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N- and S-oxygenation activity of truncated human flavin-containing monooxygenase 3 and its common polymorphic variants

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Abstract: Human flavin-containing monooxygenase 3 (FMO3) is a membrane-bound, phase I drug metabolizing enzyme. It is highly polymorphic with some of its variants demonstrating differences in rates of turnover of its substrates: xenobiotics including drugs as well as dietary compounds. In order to measure its *in vitro* activity and compare any differences between the wild type enzyme and its polymorphic variants, we undertook a systematic study using different engineered proteins, heterologously expressed in bacteria, purified and catalytically characterized with 3 different substrates. These included the full-length as well as the more soluble C-terminal truncated versions of the common polymorphic variants (E158K, V257M and E308G) of FMO3 in addition to the full-length and truncated wild-type proteins. *In vitro* activity assays were performed with benzydamine, tamoxifen and sulindac sulfide, whose products were measured by HPLC. Differences in catalytic properties between the wild-type FMO3 and its common polymorphic variants were similar to those observed with the truncated, more soluble versions of the enzymes. Interestingly, the truncated enzymes were better catalysts than the full-length proteins. The data obtained point to the feasibility of using the more soluble forms of this enzyme for *in vitro* drug assays as well as future biotechnological applications possibly in high throughput systems such as bioelectrochemical platforms and biosensors.

Keywords: flavin-containing monooxygenase; flavoprotein; polymorphic variants; biocatalysis; drug metabolism; truncation.

Abbreviations:

FMO3	human flavin-containing monooxygenase isoform 3
tr-FMO3	truncated form of FMO3 by deletion of 17 residues of C-terminal
IGEPAL	octylphenoxy poly(ethyleneoxy)ethanol
DEAE	diethyl-aminoethyl
ROS	reactive oxygen species

1. Introduction

Flavin-containing monooxygenase 3 (FMO3) is the most important isoform of the human FMO family associated with the majority of FMO-mediated hepatic metabolism. This enzyme binds non-covalently one molecule of FAD and is reduced by NADPH before exerting its catalysis [1-3]. Human FMO3 catalyzes the monooxygenation of nucleophilic heteroatom containing chemicals (drugs, pesticides, and xenobiotics) [4-6] through an unusual mechanism of activation of molecular oxygen via a stable FAD intermediate in the absence of the bound substrate. This mechanism accounts for the broad substrate range as well as uncoupling of this enzyme [7, 8] i.e., the wastage of electrons without oxygenation of the substrate leading to the formation of reactive oxygen species (ROS) such as the superoxide radical and hydrogen peroxide.

Human FMO3 is highly polymorphic, with characterized mutations ranging from missense, nonsense and deletion to truncation [9-13]. Both *in vitro* and *in vivo* studies have shown that some mutations affect the structure and function of the enzyme by interfering with protein folding or disrupting its structure whereas others have little or no consequences for the activity of the enzyme [11, 14]. Polymorphisms of FMO3 are clinically relevant because they are involved in the sensitivity of individuals to various chemicals and may contribute to adverse reactions to drugs and increase the susceptibility of individuals to exaggerated clinical responses when exposed to drugs that are FMO3 substrates [15].

Of the many polymorphisms of FMO3 reported in literature, three are designated as common polymorphic variants due to their prevalence in the general population; these are E158K, V257M and E308G (Figure 1). Using a population-based analysis, statistically significant differences have been demonstrated in the frequency of these common polymorphic variants among ethnically diverse populations: for instance, the E158K variant was found to be mostly expressed in African-American and Europeans, the E308G widely distributed in Asians and Europeans, whilst the V257M polymorphic variant was predominantly present in Asians [9, 12, 16].

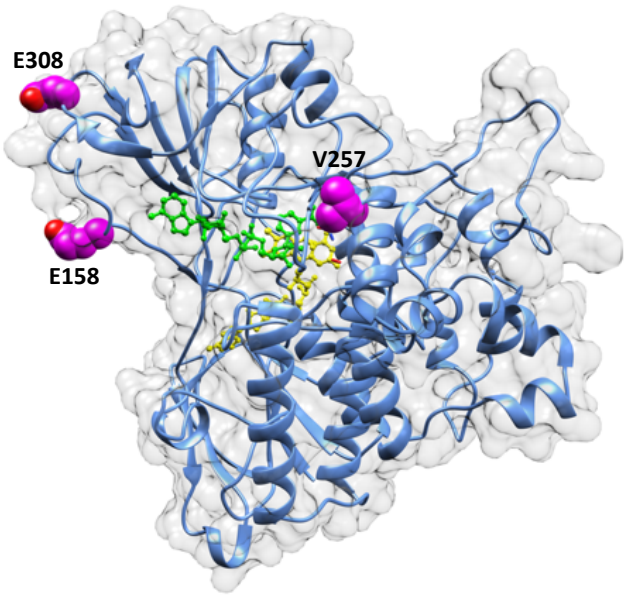

Figure 1. A 3D model of FMO3 shown in blue ribbon. The model was produced by SWISS-MODEL (swissmodel.expasy.org) protein structure homology-modelling server using PDB: 6SE3 as the template [17]. The location of the three polymorphic variants (in magenta) within the FMO3 structure is shown as well as the FAD (in yellow) and NADPH (in green). The figure was generated using CHIMERA software [18].

In this work we set out to investigate differences in the activity of the wild-type FMO3 (isoform a NP_008825.4) and its three polymorphic variants towards different drugs. In addition, the mammalian

FMOs were predicted to contain a highly hydrophobic C-terminal transmembrane helix which was only recently confirmed by solving the crystal structures of the ancestral FMO3-6 [17]. However, before the latter publication, we had already shown that a 17 amino acid truncation at the C-terminal of FMO3 results in a more soluble protein [19] and therefore the same truncation was also introduced in the common polymorphic variants. This resulted in 6 different proteins which all have been expressed in *E. coli* and purified for *in vitro* activity assays together with the existing wild type and truncated wild type FMO3 (tr-FMO3) that were measured as controls.

For the activity assays, three different known drug substrates of FMO3 were selected. These include benzydamine, tamoxifen and sulindac sulfide. The former two are converted to their corresponding N-oxide by the action of FMO3 [20-26], whereas the latter is converted to the S-oxide [27-29], as shown in Figure 2. Benzydamine is an anti-inflammatory drug that acts pharmacologically by weakly inhibiting both prostaglandin G/H synthase 1 and 2 enzymes. Benzydamine is extensively metabolized to benzydamine N-oxide by FMO3 [21]. Studies with recombinant FMO enzymes and human liver microsomes have demonstrated that FMO3 is the primary catalyst of benzydamine N-oxygenation with minimal contribution from cytochrome P450 enzymes [20] and that N-oxygenation is the major pathway of benzydamine biotransformation in various species *in vitro*. In general, these features have rendered the use of benzydamine as the marker substrate for FMO3 activity.

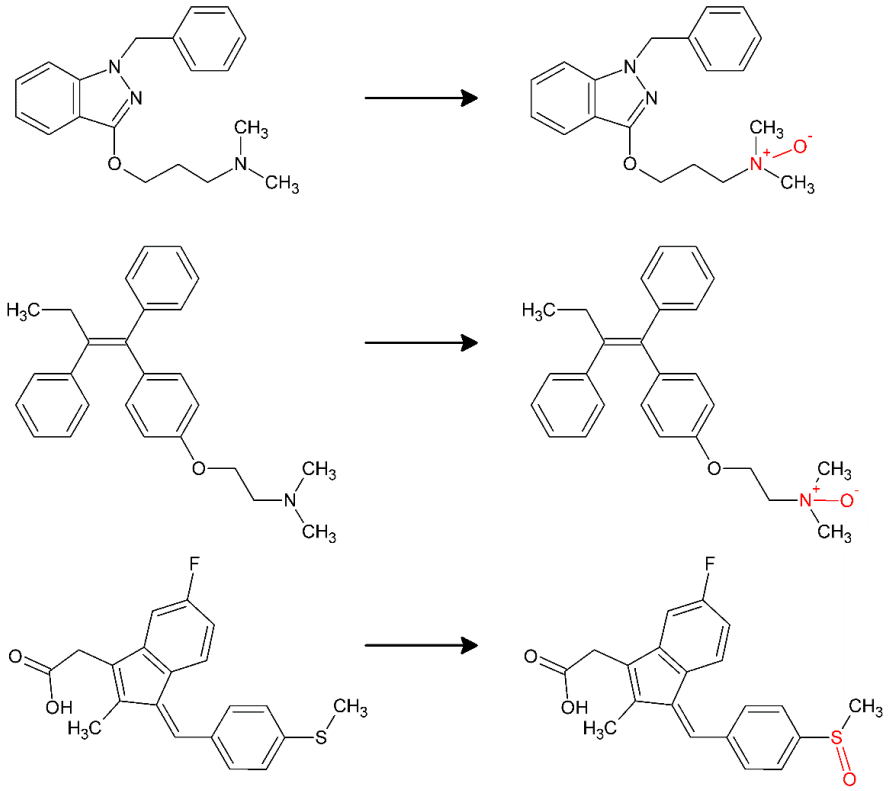

Figure 2. Chemical structures of the three FMO3 substrates used within this work; benzydamine (top), tamoxifen (middle), sulindac sulfide (bottom) and their corresponding N- or S-oxide products.

Tamoxifen is used for breast cancer therapy. Earlier studies have demonstrated that FMO3 catalyzes the formation of tamoxifen N-oxide [24]. Tamoxifen N-oxide represents a detoxification pathway, after the metabolic activation carried out by cytochrome P450 (tamoxifen α-hydroxylation) that leads to products capable of DNA adduction, with potentially significant toxicological consequences [24].

Finally, sulindac sulfide is used in the management of chronic pain and inflammation and most importantly in reducing the growth of polyps and precancerous lesions in the colon [30]. Administered orally, sulindac is a pro-drug which is reduced by the gut flora to the active sulfide form before

absorption. *In vivo*, the sulfide form is re-oxidized to sulindac, the latter compound is relatively inactive compared to the sulfide form: this oxidation step leading from sulindac sulfide to sulindac is carried out by FMO3 [27].

In order to compare the *in vitro* drug conversion by the FMO3 and its variants, the polymorphic variants (E158K, V257M and E308G) were engineered by site-directed mutagenesis in both full-length and truncated proteins for heterologous expression in *E. coli*. Spectroscopy and redox potentiometry were carried out before *in vitro* activity assays in the presence of the selected drugs.

2. Materials and Methods

NADPH and triethylamine were purchased from Merck. Methanol and acetonitrile were purchased from Appenlab (Italy). Salts, ethylacetate, phosphoric acid, benzydamine, benzydamine N-oxide, sulindac sulfide, sulindac, tamoxifen and all bacterial growth media were purchased from Sigma Aldrich (Italy). Tamoxifen N-oxide was purchased from Toronto Research Chemicals Inc. (Canada).

2.1. Mutagenesis and preparation of the six polymorphic clones of FMO3

The desired mutations resulting in the polymorphic variants E158K, V257M and E308G were generated using the QuikChange site-directed mutagenesis kit (Stratagene, USA). The forward and reverse primers used for each variant were respectively, 158K mutation: 5′ CAACCTACCAAAA**AAG**TCCTTTCCAGGAC 3′, 5′ GTCCTGGAAAGGA**TTC**TTTTGGTAGGTTG 3′; 257M mutation: 5′ CTGACTGGTTGTAC**ATG**AAGCAGATGAATGC 3′, 5′ GCATTCATCTGCTT**CAT**GTACAACCAGTCAG 3′; 308G mutation: 5′ CGTGAAGGAATTCACA**GGG**ACCTCGGCCATTTTTG 3′, 5′ CAAAAATGGCCGAGGT**CCC**TGTGAATTCCTTCACG 3′. The location of the mutated amino acid codon is underlined for clarity.

The presence of the wild type *FMO3* sequence (either full-length or truncated form) in the pJL2 plasmid [31] was confirmed by restriction analysis and sequencing prior to performing the *in vitro* site-directed mutagenesis. The presence of the correct mutation in all the resulting 6 different clones (full-length and truncated clones of E158K, V257M and E308G), was confirmed by DNA sequencing.

2.2. Expression and purification of FMO3 polymorphic variants

Wild type FMO3 and its polymorphic variants were all expressed in *E. coli* JM109 at 24 °C for 24 hours post-induction. The experimental procedures for expression and purification of the variants were identical to that previously published for the wild type FMO3 [19, 32]. The purification step included the extraction of the proteins in the presence of 1% IGEPAL (octylphenoxy poly(ethyleneoxy)ethanol). Purification in all cases followed a two-step chromatography including a DEAE (diethyl-aminoethyl) ion exchange column followed by Ni-affinity. All proteins were eluted from the affinity column using 40 mM histidine. Eluted fractions containing the flavoprotein, detected by a diode array HP-8453E spectrophotometer, were collected and exchanged to storage buffer (100 mM phosphate buffer at pH 7.4, 20% glycerol and 1 mM EDTA) by 30 kDa cutoff Amicon membranes and stored at −80 °C. The purity of each of the proteins was confirmed by 10% SDS-PAGE. Protein concentration was determined spectroscopically as described previously [19, 32].

2.3. NADPH reduction

Purified FMO3 and its variants were reduced with NADPH under aerobic conditions. UV-visible absorbance spectra were recorded in quartz cuvettes with a Hewlett-Packard diode array spectrophotometer. The protein was diluted to 7 μM in 50 mM potassium phosphate buffer, pH 7.4 at 24 °C and reduction was performed by adding NADPH. The oxidized-reduced state of the enzyme was detected by measuring the decrease in absorbance at 450 nm.

2.4. Potentiometric titration (Reduction potential measurements)

Potentiometric titration experiments are based on the measurement of the potential difference between two electrodes in solution: a reference electrode (Ag/AgCl electrode (BASi), with a constant potential) and a laminar gold working electrode with a potential that depends on the chemical species present i.e. the protein in solution. The experiment was carried out at 10 °C in a quartz cuvette placed in a cuvette holder adaptor connected by a fiber optic cable to a spectrophotometer that recorded the spectra and simultaneously to a potentiostat (Autolab PGSTAT12 potentiostat (Ecochemie, The Netherlands) controlled by GPES3 software) to measure the reduction potential. The measurements were performed in anaerobiosis (O_2 concentration less than 6 ppm) by a continuous flow of nitrogen gas within the otherwise sealed glove box (Belle Technology, UK).

Sodium dithionite was used as the reducing agent for the titrations, aliquots of 0.70 μM were added each time to the protein solution until complete reduction was observed. Protein and sodium dithionite solutions were prepared in 50 mM phosphate buffer pH 7.4 buffer. After each addition of sodium dithionite, the sample was stirred and the system was allowed to equilibrate for several minutes. After equilibration at each potential the optical spectrum was recorded [33].

Redox mediators methyl viologen (Em -440 mV), methylene blue (Em +11 mV), phenazine methosulfate (Em +80 mV), safranine O (Em -280 mV), anthraquinone-2,6-disulfonic acid (Em -185 mV) and benzyl viologen (Em -359 mV) were also added to the sample to facilitate electrochemical communication between the protein and the electrode. The concentration of the mediators was 30 times less compared to that of the protein (17 μM protein versus 0.5 μM mediator) so the absorption of the mediators was negligible compared to that of the protein. All the redox mediators (except benzyl viologen and methyl viologen that were soluble in buffer) were prepared in methanol to a final concentration of 5 mM and subsequently diluted to 0.05 mM in 50 mM phosphate buffer pH 7.4.

2.5. Enzyme assays and HPLC product separation

Turnover of the three selected substrates of FMO3 were carried out using the purified wild type and the polymorphic variants in order to determine the effect of the mutations on the activity of the resulting enzymes.

For the enzymatic assays, the purified proteins (which were stored in buffer containing 20% glycerol) were diluted in 50 mM phosphate buffer pH 7.4 with no addition of detergent. Sample preparation and all the experimental conditions were kept the same for both full-length and truncated FMO3 proteins. The concentration of the proteins was the same, 1.7 μM, based on holoprotein absorbance at 450 nm.

N-oxygenation of benzydamine and tamoxifen by the wild type and polymorphic variants of FMO3 together with the S-oxygenation of sulindac sulfide, were carried out as previously described [28, 29, 34] and the amount of product(s) determined by HPLC (Agilent-1200, Agilent Technologies, USA). For benzydamine N-oxygenation, the reaction mixture consisted of 1.7 μM enzyme, 0.5 mM NADPH, substrate (0–0.30 mM) in a final volume of 0.20 ml (50 mM phosphate buffer pH 7.4). While for tamoxifen N-oxygenation, the incubation mixture contained 0.5 mM NADPH, 1.7 μM enzyme and tamoxifen (0–100 μM). Finally, for sulindac sulfide the reaction mixture contained 0.5 mM NADPH, 1.7 μM enzyme and sulindac sulfide (0–200 μM). All reactions were performed at 37 °C for 10 min and terminated by the addition of 100 μl ice-cold acetonitrile. Reaction products were separated from the substrates using the HPLC apparatus (Agilent -1200 series) equipped with a 4.6 x 150 mm 5 m Eclipse XDB-C18 reverse phase column. The UV–visible detector was set at 308 nm for benzydamine N-oxide, 276 nm for tamoxifen N-oxide and 360 nm for sulindac sulfide. Reaction mixtures in the absence of FMO3 or substrates and/or NADPH were used as controls and the metabolites were identified by comparison with commercially available synthetic standards (see Figure S1). In all cases, enzyme kinetic parameters were obtained by nonlinear fit of the experimental data points (amount of product formed) using the Michaelis-Menten equation. All the measurements were performed in triplicate.

3. Results and Discussion

In order to compare the *in vitro* activity of FMO3 and its three common polymorphic variants (E158K, V257M and E308G) and to investigate the role of the hydrophobic C-terminal of this enzyme, new clones were generated using the wild type and truncated (tr-FMO3) clones already existing within our lab [19]. The mutagenesis experiments detailed in the methods and materials section resulted in the following 6 clones; E158K and tr-E158K; V257M and tr-V257M; E308G and tr-E308G. The successful generation of the latter versions of the three common polymorphic variants of FMO3 i.e. full-length (membrane-bound) and truncated (tr-, more soluble) allowed for the characterization of these enzymes in terms of their activity towards three known substrates of FMO3 as well as redox potentiometry to ascertain whether the mutations have had any effect on the redox potential and as a consequence the first step of this enzyme's catalytic cycle, reduction by NADPH.

3.1. Purification and spectral characterization of the 8 different FMO3 proteins

Once all clones were confirmed by DNA sequencing to contain the correct mutation, they were used for expression in *E. coli* and the subsequent purification of each resulting protein by affinity chromatography. As mentioned earlier, in a previous publication [19] it was shown that the 17 amino acid truncations of the C-terminal do not affect the activity of the FMO3 protein. Moreover, the more soluble protein is to be used not only for *in vitro* activity assays but also in biotechnological applications such as high throughput systems in drug discovery and electrochemical biosensors [35, 36].

The yields per liter of expression were calculated to be ~15 mg for the full-length proteins. Interestingly, also the enzymes in the truncated form were well-expressed, though with a slightly lower yield (~10 mg). All proteins were concentrated and exchanged to storage buffer without the surfactant used during the initial purification step and stored at -80°C until use.

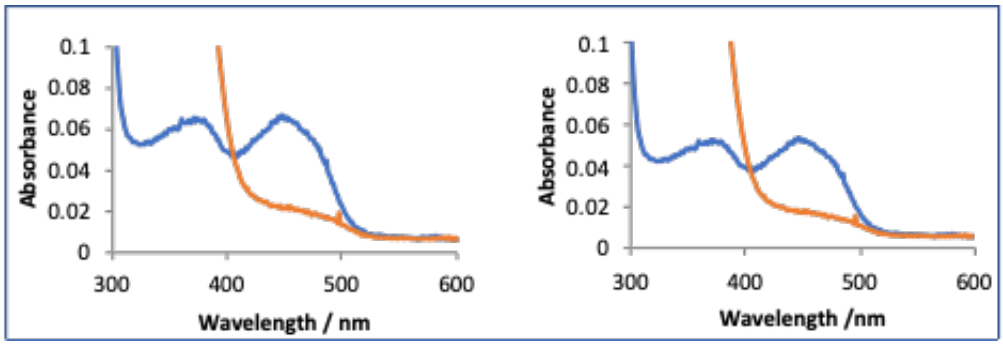

Figure 3. UV-visible spectra of purified FMO3 in the fully oxidized (in the absence of NADPH, blue line) and the fully reduced (orange line) form for wild-type (left) and V257M polymorphic variant (right). 6 μM of WT and 5 μM of V257M in 50 mM potassium phosphate buffer pH 7.4 were reduced by addition of 7 μM NADPH at room temperature.

After purification, wild type protein as well as the polymorphic variants in both full-length and truncated forms were initially characterized by UV-vis spectroscopy. This was done in order to confirm that the proteins were purified in the holo-form and due to the presence of the FAD cofactor, displayed the characteristic absorption spectrum of flavoproteins exhibiting typical maxima at 375 and 450 nm. The ability of NADPH to reduce the proteins was also investigated spectrophotometrically by following the rapid bleaching of the FAD absorbance at 450 nm (formation of two-electron reduced FAD hydroquinone) in the presence of excess amounts of this electron donor under aerobic conditions at room temperature. Figure 3 shows the oxidized and fully reduced wild type and one of the

polymorphic variants. In all cases, no differences were observed in the spectra of the variants compared to the wild type FMO3 demonstrating that the mutations have had no major effect on the interaction of the FAD cofactor with the protein.

3.2. Redox Potentiometry

The initial step of the catalytic cycle of FMO enzymes consists of the binding of NADPH and the consequent two-electron reduction of the FAD cofactor within the active site of these proteins. In order to confirm that the mutations have had no effect on the redox activity of their non-covalently bound FAD redox potentiometric experiments were carried out. In general, measurements of the reduction potential may indicate changes in the characteristics of the redox center since different key factors could influence this potential such as the contributions to the Gibbs free energy difference between the two redox states (resulting from bonding interactions at the redox center), electrostatic interactions between the redox-center charge and polar groups within the protein and solvent, and redox-state conformational changes [37].

Titrations were performed in anaerobiosis using the strong reducing agent sodium dithionite and by following the spectral changes at 450 nm of the flavin cofactor at each different potential as detailed in the methods and materials section. The reduction process led to a totally bleached protein. Reduction potentials, expressed relative to the Ag/AgCl reference electrode, were obtained from the experimental data points fitted to the theoretical Nernst equation using least-squares analysis by fitting the oxidized fractions of the protein versus the potential and are shown in Figure S2 [33]. The results of the potentiometric titrations of the flavin cofactor of FMO3 and the calculated reduction potentials for full-length wild type and polymorphic variants of FMO3 together with that of the truncated form of the wild type are summarized in Table 1.

Table 1. Reduction potential of FMO3 expressed relative to the Ag/AgCl reference electrode. The measurements were performed in anaerobiosis and in presence of chemical mediators.

Protein	**Em (vs Ag/AgCl)(mV)**
WT	- 390 ± 3
Tr-WT	- 394 ± 7
E308G	- 385 ± 2
E158K	- 384 ± 3
V257M	- 385 ± 3
WT	- 393 ± 1[+] [28]
Free FAD	- 416 [38]

[+] Em calculated for the wild type FMO3 immobilized on carbon electrode see reference [28] for experimental details.

As can be seen in the above Table, the wild type and truncated FMO3 have very similar reduction potentials (around -390 mV), which is more positive comparable to the value published for free FAD [38]. The value obtained for wild type FMO3 is also very similar to the published value of the mid-point potential -393 mV which was determined by cyclic voltammetry for the immobilized enzyme [28]. Moreover, the three variants show near identical reduction potentials, around -385 mV, although this value is slightly but not significantly different to that of the wild type, i.e. -385 versus -390 mV (versus Ag/AgCl). In general, the reduction potentials measured indicate that mutation(s) as well as the truncation have not altered the properties of the FAD redox center of the polymorphic variants of FMO3.

3.3. In vitro activity assay

As the above data demonstrate that FMO3 proteins, full-length and truncated, expressed and purified from bacteria are spectroscopically similar to those of the microsomal FMO3, we proceeded with *in vitro* activity assays to systematically study the effect of the truncations as well as the mutation responsible for polymorphism, against three known substrates, benzydamine, tamoxifen and sulindac sulfide. For some of these samples, this is the first systematic study to date.

Human FMO3 wild type and polymorphic variants in both full-length and truncated versions were incubated in the presence of NADPH and each of the three substrates at 37°C for 10 minutes, as detailed in the methods and materials section. The first drug to be tested was benzydamine and the enzymatic product(s) were analyzed by HPLC where for all the proteins, two major peaks were observed with retention times of 5.3 minutes and 8.0 minutes corresponding to benzydamine N-oxide and benzydamine, respectively. In order to calculate the K_M values for each protein, different concentrations of benzydamine were tested. The kinetic parameters calculated from these *in vitro* activity assays in presence of benzydamine are summarized in Table 2.

Table 2. Kinetic parameters calculated for the different FMO3 proteins with benzydamine as substrate.

PROTEIN	K_M (μM)	kcat (min^{-1})	v_{max} (nmol product min^{-1} mg^{-1} FMO3)	Activity %
Full-length				
Wild type	52.0 ± 9.0	3.12 ± 0.20	53.90 ± 3.50	100.0 ± 6.5
V257M	37.3 ± 4.8	3.03 ± 0.11	52.30 ± 1.96	96.0 ± 3.5
E158K	59.3 ± 5.8	1.09 ± 0.04	18.96 ± 0.67*	35.1 ± 1.2
E308G	34.5 ± 5.4	0.20 ± 0.01	3.51 ± 0.13*	6.5 ± 0.2
Truncated				
Tr-wild type	53.0 ± 6.1	4.51 ± 0.18	77.80 ± 3.20	100.0 ± 4.2
Tr-V257M	38.0 ± 6.4	3.50 ± 0.10	60.50 ± 2.70#	77.0 ± 3.5
Tr-E158K	65.9 ± 7.1	1.75 ± 0.06	30.20 ± 1.10##	38.8 ± 1.4
Tr-E308G	39.7 ± 5.5	0.47 ± 0.02	8.08 ± 0.31##	10.0 ± 0.4

*P<0.001 compared to WT when performing Student's T –Test
# P<0.01 compared to tr-WT when performing Student's T –Test
##P<0.001 compared to tr-WT when performing Student's T -Test

The calculated K_M values for wild type and the polymorphic variants fall within the range of K_M values already published [9, 13]. The v_{max} values of the variants obtained for the N-oxygenation of benzydamine were expressed as the function of wild type. These data demonstrate that the V257M variant has an activity similar to that of the wild type, and therefore the single amino acid substitution (valine to methionine) does not seem to affect the enzyme's activity for this given substrate. Previous studies of N-oxygenation of benzydamine by V257M variant have also confirmed this observation [9, 13].

On the other hand, different results were obtained for E158K and E308G variants with respectively 35% and 6.5% activity when compared to the wild type enzyme, hence these two SNPs can actually affect the N-oxygenation activity of the enzyme. Previously published data also point to a decreased activity of E158K and of the haplotype E158K/E308G [9, 13].

When comparing the full-length proteins to their corresponding truncated versions, the data suggest that the truncated enzymes are not only fully active, but also have a higher v_{max} compared to their full-length counterparts, the latter observation might be the result of their higher solubility.

The same set of *in vitro* activity experiments were carried out with the second drug, tamoxifen. The calculated kinetic parameters for FMO3 and its polymorphic variants with tamoxifen are reported in Table 3.

Table 3. Kinetic parameters calculated for the different FMO3 proteins with tamoxifen as substrate.

PROTEIN	K_M (μM)	kcat (min^{-1})	v_{max} (nmol product min^{-1} mg^{-1} FMO3)	Activity %
Full-length				
Wild type	6.40 ± 0.70 [a]	1.13 ± 0.70 [a]	29.00 ± 0.10	100.0 ± 0.3
V257M	8.10 ± 0.50 [a]	0.59 ± 0.02 [a]	15.40 ± 0.50*	53.0 ± 1.7
E158K	1.56 ± 0.03 [a]	0.45 ± 0.01 [a]	11.50 ± 0.04*	40.0 ± 0.1
E308G	2.50 ± 0.30 [a]	0.37 ± 0.02 [a]	9.80 ± 0.40*	34.0 ± 1.4
Truncated				
Tr-wild type	5.60 ± 0.30	1.63 ± 0.01	42.10 ± 0.40	100.0 ± 0.9
Tr-V257M	7.10 ± 0.30	0.85 ± 0.01	26.30 ± 0.20#	62.0 ± 0.5
Tr-E158K	1.34 ± 0.30	0.68 ± 0.01	17.64 ± 0.30#	42.0 ± 0.7
Tr-E308G	2.90 ± 0.30	0.61 ± 0.02	16.20± 0.44#	38.0 ± 0.9

[a] These values are from [26].

*P<0.001 compared to WT when performing Student's T –Test

# P<0.001 compared to tr-WT when performing Student's T -Test

Overall as can be seen in the above Table, the K_M values as well as the relative activities of the truncated proteins show a similar trend to those of the corresponding full-length enzymes.

To date, there are no published studies on the impact of V257M and E308G on tamoxifen N-oxidation by human FMO3 enzyme. Literature data regarding tamoxifen conversion by FMO3 and its polymorphic variants is scarce. Krueger and co-workers [39], the only group which have published kinetic parameters for tamoxifen, determined the K_M and kcat values of tamoxifen N-oxygenation by the indirect method of NADPH consumption. The values reported in Table 3 have been determined directly by measurement of the amount of product formed and are significantly different compared to those determined by Krueger and co-workers [39] i.e. the K_M values are two orders of magnitude smaller pointing to a better affinity in our system whereas the kcat values obtained are much lower. Due to the differences in the methodology, the expression system and the poor solubility of tamoxifen, it is very difficult to make a direct comparison between the two studies. In general, more studies are required on the effect of FMO3 polymorphism on tamoxifen conversion.

Finally for the last substrate to be tested, sulindac sulfide, the same set of activity assays were carried out. Again in this case, the kinetic parameters for FMO3 and its polymorphic variants were calculated as detailed in the methods and materials section and are summarized in Table 4.

In this study all three polymorphic variants, V257M, E308G and E158K significantly affected the catalytic efficiency of the enzyme with sulindac sulfide, with a decrease in activity of 26%, 34% and 54%, respectively as compared with wild type enzyme. Two previously published studies have also reported this lower activity; (a) Shimizu and co-workers using a recombinant FMO3 protein expressed in *E. coli* demonstrated that V257M and E158K have a lower catalytic efficiency with respect to that of

the wild type [13]; (b) Hisamuddin and co-workers in their *in vivo* study found an increased bioavailability of sulindac in patients with FAP carrying the double mutations E308G and E158K. These patients showed a strong regression of their polyposis which was attributed to the lower metabolism of sulindac by the variant forms of FMO3 found in these patients [40, 41].

Table 4. Kinetic parameters calculated for the different FMO3 proteins with sulindac sulfide as substrate.

PROTEIN	K_M (μM)	kcat (min^{-1})	v_{max} (nmol product min^{-1} mg^{-1} FMO3)	Activity %
Full-length				
Wild type	15.6 ± 2.2	0.56 ± 0.02	9.60 ± 0.44	100.0 ± 4.6
V257M	14.3 ± 0.6	0.41 ± 0.01	7.10 ± 0.10*	74.0 ± 1.0
E158K	15.1 ± 2.6	0.25 ± 0.01	4.40 ± 0.30*	46.0 ± 3.1
E308G	10.1 ± 1.3	0.37 ± 0.02	6.40 ± 0.35*	66.0 ± 3.6
Truncated				
Tr-wild type	12.7 ± 1.6	1.60 ± 0.06	27.60 ± 1.00	100.0 ± 3.6
Tr-V257M	10.3 ± 1.2	1.10 ± 0.03	19.10 ± 0.60#	69.0 ± 2.2
Tr-E158K	16.3 ± 1.3	0.69 ± 0.02	11.90 ± 0.30#	43.0 ± 1.1
Tr-E308G	10.3 ± 1.4	0.89 ± 0.02	15.60± 0.40#	56.0 ± 1.4

*P<0.001 compared to WT when performing Student's T –Test
# P<0.001 compared to tr-WT when performing Student's T -Test

Again, for this drug the catalytic efficiency of all the truncated polymorphic variants was lower compared with that of the truncated wild type. These truncated polymorphisms reflected the activity changes found in the drug metabolism studies of their full-length counterparts. Therefore, it was confirmed once more that the truncated enzymes showed the same trend in activity compared to the wild type enzyme i.e. E158K has the lowest activity followed by E308G and finally V257M. Moreover, as seen for the conversion of the other two selected drugs, the more soluble truncated enzymes show an increased activity compared to their full-length membrane-bound counterparts, confirming the suitability of their use in *in vitro* assays.

In general, kinetic parameters determined for oxygenation of benzydamine, tamoxifen and sulindac sulfide all demonstrated that the polymorphic variants (each only a single amino acid mutation) had a lower activity towards the three selected drugs as compared to the wild type FMO3, in some cases as low as 10% (Figure 4). In the majority of cases, the E308G variant showed the largest decrease in the measured activities with all the three drugs, followed by E158K and V257M variants.

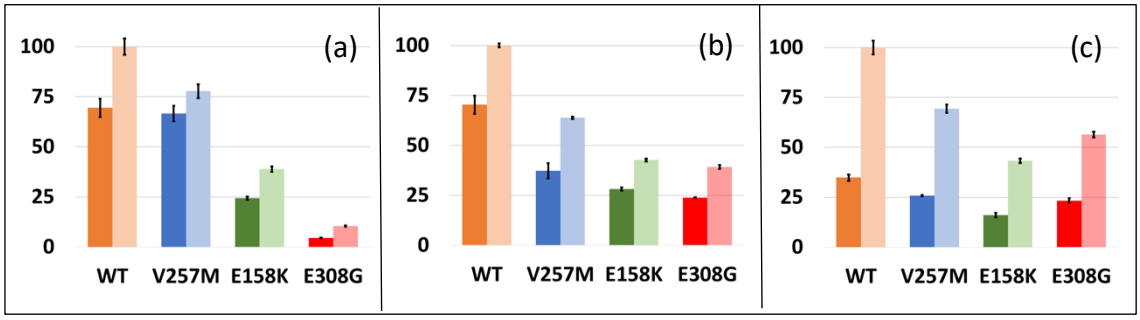

Figure 4. Comparison of the percentage activity of the different proteins normalized to the truncated wild type in the present of the three selected drugs; (a) Benzydamine, (b) tamoxifen and (c) sulindac sulfide. In each case, the darker and the lighter colors correspond to full-length and truncated proteins, respectively.

Furthermore, the same trend of decreased activity of the polymorphic FMO3 enzymes was seen in the more soluble truncated versions of these enzymes (Figure 4). Interestingly, the truncation by deletion of the C-terminal hydrophobic amino acids, constantly resulted in an enhancement in the activity of enzymes as compared to their membrane-bound counterparts. The latter confirms the feasibility of using these engineered more soluble enzymes for *in vitro* assays.

In general, the data demonstrate that polymorphic variants do influence the activity of the enzyme. The influence on a person's ability to metabolize or clear a drug will depend on whether or not they are homozygous for the variant.

4. Conclusions

The aim of this study was to systematically study how the most common polymorphic variants of FMO3 (V257M, E308G and E158K) affect the activity of the enzyme by measuring the kinetic parameters by *in vitro* assays with purified proteins against three well known substrates. In addition, the same parameters were measured with the truncated, more soluble versions of the same variants in order to draw some conclusions regarding the suitability of using these engineered versions in *in vitro* assays.

From the activity comparisons (Tables 2-4), it can be concluded that all mutations resulted in a lower activity as compared to the wild type FMO3. Why single amino acid changes could have this effect can be dependent on many factors including: (1) differences in incorporation of the redox cofactor and changes in the active site. In this respect, we based all our measurements on the holoenzyme (450 nm absorbance for concentration measurements) and therefore the apoenzyme was never considered. In addition, the environment of the FAD was probed by any changes in the redox potential and again no changes were observed. (2) The mutation might affect the affinity of substrate binding, K_M values. From the data obtained it can be seen that in some cases it is actually true that the K_M values have been changed by the mutation. However, differences in the K_M values are not sufficient in explaining the different activities observed i.e., even when similar K_M values are observed, activity is very different for example in the case of wild type and E158K variant with benzydamine i.e., both have K_M values around 55 μM but E158K shows only 35% activity compared to wild type (Table 2). (3) The presence of competing uncoupling reactions of FMO3 which lead to the production of reactive oxygen species such as hydrogen peroxide and superoxide [7, 8]. In this respect further studies are required to determine the effect of each mutation on the uncoupling reactions in the presence of the different substrates. All that can be said here is that the results definitely indicate a contribution from these ROS reactions.

Table 5. Comparison of the catalytic efficiencies of the full-length and truncated FMO3 with the three different substrates.

	Benzydamine	**Tamoxifen**	**Sulindac Sulfide**
PROTEIN	Catalytic Efficiency min^{-1} μM^{-1}	Catalytic Efficiency min^{-1} μM^{-1}	Catalytic Efficiency min^{-1} μM^{-1}
Wild type	0.06 ± 0.011	0.18 ± 0.111	0.04 ± 0.005
Tr-wild type	0.09 ± 0.010	0.29 ± 0.016	0.13 ± 0.017
V257M	0.08± 0.011	0.07± 0.005	0.03 ± 0.001
Tr-V257M	0.09 ± 0.012	0.12 ± 0.005	0.11 ± 0.013

E158K	0.02 ± 0.002	0.29 ± 0.008	0.02 ± 0.003
Tr-E158K	0.03 ± 0.003	0.51 ± 0.114	0.04 ± 0.004
E308G	0.006± 0.001	0.15 ± 0.019	0.04 ± 0.005
Tr-E308G	0.012± 0.002	0.21 ± 0.023	0.09 ± 0.012

As in some cases both the K_M and the kcat values are affected by the mutations for the different substrates, the efficiency of the catalysis is better expressed as kcat/K_M ratio, the catalytic efficiency (Table 5). Although the efficiency values may appear modest, they are in line with the results available in literature from other groups for wild type measured against benzydamine [13, 42] and, wild type and E158K against tamoxifen [39]. However modest, the catalytic efficiency results demonstrate that for all mutants with respect to all substrates tested, the truncated forms are better catalysts.

Finally, these observations are highly significant from a biological point of view; patients carrying the polymorphic variants of FMO3 with lower activity should be prescribed with a different dose of a drug (that is a substrate of FMO3) since the normal dose can lead to higher bioavailability of the circulating drug, causing undesired side-effects and/or toxicity. However, more systematic studies must be conducted to give a better picture of how the genetic variation in FMO3 might result in a diminished or enhanced drug efficacy and how these differences should be considered when prescribing drugs in the current era of "personalized medicine".

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Author Contributions

Conceptualization and methodology, G.G. and S.J.S.; formal analysis, S.B. and G.C.; investigation, S.B.; resources, G.G. and S.J.S.; data curation, S.B. and G.C.; writing—original draft preparation, S.B. and S.J.S.; writing—review and editing, S.B., G.C., G.G. and S.J.S.; supervision, S.J.S.; funding acquisition, S.J.S.

Declaration of Conflicts of Interest:

The authors declare no conflict of interest.

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