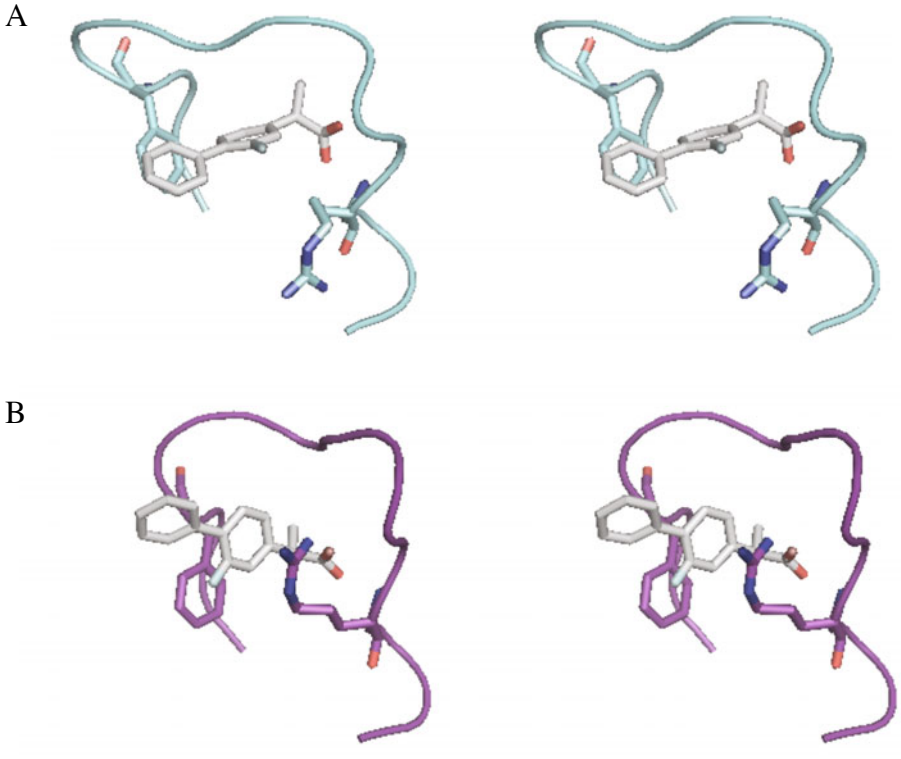

Fig. 2. Stereoview of flurbiprofen binding orientation under BC loop in CYP2C9*1 (A) and CYP2C9*13 (B). Arginine108 of CYP2C9*1 bends away from flurbiprofen, which is in contrary to CYP2C9*13, and leads to additional aromatic interactions of CYP2C9*13-flurbiprofen complex. Phe106 is also implicated in this interaction, reinforcing the aromatic interactions to be stronger.

3.3 Pharmacophore Model of CYP2C9*1 and CYP2C9*13

The conformation of CYP2C9-flurbiprofen complex from the molecular dynamics simulation was analyzed by using LigandScout2.03, which can describe the interaction patterns of drug-enzyme complex.

Pharmacophore modeling of CYP2C9*1-flurbiprofen complex and CYP2C9*13-flurbiprofen complex are shown in figure 3 and figure 4, respectively. In the complex of CYP2C9*1-flurbiprofen, hydrophobic interactions are formed between two aromatic rings of flurbiprofen and non-polar amino acid resides that line the catalytic cavity of the enzyme. These interactions were also observed in CYP2C9*13-flubiprofen complex. Nevertheless, since Arg108 of CYP2C9*13 roars upwards, aromatic interactions between one aromatic ring of flurbiprofen and amino group of Arg108 distinguishingly differentiates drug interaction pattern of CYP2C9*13 from that of CYP2C9*1. Moreover, the aromatic interactions were strengthened by stacking aromatic interactions between aromatic rings of flurbiprofen and Phe106, although Phe106 conformation is similar to that of wild type enzyme.

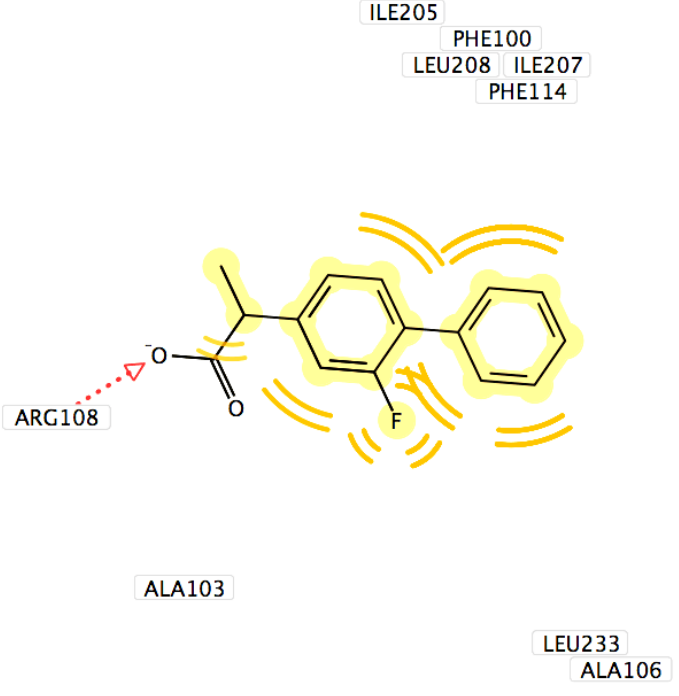

Fig. 3. Pharmacophore modeling of flurbiprofen bound in catalytic cavity of CYP2C9*1, hydrophobic interactions are represented by yellow color and hydrogen bond acceptor represented by a red arrow.

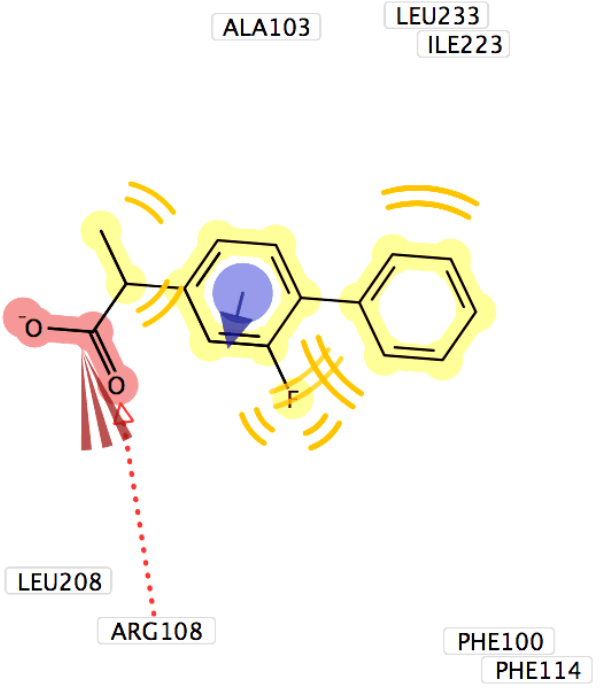

Fig. 4. Pharmacophore modeling of flurbiprofen bound in catalytic cavity of CYP2C9*13. Hydrophobic interactions are represented by yellow color, hydrogen bond acceptor is represented by red arrow, aromatic ring interactions are represented by blue arrow and negative ionizable area is represented by red area.

4 Discussion

CYP2C9 has an important role on both metabolic clearance and the response of a wide range of therapeutic agents. CYP2C9 polymorphisms are associated with reduced enzymatic activity and cause a risk of serious toxicity in poor metabolizers who carry the mutant alleles. Several studies have been indicated that CYP2C9*13, one of the CYP2C9 polymorphism variants caused by a single amino acid substitution of Leu90Pro, exhibits a reduced tolbutamide metabolic activity in some studied CYP2C9 substrates as Michaelis-Menten constant (K_m) of CYP2C9*13 was found to be increased while maximal reaction velocity (V_{max}) was not altered [15]. Surprisingly, V_{max} was reduced in diclofenac metabolism although K_m was also increased [15]. Consequently, drug K_m of CYP2C9*13 tend to be increased, indicating its decline in rate of the reaction. This change in kinetics is probably originated from amino acid substitution that alters the 3D structure of the protein. In an attempt to investigate the structure and metabolism relationship of mutant enzyme, molecular dynamics simulation of lornoxicam as well as diclofenac binding in CYP2C9*13 were performed in comparison to CYP2C9*1 as substrate entrance of CYP2C9*1 is considerably larger than that of CYP2C9*13 [28]. This tremendous change is caused by turnover of

residue 106-108 backbones in CYP2C9*13 [28]. Corresponding to the study by Zhou et al., we also observed the turnover of these residues that results in distinct conformation of Arg108 on BC loop of CYP2C9*13. In addition, they remarked that less hydrogen bonds were formed to stabilize diclofenac and lornoxicam in CYP2C9*13 cavity, affecting distances between the drugs and the heme iron of the mutant enzyme [28]. Herein, we simulated binding of flurbiprofen, which is one of anti-inflammatory drug (NSAIDs) metabolized by CYP2C9, to determine the consequence of this conformational change, which is caused by amino acid substitution. We found that orientation of fluriprofen located below the BC loop of CYP2C9*13 differs from that of CYP2C9*1. To illustrate the interactions more evidently, pharmacophores of flurbiprofen bound in different CYP2C9 variants were constructed and compared. CYP2C9*13-flurbiprofen complex had additional aromatic interactions between aromatic ring of flurbiprofen and amino group of Arg108. These interactions were not observed in CYP2C9*1. Therefore, the aromatic interactions might hinder metabolism rate of flurbiprofen in the mutant enzyme by strengthening the binding of flurbiprofen beneath the BC loop. Consequently, the drug might participate in monooxygenation reaction with difficulty, resulting in reduced metabolic rate. Accordingly, conformation of Arg108 is crucial in binding of flurbiprofen in CYP2C9.

In order to comprehend defective drug metabolism caused by single nucleotide polymorphism, structural insight is demanding. Herein, molecular dynamics simulation may be an alternative approach. It can be applied to investigate structural pathology caused by amino acid substitution of mutant enzymes regardless of the simulation system (Discovery-3 module by Zhou et al. and SANDER program in this study). Furthermore, this study strategy can be applied to other polymorphic variants of CYP2C9 in order to elucidate effects of structural changes that underlie poor metabolic activities in drug clearance among individuals carrying mutant allele(s).

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