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Human flavin-containing monooxygenase 3: Structural mapping of gene polymorphisms and insights into molecular basis of drug binding

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ABSTRACT

Human hepatic flavin-containing monooxygenase 3 (hFMO3) catalyses the monooxygenation of carbon-bound reactive heteroatoms and plays an important role in the metabolism of drugs and xenobiotics. Although numerous hFMO3 allelic variants have been identified in patients and their biochemical properties well-characterised *in vitro*, the molecular mechanisms underlying loss-of-function mutations have still not been elucidated due to lack of detailed structural information of hFMO3. Therefore, in this work a 3D structural model of hFMO3 was generated by homology modeling, evaluated by a variety of different bioinformatics tools, refined by molecular dynamics simulations and further assessed based on *in vitro* biochemical data. The molecular dynamics simulation results highlighted 4 flexible regions of the protein with some of them overlapping the data from trypsin digest. This was followed by structural mapping of 12 critical polymorphic variants and molecular docking experiments with five different known substrates/drugs of hFMO3 namely, benzydamine, sulindac sulfide, tozasertib, methimazole and trimethylamine. Localisation of these mutations on the hFMO3 model provided a structural explanation for their observed biological effects and docked models of hFMO3-drug complexes gave insights into their binding mechanism demonstrating that nitrogen- and sulfur-containing substrates interact with the isoalloxazine ring through Pi-Cation interaction and Pi-Sulfur interactions, respectively. Finally, the data presented give insights into the drug binding mechanism of hFMO3 which could be valuable not only for screening of new chemical entities but more significantly for designing of novel inhibitors of this important Phase I drug metabolising enzyme.

1. Introduction

The mammalian flavin-containing monooxygenases (FMO) belong to the oxidoreducatase subclass 1.14 and comprise a family of five functional enzymes that catalyse the oxygenation of a wide variety of nucleophilic heteroatom-containing compounds and have specific endogenous biological roles (Cashman and Zhang, 2006). In humans, FMO3 (hFMO3) has been described as playing a prominent role in the catabolism of drugs by selective oxygenation of nitrogen- and sulfur-containing xenobiotics resulting in their detoxification *via* excretion (Cashman and Zhang, 2006). The latter reactions are part of human hepatic Phase I drug metabolism mainly carried out by cytochromes P450. However, unlike cytochromes P450, the hFMO3 reaction produces few toxic metabolites and therefore poses considerable clinical advantages (Cashman, 2005, 2008). Moreover, in addition to its role in drug metabolism, FMO3 has also been shown more recently to act as an important modifier of human health in atherosclerosis and cholesterol/glucose/lipid metabolism (Leiser et al., 2015; Schugar and Brown, 2015).

Certain mutations within the hFMO3 gene can lead to the abnormal drug or chemical metabolism. One such genetic disease, trimethylaminuria (TMAU or fish odour syndrome), is due to genetic polymorphisms in the hFMO3 gene that cause defective trimethylamine (TMA) *N*-oxygenation (Dolphin et al., 1997). In addition, mutations can also affect the substrate selectivity with some abolishing or lowering both *N*- or *S*-oxygenation capacity of the enzyme, while other mutations only abolish the *N*-oxygenation activity (Zhou and Shephard, 2006).

In spite of growing knowledge of genetic polymorphisms in hFMO3, the molecular mechanisms underlying the loss of function or substrate specificity of genetic variants is still unclear due to lack of detailed structural information for hFMO3. However, the crystal structures of a eukaryotic FMO (yFMO) from *Schizosaccharomyces pombe* (Eswaramoorthy et al., 2006) and a prokaryotic FMO (bFMO) from *Methylophaga* sp. Strain SK1 (Alfieri et al., 2008) have been solved recently providing useful templates for the construction of a hFMO3 model. In previous years our group (Sadeghi et al., 2010) and others have published 3D models of hFMO3 (Borbás et al., 2006; Yeung et al., 2007) however, in most cases the templates selected were different. Cashman's group (Borbás et al., 2006) used

Abbreviations: hFMO3, human Flavin-containing monoxygenase.

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four different PDB files namely glutathione reductase (1GET), NADPH-peroxidase (1NPX), phenylacetone monooxygenase (1W4X) and a protein with similarity to FMOs (1VQW) whereas Rettie's group (Yeung et al., 2007) used the yeast FMO (2GVC and 2GV8) together with cytochrome C oxidase from Rhodobactor sphaeroides (1M56).

The goal of the present study is to build a hFMO3 model used for structural mapping of critical polymorphic variants to provide a structural explanation for their biological effects. In addition, molecular docking is performed to investigate the possible molecular basis of binding of different drugs.

2. Materials and methods

2.1. Sequence analysis and molecular modeling

The search and the identification of templates suitable for homology modeling were performed by BLAST (http://blast.ncbi.nlm.nih.gov/). The hFMO3 sequence (NCBI CCDS ID: CCDS1292.1) was defined as the target sequence, and the crystal structures of bFMO (PDB ID: 2VQ7) (Alfieri et al., 2008) and yFMO (PDB ID: 2GV8) (Eswaramoorthy et al., 2006) served as templates. Sequence identities and homologies to hFMO3 are 23.0% and 40.4% for bFMO and 21.5% and 37.5% for yFMO, respectively. Multiple sequence alignment was performed with ClustalW (Chenna, 2003) and ESPript 3.0 (Gouet et al., 1999). This alignment was used for the model construction by YASARA Model package (Krieger and Vriend, 2002). A long insertion of 70 amino acids (D226-G295) together with the extended C-terminal portion which could not align to the templates were constructed by Phyre2 server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and integrated into the final model by YASARA (Krieger and Vriend, 2002). The quality of the obtained hFMO3 structural model was evaluated using PROCHECK (http://services.mbi.ucla.edu/PROCHECK/), ProSA (https://prosa.services.came.sbg.ac.at/prosa.php) and ERRAT (Colovos and Yeates, 1993; Wiederstein and Sippl, 2007).

2.2. Molecular dynamics (MD) simulation

The molecular dynamics simulation was performed by YASARA structure package (Krieger and Vriend, 2002). This software package contains all the functions needed to predict and validate macromolecular structures, including ligand docking and highly accurate force fields with knowledge-based potentials. Prior to MD simulation, the hFMO3 model was first optimized for its hydrogen bonding network to achieve more stable trajectories. The optimized structure was then placed in a simulation cell that was 10 Å larger than the protein. The force Field AMBER 03 was assigned and the cell was then filled with water molecules and NaCl at the physiological concentration of 0.9%. Counter ions were also placed in the cell, predicting pKa values and assigning protonation states and the temperature was set to 298 K. The simulation cell was subsequently rescaled to obtain a density for water residues of 0.997 g/L. The initial structure was subjected to energy minimization performing a simulated annealing minimization which moves the structure to a nearby stable energy minimum. Initial atom velocities were set according to a Boltzmann distribution and the molecular dynamics was then followed for 5 ns recording snapshots at regular time intervals of every 25 ps. The AMBER 03 force field was employed throughout all simulations. The cut-off for van der Waals interactions was 7.86 Å and the Particle Mesh Ewald (PME) algorithm was applied to compute the long range electrostatic interactions. The results of the molecular dynamics simulation were analysed with YASARA using an embedded pre-written script that, on the basis of the time course, is able to analyse structure stability creating a per residue table with average RMSFs and RMSDs from the starting structure.

2.3. Protein expression and purification

E. coli JM109 cells transformed with pJL2-hFMO3 were used for the expression of hFMO3 protein as previously described (Catucci et al., 2012). The cells were harvested 22 h post-induction and the protein was extracted from membrane fractions using 1% IGEPAL (octylphenoxypolyethoxyethanol) and purified by Ni affinity chromatography. The purified protein was collected and analysed by 12.5% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Protein concentration was determined by UV–vis spectroscopy with the peak absorbance at 450 nm and an extinction coefficient of 11,900 M^{-1} cm^{-1} as described previously (Catucci et al., 2012).

2.4. Trypsin digestion

Trypsin digestion was carried out to analyse the banding patterns of peptides generated (Coulter-Mackie and Lian, 2008). Briefly, 116 μg hFMO3 in 80 μL phosphate buffer (pH 7.4) was mixed with 60 μg trypsin and incubated for 2, 5, 10, 15, 30, 60, 120 min at 37 °C. At each time interval, 10 μL of the reaction mixture was removed and mixed with 10 μL SDS loading buffer (60 mM Tris pH 6.8/2% SDS/12% glycerol/0.001% bromophenol blue) and incubated at 98 °C for 10 min. All samples collected in this manner were subsequently subjected to SDS-PAGE analysis.

2.5. Molecular docking

Autodock version 4.0 (Goodsell et al., 1996) incorporated into the YASARA Structure package was used for molecular docking studies. Five different known substrates of hFMO3, namely benzydamine, methimazole, sulindac sulfide, tozasertib and trimethylamine were docked into the substrate binding pocket of the hFMO3 model. Each of these substrates (ligands) were initially built and optimized using Discovery Studio Visualizer (Dassault Systèmes, 2016) prior to the docking experiments. First a global docking experiment was performed by executing a total of one hundred runs of Global Docking centring a 15 × 15 × 15 Å simulation cell on the FAD group. In this experiment, the substrate is originally outside the simulation box and is placed inside the cell by exploiting the Autodock algorithm, resulting in a series of binding modes classified by the binding energy outputs. Among these binding modes the complex protein-ligand bearing the highest binding energy, calculated by YASARA as the mechanical energy required for disassembling a whole into separate parts (where positive energies indicate stronger binding and negative energies equate to no binding), was selected and further refined by local docking. After global docking the best binding mode (pose) was selected based on the best binding energy. The complexes were then subjected to 999 runs of Local Docking yielding the final docked binding modes. In local docking experiments the ligand is within the simulation cell and the possible conformations are assayed around the starting pose. The results are again sorted by binding energy, and the most suitable binding mode with the highest binding energy was finally selected and optimized by energy minimization using YASARA.

3. Results and discussion

3.1. Sequence alignment and homology modeling

As mentioned earlier, currently there are only two FMO proteins with known crystal structures: one bacterial (bFMO) (Alfieri et al., 2008), the other from yeast (yFMO) (Eswaramoorthy et al., 2006). When these two protein structures are superimposed (Cα backbone) in spite of only 23.4% sequence identity, they present a highly conserved overall structure. The primary amino acid sequence of hFMO3 was aligned with the sequences of these two proteins and the FMO-identifying sequence motif together with the two Rossmann-fold motifs