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In vitro drug metabolism by C-terminally truncated human flavin-containing monooxygenase 3

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ABSTRACT

Human flavin-containing monooxygenase 3 (hFMO3) is a microsomal drug-metabolizing monooxygenase that catalyzes the NADPH-dependent oxygenation of a wide range of drugs and xenobiotics which contain a soft-nucleophiles, usually sulfur or nitrogen. As the release from the microsomal membranes can facilitate the *in vitro* experimental determination of drug metabolism by hFMO3, in this work we identified and eliminated the membrane anchoring sequence without affecting the activity of the enzyme and producing a soluble active enzyme. The truncated hFMO3 carrying a C-terminal deletion of 17 amino acids (tr-hFMO3) was expressed and purified from the cytosolic fraction. The tr-hFMO3 proves to be detached from the membrane, properly folded and fully active towards well-known marker substrates such as benzydamine and sulindac sulfide with measured apparent K_m values of 45 ± 8 μM and 25 ± 4 μM, respectively. Its activity was further tested with newly discovered Aurora kinase inhibitors, Tozasertib and Danusertib, and compared to those of the wild type enzyme.

The use of this soluble form of the hFMO3 enzyme as opposed to the usual microsomal preparations is advantageous for *in vitro* drug metabolism studies that are a requirement in the early phases of drug development by pharmaceutical industry.

1. Introduction

The flavin-containing monooxygenases comprise a family of five functional enzymes and are the second most important phase 1 drug-metabolizing enzymes after cytochromes P450 [1,2]. One member of the family, human flavin-containing monooxygenase 3 (hFMO3) is predominantly expressed in the liver where its substrates, generally nitrogen-, sulfur- and phosphorous-containing soft nucleophiles are transformed into more polar and excretable metabolites [3]. Human FMO3 contributes to the metabolism of many drugs such as ranitidine, cimetidine, tamoxifen, clozapine, benzydamine, amphetamine and mutations in its gene sequence have revealed polymorphisms that can cause significant differences in metabolism and lead to disease, one example of which is trimethylaminuria [4–6]. The enzyme is anchored to the smooth endoplasmic reticulum membrane, where its reduced flavin group binds molecular oxygen and is thought to act as a loaded gun ready to perform nucleophilic attack on the substrates [7–9].

To date the three-dimentional structure of hFMO3 has not been solved due to difficulties in the crystallization of microsomal, membrane-bound proteins [10]. In the past, several reports have demonstrated a successful increase in the solubility of membrane proteins by deleting the anchor that binds them to the membranes [11–13]. In 1991 Ozols [14] revealed that the C-terminus of FMO2 is very hydrophobic and it could anchor the protein to the membrane. More recently, Krueger et al. [15] reported on the expression and purification attempts of a soluble rabbit FMO2 by deleting 26 amino acids from the C-terminus region presumed to function as the membrane anchor.

In the present study, based on the hydrophobic nature of the C-terminus of the protein, a truncated hFMO3 was engineered by deleting the C-terminal region at different amino acid positions. The protein sequence with a 17 amino acid deletion (tr-hFMO3) was expressed and purified. The tr-hFMO3 proves to be detached from the membrane and it is not only fully active towards well-known marker substrates such as benzydamine and sulindac sulfide, but also newly discovered Aurora kinase inhibitors such as Tozasertib [16] and Danusertib [17].

The use of this soluble form of the hFMO3 enzyme as opposed to the usual microsomal preparations is advantageous for *in vitro* drug metabolism studies that are a requirement in the early phases of drug development by pharmaceutical industry. These studies are important not only in revealing potential metabolites but also

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allowing for elucidation of their toxicity and any possible adverse drug reactions.

2. Experimental procedures

2.1. Reagents

Methimazole (MMI), sulindac, sulindac sulfide, benzydamine, benzydamine N-oxide, FAD, Igepal, acetronitrile, methanol, NADPH and 5,5′-dithiobis(2-nitrobenzoate) and salts were purchased from Sigma–Aldrich (Italy). Tozasertib and Danusertib were purchased from Aurogene (Italy).

2.2. Molecular dynamics

Molecular dynamics in membrane was done using the YASARA [18] "md run membrane" for 4 ns. YASARA automatically identified the C-terminus of the hFMO3 as a hydrophobic exposed region bound to the membrane and constructed a membrane environment accordingly. YASARA unequivocally assigned the sequence between residues ARG 504 and THR 532 to a putative transmembrane helix and the protein was automatically rotated in order to fill the simulation cell volume with physiological solution and lipid content accordingly to the C-terminal helix. For all simulations, AMBER 03 force field was applied.

2.3. Protein–ligand interaction study

AutoDock 4.0 [19], embedded into the YASARA Structure package, was used to dock benzydamine, sulindac sulfide, tozasertib and danusertib to the refined model. Danusertib and tozasertib were obtained from the HIC-UP [20] database for hetero-compounds and were utilized with the same coordinates as they can be found in the co-crystals with the kinase domain of Abl [21] and Aurora kinase AurA [22], respectively. Benzydamine and sulindac sulfide were obtained from PubChem (www.ncbi.nlm.nih.gov/pccompound) and were geometrically optimized using MOPAC [23] also embedded in the YASARA package. The optimized ligand molecules were docked into the refined protein model by running twenty-five of Global Docking centering a $15 \times 15 \times 15$ Å simulation cell on the FAD group. In YASARA, docking runs of the ligand to receptor yield results sorted by binding energy where more positive energies indicate stronger binding and negative energies equate to no binding. 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.

The global docking experiment, in which the drug is originally outside the simulation box and is placed inside the cell by exploiting the autodock algorithm, resulted 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 [18] as the mechanical energy required for disassembling a whole into separate parts, was selected and refined by local docking. In local docking experiments the ligand is within the simulation cell and the possible conformations are assayed for a maximum of 999 runs and the results are again sorted by binding energy. In this case, since the starting positions of the ligand inside the simulation box are often random and it is probable that a certain pose/binding mode could never be detected, molecular dynamics simulations were applied to better accommodate the ligand inside the pocket.

2.4. Cloning, expression and purification

WT hFMO3 was cloned in the expression vector pJL2 [24] using the two restriction enzymes XbaI and HindIII previously [25]. The plasmid was digested by these two enzymes and the gene was amplified with the primers: 5′ AGT TCC CAG AAC TCT AGA ATG GGG AAG AAA GTG GCC ATC 3′ as forward for all the reactions, 5′ GCT CAT CGA AGC TTT TAA TGG TGA TGG TGC CGG TCC CAC TGG GTC AGT AT 3′ as a reverse for 493X, 5′ GCT CAT CGA AGC TTT TAA TGG TGA TGG TGT CTC CCG ACC ACT CGT GTC TG 3′ as a reverse for 505X and 5′ GCT CAT ATA AGC TTT TAA TGG TGA TGG TGC AGC CAA TGG AAA AAG AAG CAA GGC 3′ as a reverse for 516X (tr-hFMO3). All the reverse primers contained the sequence coding for a 4His-tag (5′ ATGGTGATGGTG 3′), to assist in the purification. The amplified genes were then digested with the restriction enzymes XbaI and HindIII and cloned back into pJL2.

Wild type and the tr-hFMO3 were expressed in *E. coli* JM109 cells and grown 24 h post-induction. WT protein was purified from the membrane fractions whereas the truncated hFMO3 was purified from the cytosolic fraction. All other purification steps were identical. Both proteins were purified via Ni affinity chromatography. Spectra of the eluted fractions (with 40 mM histidine) were recorded using a diode array HP-8453E spectrophotometer. FAD containing fractions with the characteristic absorption peaks at 375 and 442 nm were pooled and exchanged to storage buffer (100 mM potassium phosphate buffer pH 7.4, 20% glycerol and 1 mM EDTA) by 30 kDa cutoff Amicon membranes and stored at −20 °C.

2.5. FAD content determination

The concentration of holo hFMO3 was determined by spectroscopy with the peak absorbance at 450 nm and an extinction coefficient of 11,900 M^{-1} cm^{-1}, as previously described for other flavin-containing enzymes [26]. This value was also used for the determination of the enzyme concentration under non-denaturing conditions. In addition to the active enzyme (flavin-bound) concentration, the total hFMO3 protein concentration for the final purified protein was determined using both absorbance at 280 nm (extinction coefficients of 87,520 M^{-1} cm^{-1} and 87,485 M^{-1} cm^{-1} for WT and tr-hFMO3, respectively) and Bradford assay.

2.6. Far-UV circular dichroism analysis

Far UV circular dichroism experiments were performed on the WT and tr-hFMO3 at room temperature (Jasco-J600 spectropolarimeter). In order to improve the signal-to-noise ratio, several (3–4) spectra were accumulated and averaged for each sample. The measurements were carried out using 5 μM of protein and quartz cuvettes with a path length of 0.1 cm for the far UV (200–250 nm). Secondary structure prediction for both enzymes was performed using the K2D2 algorithm [27].

2.7. FAD aerobic reduction

The protein was diluted to 7 μM in 50 mM potassium phosphate buffer pH 7.4 at 10 °C. After the addition of aerated NADPH spectra were recorded using a Hewlett-Packard diode array spectrophotometer. The re-oxidation of the enzyme was followed by measuring the increase in absorbance at 442 nm.

2.8. Enzyme-substrate incubations and HPLC analysis

WT and tr-hFMO3 catalyzed S-oxygenation of MMI was monitored using the method previously described [28,29] at 37 °C. Rates of N-oxygenation of benzydamine [30] and