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(Article begins on next page)

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Flavin-containing monooxygenase 3 polymorphic variants significantly affect clearance of tamoxifen and clomiphene

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Running Title: *Metabolism of tamoxifen analogs by FMO3*

The authors declare no conflict of interest.

Abstract

Human flavin-containing monooxygenase 3 (hFMO3) is a drug metabolising enzyme that oxygenates many drugs and xenobiotics in the liver. This enzyme is also known to exhibit single nucleotide polymorphisms (SNPs) that can alter the rates of monooxygenation of therapeutic agents. The purpose of this study was to investigate the effect of the three common polymorphic variants of hFMO3 (V257M, E158K and E308G) on the metabolism and clearance of 3 structurally similar compounds: tamoxifen (breast cancer medication), clomiphene (infertility medication) and GSK5182 (anti-diabetic lead molecule). For GSK5182, none of the three variants showed any significant differences in its metabolism when compared to the wild-type enzyme. In the case of clomiphene, two of the variants, V257M and E308G, exhibited a significant increase in all the kinetic parameters measured with nearly two times faster clearance. Finally, for tamoxifen a mixed behavior was observed; E158K variant showed a significantly higher clearance compared to the wild-type whereas V257M mutation had the opposite effect. Overall, the data obtained demonstrate that there is no direct correlation between the SNPs and the metabolism of these three hFMO3 substrates. The metabolic capacity is both variant- as well as substrate-dependent and therefore when testing new drugs or administering already approved therapies these differences should be taken into consideration.

Introduction

Over the last few years it has become increasingly clear that there are significant differences in drug metabolism related to polymorphisms of drug metabolising enzymes including the hepatic hFMO3 (Human flavin-containing monooxygenase isoform 3) [1-5]. This enzyme is known to be highly polymorphic with more than 20 single nucleotide polymorphisms (SNPs) deposited in the SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP). A small portion of these SNPs are associated with inter-individual differences in the expression and/or function of hFMO3 enzyme that can contribute to an individual's susceptibility to toxicants and drug response [6]. Mutants may affect activity towards given substrates by decreasing, increasing or even totally abolishing it [1-8].

The most common hFMO3 genetic variants are E158K, V257M and E308G, and have been shown to have a relatively high population distribution [7]. Cashman and colleagues demonstrated a frequency of V257M polymorphism among African-Americans which is 8% versus a frequency of 20% for Asians [7]. Similarly, Lattard and colleagues demonstrated a higher frequency of the E308G polymorphisms in Asians and Caucasians, while the E158K is found at a higher frequency in African-Americans and Caucasians [9]. In Europeans and Asians, the haplotype E158K/E308G is present in 20.6% of individuals [10] and has been shown to reduce the activity of the enzyme with different substrates including methamphetamine, tamoxifen, thiacetazone and sulindac sulfide [3].

In this work, the metabolism of three different drugs namely tamoxifen, clomiphene and GSK5182 by the recombinant wild-type (WT) hFMO3 (Uniprot Code: P31513, Gene

name: FMO3_HUMAN) and its three common polymorphic variants V257M hFMO3 (rs1736557, Global minor allele frequency - GMAF = 0.0976), E158K hFMO3 (rs2266782, GMAF = 0.3478), E308G hFMO3 (rs2266780, GMAF = 0.0915) was investigated. These three compounds are at present used for the treatment of different diseases but share the same carbon backbone (Fig. 1): tamoxifen (a breast cancer drug, [11, 12]), clomiphene (a well-known drug used for the treatment of infertility in woman, [13]) and GSK5182 (currently being evaluated in preclinical studies as a new anti-diabetic agent for Diabetes mellitus type 2, [14]). These three selected drugs have been reported to be converted to the N-oxide product by WT hFMO3 [11,12,15-17] but differences in their metabolism due to polymorphisms have not been reported so far.

Materials and Methods

Materials

NADPH was purchased from Merck Group, Milan, Italy. Methanol and acetonitrile were purchased from Appenlab, Turin, Italy. Salts and tamoxifen were purchased from Sigma Aldrich, Milan, Italy. Tamoxifen N-oxide was purchased from Toronto Research Chemicals Inc., New York, ON, Canada. Clomiphene, clomiphene N-oxide and GSK5182 were purchased from D.B.A. Italia S.r.l, Segrate, Italy.

Purification of Recombinant Polymorphic proteins

The common polymorphic variants of hFMO3 were previously prepared in our lab using the QuikChange site-directed mutagenesis kit (Stratagene, USA) [4, 8].

All steps of expression and purification were performed according to previously published protocols [18, 19]. WT and polymorphic variants were expressed in *E. coli* JM109 and after the induction with IPTG cells were furtherly grown for 24 hours. The proteins were purified using negative anionic exchange chromatography (the protein is not retained by the DEAE resin at pH 7.4) and Nickel affinity chromatography. Spectra of the eluted fractions (with 40 mM histidine) were obtained using a diode array HP-8453E spectrophotometer. IGEPAL at a concentration of 0.1% was present during all steps of protein purification and diluted by means of ultrafiltration after affinity chromatography till histidine was below 40 µM. 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 -80 °C.

Enzyme-substrate incubations

Enzyme incubations were performed at 37 °C using 1 mM NADPH, tamoxifen (2.5 – 100 µM) or clomiphene (5 – 100 µM) or GSK5182 (0 – 80 µM) in 200 µL of 50 mM KPi pH 7.4. The reactions were initiated by adding 0.64 µM of the pure enzyme for tamoxifen and GSK5182 and 1 µM for clomiphene.

Enzymatic N-oxide products analysed by HPLC

In all cases, rates of N-oxygenation of the 3 substrates were calculated by quantifying the product using a 4.6 x 150 mm 5 µm Eclipse XDB-C18 column mounted on an Agilent 1200 HPLC system.

WT and polymorphic variants of hFMO3 with tamoxifen

Tamoxifen was dissolved in ethanol (solubility 10 mg/ml) and stored at 4 °C in dark containers. Tamoxifen N-oxide was dissolved in methanol at a final concentration of 20 mM and stored at -20 °C. The enzymatic reactions were carried out at 37 °C for 10 minutes and were terminated by adding 100 μl of ice cold acetonitrile. After this time, the samples were spun at 14,000 rpm for 5 min in order to eliminate the precipitated protein. A 100 μl sample of each resulting supernatant was subsequently analysed by HPLC according to the previously published methods [11, 17]. Reaction mixtures in the absence of hFMO3, tamoxifen and/or NADPH were used as controls and the metabolites were identified by comparison with commercially available synthetic standards.

WT and polymorphic variants of hFMO3 with clomiphene

Solutions of clomiphene and clomiphene N-oxide were prepared by dissolving the powder in methanol. Incubations with the enzyme were kept at 37 °C for 30 minutes and the then terminated by the addition of 100 µL of ice-cold methanol. Samples were spun at 14,000 rpm for 5 min to eliminate the precipitated protein and the resulting supernatants were analysed by HPLC. A 50 μl sample of each reaction was injected in the HPLC system and the separation was performed according to previously published method [15]. For clomiphene, the metabolites were identified by comparison with purchased synthetic standards. Reaction mixtures in the absence of FMO3 or clomiphene and/or NADPH were used as controls.

WT and polymorphic variants of hFMO3 with GSK5182

Currently, the metabolite(s) of the GSK5182 reaction with hFMO3 are not commercially available and therefore the expected N-oxide product was confirmed by mass spectrometry analysis (see next section). The solution of GSK5182 N-oxide was prepared enzymatically by incubating the substrate with the pure enzyme at 37°C for 3 hours to reach complete conversion. For the determination of the Michaelis-Menten parameters, reactions were carried out at 37 °C for 20 minutes and terminated by addition of 100 μl acetonitrile. Reactions in the absence of hFMO3 or GSK5182 and/or NADPH were used as controls. GSK5182 and its product were separated on a 4.6 x 150 mm 5 μm Eclipse XDB-C18 column at room temperature and with the UV- visible detector set at 340 nm. A 30 μl sample of was injected in the HPLC system and the separation was performed using isocratic elution with 75% of acetonitrile 25% of 0.1% formic acid in water.

Mass spectrometry

The identification of GSK5182 N-oxide product was carried out in collaboration with Ion Source & Biotechnologies Srl (Milan, Italy) employing a SACI/ESI source coupled to an Orbitrap mass spectrometer (ThermoFisher, San Jose, USA) working in positive ion mode [20]. Spectra acquisition was performed in the 40–3500 m/z range. ESI capillary voltage was kept at 1500 V, SACI surface voltage was set to 47 V, drying gas: 2 L/min, nebulizer gas: 80 psi and temperature: 40°C. Fragment and precursor ions isolation was performed using an ion trap (isolation windows ±0.3 m/z, collision energy 30% of its maximum value (5 V peak to peak) and the Orbitrap mass analyser was used to obtain the fragments at high accurate m/z ratio (resolution 15,000, m/z error <10 ppm).

Results

Tamoxifen, clomiphene and GSK5182 were incubated with the purified WT hFMO3 and its three common polymorphic variants. Recombinant WT, V257M, E158K and E308G were heterologously expressed in *E. coli* and purified using ion exchange and affinity chromatography. The activity of the enzymes was followed by performing steady-state kinetics experiments at 37°C in physiological buffer at pH 7.4 with the three selected compounds. Michaelis-Menten parameters were determined for the N-oxygenation of GSK5182, tamoxifen and clomiphene, and the data are shown in Figure 2.

For tamoxifen and clomiphene, the products were separated and identified using HPLC as reported in the Methods and materials section. However, GSK5182 has only recently been reported to be metabolised to the corresponding N-oxide by hFMO3 [16] and no standards of the latter product are commercially available. Therefore, mass spectrometry was used to verify the conversion of this compound to the corresponding N-oxide product by the purified recombinant hFMO3. As expected GSK5182, with a mass of 418.3, was indeed converted by the purified enzyme to GSK5182 N-oxide, with a mass of 434.3 (Figure 3B). Fragmentation of the 434.3 ion yielded 2 different ions: 252 and 182.0 that contains the N-oxide as further proof of the *in vitro* enzymatic catalysis (Figure 3A, B).

Nonlinear regression analysis of the data obtained allowed for the calculation of the kinetic parameters for the three variants and their comparison to WT (Table 1). Data analysis demonstrated that for GSK5182, the k_{cat} value of V257M variant was 2.5 times higher compared to that of the WT, whereas both E158K and E308G variants showed 50% decrease in activity compared to the WT enzyme (Table 1). Overall, there were no significant differences among the variants for the GSK5182 affinity to the enzymes, as

can be seen by the K_m values that are between 4.5-9.82 μM, except for V257M with a K_m of 28.5 μM. Although the turnover numbers calculated for the three variants were all significantly different to that of the WT hFMO3, overall no significant differences were observed in terms of k_{cat}/K_m or the relative clearance of GSK5182 as reported in Table 1. For tamoxifen, the calculated k_{cat} values of the three polymorphic variants are in general 2-3 times lower than those of the WT enzyme (Table 1). On the other hand, K_m values obtained demonstrate a higher affinity for tamoxifen for E158K and E308G variants. Moreover, both k_{cat}/K_m and relative clearance are affected but in different ways in the variants; significantly decreased for V257M but significantly increased for E158K variant. In this case, the metabolism is both variant- as well as substrate-dependent.

The enzymatic N-oxygenation of clomiphene was also evaluated and the kinetic parameters compared to those of the WT hFMO3. One of the polymorphic variants, E158K, showed no significant differences compared to the WT enzyme in terms of the kinetic parameters calculated i.e. $K_{m,}$ k_{cat} and k_{cat}/K_m. For the other two variants, the turnover numbers for clomiphene were increased around 4 times compared to the WT enzyme (Table 1). These two variants also showed a significantly lower substrate affinity. As can be seen in Table 1, both k_{cat}/K_m and relative clearance are significantly impacted by the mutations: E158K shows lower clearance whereas the other two variants show markedly increased clearance, more than tamoxifen or GSK5182.

Discussion

The data demonstrate that depending on the specific hFMO3 polymorphic variant the metabolism of the three structurally similar drugs can be slightly or severely affected when

compared to the WT form of the enzyme (Table 1). Therefore, it is not possible to predict the protein functionality on the basis of the mutation, but it is crucial to evaluate the oxygenation rates for each specific substrate i.e., the metabolic capacity of the variants is substrate dependent. Nevertheless, by comparing each variant to the WT enzyme, E158K seems to be the most affected as shown by the constant decrease in the k_{cat} values for all three substrates studied. For E308G a substantial decrease in k_{cat} was found for tamoxifen and GSK5182 as opposed to a moderate increase for clomiphene. V257M showed decreased k_{cat} for tamoxifen and increased k_{cat} for both clomiphene and GSK5182.

To explain differences in drug metabolism rates a key factor is the identification of the location of the mutations in the structure of hFMO3 leading to a possible justification for the observed functional differences, structure-function relationship. Unfortunately, at present such a structure is lacking and therefore we can only rely on *in silico* homology models (Figure 4) [21, 22]. According to the latter 3D models, E308 is located on the surface of the enzyme in the $NADP^+$ binding domain in a position that could potentially affect the stability and correct positioning of the cofactor in the active site [21, 22], while E158 is situated in a putative access channel to the catalytic domain near the NADPH binding motif. Finally, V257 is not in the immediate vicinity of the active site and is part of the hFMO3 “insert” region in a loop that protrudes from the $NADP^+$ binding domain across the FAD binding domain [21]. We have recently demonstrated the inactivation mechanism of hFMO3 by a SNP in the active site of the enzyme that impairs the efficient binding of $NADP^+$ [23]. This current work demonstrates how even surface SNPs in the $NADP^+$

binding domain of hFMO3 can affect its ability to perform catalysis by either decreasing or increasing it.

It is evident from the analysis of the location of the mutations (Figure 4), that this parameter cannot in itself justify the differences in the activity of the enzymes in relation to the different substrates. Moreover, the structural and activity data collectively demonstrate that it cannot be affirmed that a particular polymorphic variant will have a lower/higher activity per se. Indeed, the metabolic capacity is both variant- as well as substrate-dependent.

In conclusion, this work highlights the importance of considering common genetic polymorphisms of the hFMO3 gene in the population when testing new drugs or administering already approved therapies in the era of personalised medicine.

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Table 1 Kinetic parameters for GSK5182, Tamoxifen and Clomiphene of wild type and 3 hFMO3 polymorphic variants

Substrate	Variant	K_m μM	k_{cat} min^{-1}	k_{cat}/K_m $min^{-1}μM^{-1}$	Relative clearance (% of wild type)
GSK 5182	WT	9.82±1.85	2.22±0.22	0.22±0.048	100.00
	V257M	28.5±6.2*	5.69±0.47*	0.19±0.046	86.36
	E158K	4.57±0.42	1.16±0.05*	0.25±0.026	113.64
	E308G	5.87±0.92	1.20±0.08*	0.20±0.035	90.91
Tamoxifen	WT	6.4±0.7	1.13±0.7	0.18±0.02	100.00
	V257M	8.1±0.5*	0.6±0.02*	0.07±0.005*	38.89
	E158K	1.56±0.03*	0.45±0.01*	0.29±0.01*	161.11
	E308G	2.50±0.3*	0.38±0.02*	0.15±0.02	83.33
Clomiphene	WT	18.3±2.1	0.07±0.002	0.004±0.0005	100.00
	V257M	33.2±3.85*	0.30±0.01*	0.009±0.001*	225.00
	E158K	20.46±3.29	0.06±0.003	0.003±0.0005	75.00
	E308G	44.4±1.67*	0.25±0.01*	0.006±0.0003*	150.00

*$p < 0.05$ versus wild-type hFMO3

Figure 1

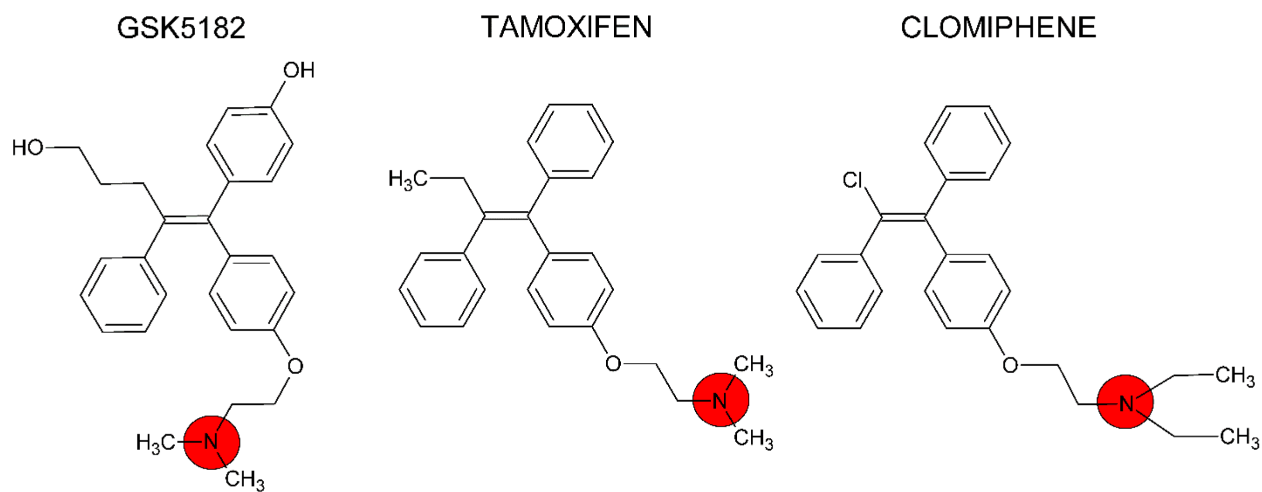


Fig.1 **Chemical structures of tamoxifen analogues used in this study**. The N-oxygenation site attacked by hFMO3 is highlighted in red.

Figure 2

A B C

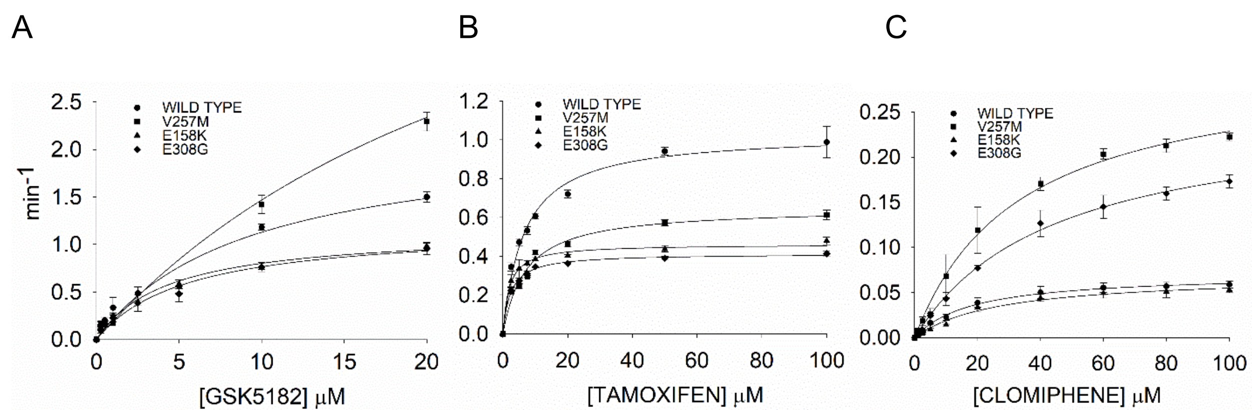
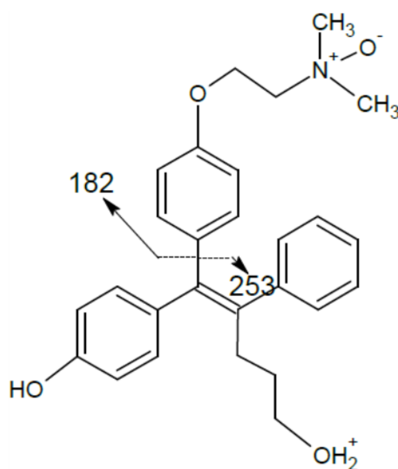


Fig. 2 **Michaelis-Menten curves of the enzymatic activity of hFMO3**. Data shown for wild-type hFMO3 and its 3 common polymorphic variants in the presence of (A) GSK5182, (B) Tamoxifen and (C) Clomiphene (each point represents the mean ± SD of three parallel experiments).

Figure 3

A



B

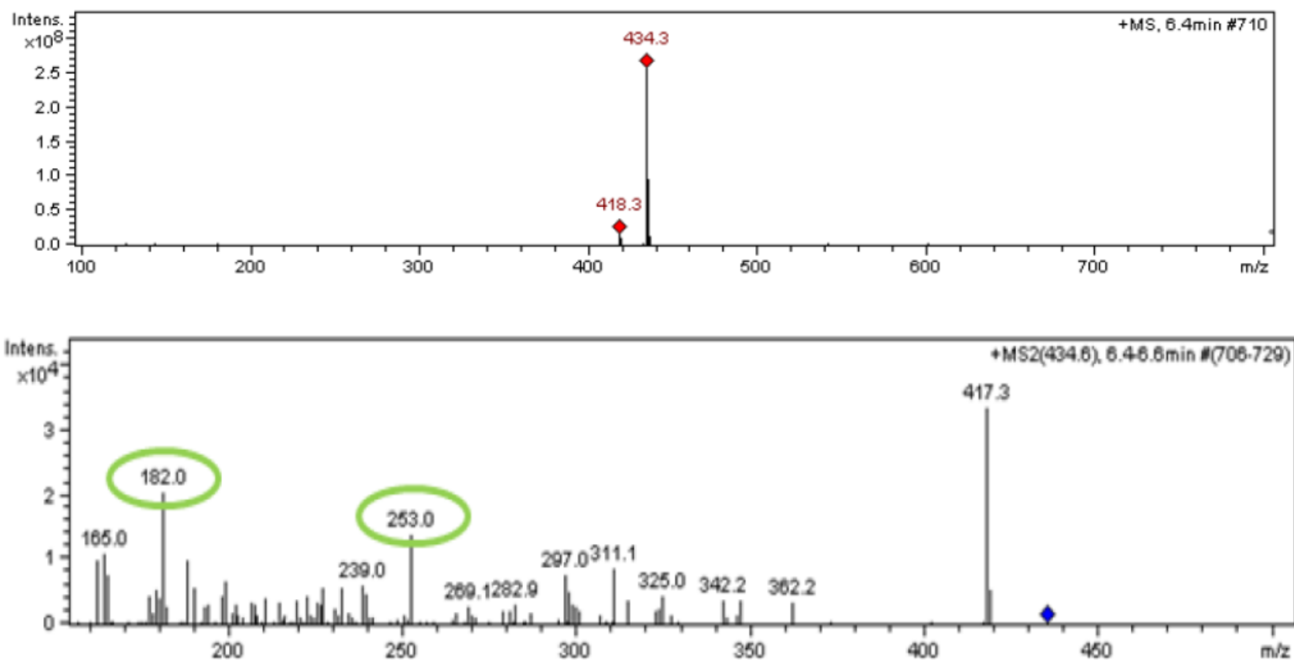


Fig.3 **Fragmentation profile of GSK5182 N-oxide**. Enzymatic N-oxide product with the molecular ion 434.2 (A) and subsequent result of the cleavage of 253.0 and 182.0 in MS (B).

Figure 4

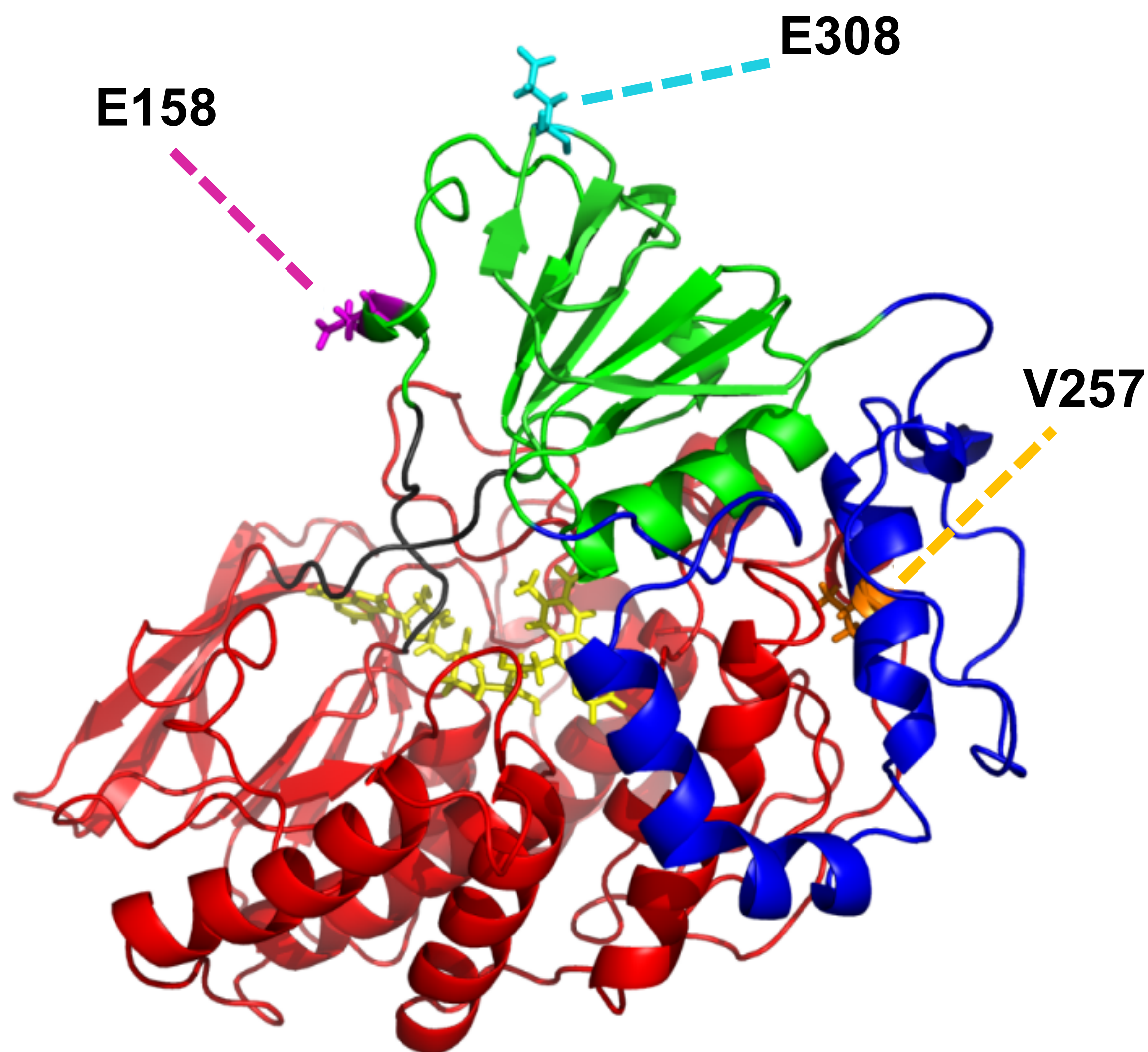


Fig. 4

Structural model of hFMO3 [22]. NADP-binding domain is shown in green with FAD-binding domain in red. The insert region is colored in blue. The FAD molecule is shown in yellow sticks together with the 3 mutation sites: V257 in orange, E158 in magenta and E308 in cyan.