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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1623744> since 2017-05-17T14:36:19Z

Published version:

DOI:10.1016/j.ab.2017.01.024

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This is the accepted version of the following article:

[Gianluca Catucci, Isabelle Polignano, Debora Cusumano, Claudio Medana, Gianfranco Gilardi, Sadeghi SJ. **Identification of Human Flavin-Containing Monooxygenase 3 Substrates by A Colorimetric Screening Assay**. Anal. Biochem. (2017) 522: 46-52.],

which has been published in final form at [<http://dx.doi.org/10.1016/j.ab.2017.01.024>]

Identification of Human Flavin-Containing Monooxygenase 3 Substrates by A Colorimetric Screening Assay

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Abstract

Human hepatic flavin-containing monooxygenase 3 is a phase I drug-metabolizing enzyme that is responsible for the oxidation of a variety of drugs and xenobiotics. This work reports on a high-throughput rapid colorimetric assay for the screening of substrates or inhibitors of this enzyme. The method is based on the competition of two substrates for access to the active site of hFMO3 whereby the enzymatic product of the first drug converts nitro-5-thiobenzoate (TNB, yellow) to 5,5'-dithiobis(2-nitrobenzoate) (DTNB, colourless). Upon addition of a competing substrate, the amount of detected DNTB is decreased. The assay is validated testing three known substrates of hFMO3, namely benzydamine, tozasertib and tamoxifen. The latter drugs resulted in 41% - 55% inhibition. In addition, two other drugs also classified as doping drugs, selegiline and clomiphene, were selected based on their chemical structure similarity to known substrates of hFMO3. These drugs showed 21% and 60% inhibition in the colorimetric assay and therefore were proven to be hFMO3 substrates. LC-MS was used to confirm their N-oxide products. Further characterisation of these newly identified hFMO3 substrates was performed determining their K_m and k_{cat} values that resulted to be 314 μM and 1.4 min^{-1} for selegiline and, 18 μM and 0.1 min^{-1} for clomiphene. This method paves the way for a rapid automated high throughput screening of nitrogen-containing compounds as substrates/inhibitors of hFMO3.

Keywords: FMO; flavoprotein; HTS; tamoxifen; doping drugs, mass spectrometry.

1. INTRODUCTION

Human flavin-containing monooxygenase 3 (hFMO3) is a phase I drug-metabolizing enzyme predominantly expressed in the liver where its substrates, generally nitrogen-, sulfur- and phosphorous-containing soft nucleophiles are transformed into more polar and excretable metabolites [1-3]. Unlike cytochromes P450 (the most important phase I drug metabolizing enzymes), hFMO3 produces few toxic metabolites and therefore designing drugs that are metabolized by hFMO3 rather than by cytochromes P450 may lead to significant clinical advantages [4]. In addition, hFMO3 is not readily induced or inhibited, like cytochromes P450, consequently minimizing potential adverse drug-drug interactions [4, 5]. However, designing such drugs requires a rapid selection method which according to our knowledge does not currently exist although our group has previously described an electrochemically based assay for hFMO3 [6].

In order to develop a high-throughput colorimetric detection method for hFMO3 substrates or inhibitors, methimazole-dependent TNB (nitro-5-thiobenzoate) oxidation assay was chosen as the starting point. Methimazole is a known substrate of hFMO3 that is S-oxygenated by the enzyme and it has been widely employed in the past for FMO activity measurement using a colorimetric assay developed by Dixit and Roche [7]. The main advantages of this assay are: (a) the specificity of methimazole S-oxide (product of hFMO3 enzyme activity) for TNB and (b) its colorimetric nature leading to possible conversion into a high throughput screening assay.

Herein we describe a competitive assay based on the concept that adding a second N-containing substrate or inhibitor to the reaction mixture, the molecule would compete with methimazole for the enzyme activity. As a result, a lower amount of methimazole is converted into methimazole S-oxide and this can be measured by the differences in absorbance. Since the Dixit and Roche assay is based on the re-oxidation of TNB to DTNB the formation of a disulfide conjugate between methimazole S-oxide and TNB is crucial and therefore only N-containing substrates that are converted by hFMO3 into N-oxide products can be tested as competitive substrates. A scheme of the different steps of the colorimetric assay are shown in Figure 1.

Initially, to prove that the assay is indeed reliable in determining whether a chemical compound is a substrate of hFMO3, three known substrates namely benzydamine (anti-inflammatory drug), tamoxifen (anti-breast cancer) and tozasertib (kinase inhibitor used in cancer treatment) were selected. All these drugs have been shown to be converted to their N-oxide product by the action of purified hFMO3 [8-10].

Subsequently, the colorimetric assay was also exploited for the identification of putative new substrates of hFMO3. Two doping drugs, clomiphene and selegiline, were tested with the colorimetric assay and after a positive result, their corresponding N-oxide products were identified by LC-MS and finally their kinetic parameters including K_m and k_{cat} measured.

2. MATERIALS AND METHODS

2.1. Materials

Methimazole (*N*-methyl-2-mercaptoimidazole), benzydamine (3-(1-benzyl-1*H*-indazol-3-yl-oxy)-*N,N*-dimethylpropan-1-amine), tamoxifen ((*Z*)-2-[4-(1,2-diphenylbut-1-enyl)phenoxy]-*N,N*-dimethylethanamine), methanol, ethanol, NADPH, DL-dithiothreitol (DTT) and 5,5'-dithiobis(2-nitrobenzoate) (DTNB) and salts were purchased from Sigma–Aldrich (Italy).

Tozasertib (N-[4-[4-(4-methylpiperazin-1-yl)-6-[(5-methyl-1H-pyrazol-3-yl)amino]pyrimidin-2-yl]sulfanylphenyl]cyclopropane carboxamide) was purchased from Aurogene (Italy). Clomiphene (2-[4-(2-chloro-1,2-diphenylethenyl]phenoxy]-N,N-diethylethanamine), selegiline ((2R)-N-methyl-1-phenyl-N-prop-2-ynylpropan-2-amine), clomiphene N-oxide and selegiline N-oxide were purchased from DBA Italia (Italy).

2.2. Protein Expression and Purification

All steps of expression and purification were performed according to previously published protocols [8,9]. Wild type hFMO3 was expressed in *E. coli JM109* cells and grown 24 h post-induction. The protein was 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.

2.3. Methimazole assay

Methimazole-dependent TNB oxidation assay was previously described by Dixit and Roche as a method to determine catalytic activities of flavin-containing monooxygenases through spectrophotometrical measurement [7]. Each reaction was performed at 37°C using 0.84 µM hFMO3 in presence of 1 mM NADPH and 1 mM methimazole in 30 mM Tris-HCl pH 8.0. DTNB (60 µM) which is colourless, is initially reduced by 30 µM dithiothreitol (DTT) to 5-thionitrobenzoate (TNB) upon incubation for 5 minutes at 37°C. TNB has a maximum absorbance at 412 nm. Methimazole itself cannot react with TNB but when it is converted by hFMO3 to its oxidised product (methimazole S-oxide), the latter forms a disulfide conjugate with TNB which is re-oxidised to DTNB.

2.4. Colorimetric competition assay

Benzydamine hydrochloride was dissolved both in 30 mM Tris-HCl buffer (pH 8.0) and ethanol. The ability of benzydamine to compete with methimazole for hFMO3 activity was evaluated at different concentrations, in the range of 0 to 1 mM. The reactions were performed as described above for the methimazole assay, but with the addition of benzydamine in the reaction mixture. The non-enzymatic reaction was performed in the absence of enzyme and in presence of 1 mM benzydamine. The same reactions were also performed in ethanol (2.5% final concentration). Ethanol was used to test the assay feasibility also in the presence of an organic solvent. The percentage of inhibition was calculated by subtracting the amount of oxidized TNB at 1 min from the amount oxidized after 31 minutes. As a negative control a drug known to be a substrate of cytochrome P450, ketamine ((*RS*)-2-(2-Chlorophenyl)-2-(methylamino)cyclohexanone), was also tested with the colorimetric assay. As expected for a non-substrate, no differences in absorbance were observed.

The second known substrate, tamoxifen, was dissolved in ethanol. The ability of tamoxifen to compete with methimazole for hFMO3 activity was evaluated at different concentrations,

in the range 0 to 100 μM . The third known substrate, tozasertib, was dissolved in methanol and evaluated in the concentration range 0 to 200 μM . The reaction in absence of tozasertib was performed replacing its volume with methanol (4% final concentration, which was equivalent to the highest concentration of methanol used in the reactions). The non-enzymatic reaction was performed in the absence of hFMO3 and in presence of 200 μM tozasertib.

Selegiline was dissolved in 30 mM Tris-HCl buffer (pH 8.0) and tested in the concentration range from 0 to 1 mM. The non-enzymatic reaction was performed in the absence of the enzyme but in presence of 1 mM selegiline. The last drug to be tested, clomiphen (0 to 50 μM), was dissolved in methanol. The control reaction in the absence of this drug was performed replacing its volume with methanol (1% final concentration, which was equivalent to the highest concentration of methanol used in the reactions). The non-enzymatic reaction was performed in absence of hFMO3 and in presence of 50 μM clomiphen.

2.5. LC-MS Analyses of selegiline, clomiphen and their corresponding metabolites

HPLC analysis was performed on a C18 column (Phenomenex Luna, 150 \times 2.0 mm, 3 μm particle size, Phenomenex, Torrance, CA, USA) applying a gradient of mobile phase composition based on an aqueous 0.05% formic acid (solvent A) and acetonitrile (solvent B). The initial A:B ratio of 5:95 was linearly brought to 100:0 in 20 min. The injection volume was 20 μL and the flow rate 0.2 mL min^{-1} . A LTQ Orbitrap hybrid mass spectrometer (Thermo Scientific, Bremen, Germany), equipped with a ESI ion source, was as high resolving power MS analyzer. The syringe pump effluent was delivered to the ion source at 10 $\mu\text{L/min}$, using nitrogen as both sheath and auxiliary gas. The source voltage was set to 4.5 kV. The heated capillary temperature was maintained at 260°C. The acquisition method used had previously been optimized in the tuning sections for the parent compound (capillary, magnetic lenses and collimating octapoles voltages) in order to achieve maximum sensitivity. The main tuning parameters adopted for the ESI source were 19 V for capillary voltage and 50 V for tube lens. Full scan spectra were acquired in the range 100-500 m/z . MS^n spectra were acquired in the range between ion trap cut-off and precursor ion m/z values. Mass resolution was set to 30000. Mass accuracy of recorded ions (vs. calculated) was ± 0.001 u (without internal calibration). Spectra were acquired in the positive HRMS and MS/MS mode, with a precursor ion normalized collision energy of 25%.

2.6. Enzyme-substrate incubations and HPLC analysis

For the N-oxygenation of selegiline a typical incubation mixture consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, different concentrations of the substrate and 0.64 μM of purified enzyme in a final volume of 0.10 ml. Incubations were carried out at 37°C for 30 min: the linearity of product formation was confirmed with purified hFMO3 preparations for 60 min. The incubation was terminated by the addition of 0.05 ml of ice-cold methanol. The aqueous supernatant was centrifuged at 14000 rpm for 10 min and was subjected to HPLC equipped with an analytical Zorbax Eclipse reverse phase C18 column (4.6 \times 150 mm, 5 μm). A 50 μL sample was injected and the separation was performed using a mobile phase of 80% 30 mM potassium phosphate buffer pH 7.2 and 20% of acetonitrile at a flow rate of 1.0 ml/min. After 6 minutes a linear gradient was applied

increasing the acetonitrile to 50% after 10 minutes. The presence of selegiline N-oxide was monitored at the 210 nm.

For the N-oxygenation of clomiphenes the same conditions described above for selegiline were followed. Incubations were carried out at 37°C for 30 min and the linearity of product formation was confirmed with purified hFMO3 preparations for 60 min. The incubation was terminated by the addition of 0.05 ml of ice-cold methanol. Again in this case, the aqueous supernatant was centrifuged at 14000 rpm for 10 min and subsequently analysed by HPLC equipped with an analytical C18 column (4.6 x 150 mm, 5 µm). A 50 µl sample was injected and the separation was performed using a mobile phase of 78% methanol and 22% of 1% triethylamine at a flow rate of 1.0 ml/min. Clomiphenes N-oxide elution was monitored at 254 nm.

3. RESULTS and DISCUSSION

3.1. Colorimetric assay applied to known substrates of hFMO3

As a proof of principle that the colorimetric assay previously described by Dixit and Roche [7] can also work as a competitive assay for N-containing substrates of hFMO3, several known substrates of the enzyme were tested. According to the scheme in Figure 1, any drug that can compete with methimazole for binding to the active site of hFMO3, can result in a decrease in methimazole S-oxide formed which will subsequently effect the amount of TNB spectrophotometrically detected at 412 nm. Human FMO3 can act on heteroatom containing substrates including sulfur or nitrogen but for the competitive colorimetric assay described here, only N-containing substrates can be used since they do not interfere with the TNB oxidation.

The first substrate of hFMO3 to be investigated was benzydamine [11-13], a locally-acting nonsteroidal anti-inflammatory drug with anaesthetic and analgesic properties for pain relief and treatment of inflammatory conditions of the mouth and throat. The kinetics of the competition assay was followed for 30 minutes or 60 minutes to assess whether longer exposure to both substrates could affect methimazole S-oxidation. The experiments were carried out with increasing concentrations of benzydamine (0 to 1 mM). The absorbance values measured at 412 nm after 30 and 60 minutes were used to evaluate the percentage of inhibition of methimazole S-oxide formation due to the presence of benzydamine. The non-enzymatic reaction was used as the 100% inhibition reference point and the reaction in the absence of benzydamine was utilized as the 0% inhibition point. In the presence of increasing concentrations of benzydamine, the S-oxygenation rates decreased.

Furthermore, since the molar extinction coefficient of DTNB is known ($13,800 \text{ M}^{-1} \text{ cm}^{-1}$ at 412 nm), the delta absorbance measured for each sample was used to calculate the moles of TNB oxidised to DTNB, which were equivalent to the moles of methimazole converted to the S-oxide product by the hFMO3 enzyme. As expected, the moles of methimazole which were converted to S-oxide product decreased as a function of benzydamine concentration. After 30 minutes and in the presence of 1 mM benzydamine, methimazole S-oxide formation was decreased to 47% (versus ~45% after 60 minutes) (Figure 2). Since inhibition percentages were found to be comparable for 30 min or 60 min reactions, subsequent experiments were carried out for the shorter time scale of 30 min without further incubation.

Most drugs are hydrophobic and therefore not easily dissolved in aqueous buffers, so the above experiments were repeated dissolving benzydamine in ethanol, to evaluate a possible extension of the competition assay to more hydrophobic molecules. In this case a 49%

decrease in methimazole conversion was observed, which is comparable to values obtained with benzydamine dissolved in buffer, i.e. 47%. Therefore, the colorimetric competition assay can be extended to less soluble drugs.

The second drug to be tested was tamoxifen which inhibits the binding of estradiol to estrogen receptors hence used in the treatment of hormone-receptor positive breast cancer and it is metabolized by hFMO3 to tamoxifen N-oxide [14]. The colorimetric competition assay was carried out using tamoxifen dissolved in ethanol and investigating its effect on methimazole S-oxygenation at concentrations ranging from 0 to 100 μM , as mentioned in the Methods section. Also in this case spectrophotometric measurements were converted to percentage inhibition of methimazole conversion, with a calculated maximum inhibition of $\sim 55\%$ (Figure 3A), slightly higher than those obtained with benzydamine.

The third substrate of hFMO3 selected to test the feasibility of the colorimetric inhibition assay was tozasertib. This drug is an inhibitor of Aurora A, Aurora B, and Aurora C serine-threonine kinases that regulate mitotic events [15]. Aurora A was found to be overexpressed in many human tumors including breast, colorectal, and ovarian cancers with Aurora B also overexpressed in various types of cancer. Recently our group has demonstrated the conversion of tozasertib to its N-oxide using purified recombinant hFMO3 enzyme [10]. Different concentrations of this drug, dissolved in methanol, were tested in the colorimetric assay and the inhibition data obtained are shown in Figure 3B. In this case, the highest inhibition of methimazole S-oxygenation was around 41%, the lowest when compared to the previous two drugs.

3.2. Extension of the colorimetric assay to probable substrates of hFMO3

The colorimetric competition assay was proven to be reliable when testing known drug substrates of hFMO3, as demonstrated above. We therefore investigated its reliability with other probable substrates. One such example is selegiline, a selective inhibitor of monoamine oxidase B (MAO-B) and currently used in the treatment of Parkinson's Disease [16] where it has been shown to provide moderate symptomatic benefit to patients at the early stages of the latter mentioned disease. Furthermore, it is also classified as a doping drug by WADA (World Anti-Doping Agency) since it acts as an amphetamine-type stimulant. Selegiline has previously been reported to be a substrate of FMO in studies carried out in rats [17]. Tsutsuni and co-workers [17] calculated the kinetic parameters of selegiline and reported a K_m of $177.5 \pm 12.3 \mu\text{M}$ with a V_{max} of $2.3 \pm 0.4 \text{ nmol/min/mg protein}$. In order to determine whether selegiline is also a substrate of the human FMO3, it was tested with the colorimetric competition assay. The results demonstrated how selegiline competes with methimazole for hFMO3 activity, but the maximum inhibition level observed was only 21% (Figure 4A), much lower than the other drugs tested. In order to identify the enzymatic product, high resolution mass spectrometry was used (Figure 5). With LC-MS analysis it was possible to confirm that the product of the enzymatic reaction was indeed selegiline N-oxide. Selegiline precursor ion has a m/z of 188.1436 (MS not shown) whereas the peak observed at 204.1386 m/z corresponds to the precursor ion of selegiline N-oxide (Figure 5, left). The interpretation of the high resolution MS spectrum of hFMO3 metabolite is reported in Figure 5 (right). The fragmentation of selegiline N-oxide (Fig. 5) resulted in three peaks at 199.0852 m/z , 91.0537 m/z and 86.0595 m/z corresponding to a loss of 85.0534 m/z , 113.0849 m/z and 118.0709 m/z , respectively.

In order to better understand the affinity of hFMO3 for selegiline, the Michaelis-Menten parameters were calculated by measuring the activity of hFMO3 with selegiline in the

absence of a competing substrate. The calculated kinetic parameters, K_m value of 314 μM with a k_{cat} of 1.4 min^{-1} (Table 1), are consistent with a lower competitive inhibition of methimazole turnover, given the low affinity of the enzyme for selegiline which is an order of magnitude lower than the other drugs tested (Table 1).

The last drug, clomiphene, is a well-known drug used for the treatment of infertility in woman [18]. However, it is also classified by WADA as a doping drug since through a complex mechanism, accelerates testosterone secretion [19]. The drug contains a mixture of two active isomers that are known as enclomiphene and zuclomiphene. Clomiphene has not been previously reported as a substrate of hFMO3 but was selected in this work due to its chemical structure similarity to tamoxifen (Figure 3) and therefore its ability to compete with methimazole binding to hFMO3, was tested again with the colorimetric assay. Surprisingly, the drug competed very efficiently with methimazole for the hFMO3 active site and was already able to inhibit methimazole conversion at a low concentration of only 10 μM . Maximum inhibition was observed using 50 μM clomiphene, where 60% inhibition was reached (Figure 4B), higher than any of the other four drugs.

As mentioned earlier, to date there are no reports of clomiphene as a substrate of hFMO3 and therefore the enzymatic product formed was identified and confirmed by high resolution mass spectrometry as the N-oxide (Figure 6). The fragmentation of clomiphene N-oxide precursor ion (422.1884 m/z) resulted in five peaks, with the main one at 313.1228 m/z ($\text{C}_{22}\text{H}_{17}\text{O}_2$). The interpretation of the high resolution MS spectrum of hFMO3 metabolite is also reported in Figure 6 (right).

Subsequently, kinetic parameters were also calculated for clomiphene to better understand both substrate affinity and turnover rates. Results obtained show a K_m value of 18 μM and a k_{cat} of 0.1 min^{-1} , demonstrating the higher affinity of hFMO3 for clomiphene, in line with the greater level of inhibition observed with the competitive assay.

4. CONCLUSIONS

Human FMO3 is a drug metabolising enzyme but within the last few years, it has also been implicated as a key enzyme outside the field of drug metabolism. Recent reports have demonstrated how FMO3 is involved in diabetes, atherosclerosis and oxidative stress [20-23] and how its inhibition can have a preventative effect in the development of these disorders. These studies have highlighted the need for novel rapid methodologies that can not only identify potential new substrates of hFMO3 but also inhibitors of this enzyme. The colorimetric competitive assay reported here, can be one such method allowing for the screening of potential substrates/inhibitors of FMO enzymes through spectrophotometric measurements.

The results obtained clearly demonstrate that all the five substrates tested in this study can compete with methimazole for binding to hFMO3. The already known substrates, benzydamine, tamoxifen and tozasertib, show percentage inhibition values between 40-55%. In the case of selegiline, this inhibition value was decreased to 21%, less than half the value measured for the other drugs and in order to rationalize this lower value the kinetic parameters of selegiline metabolism, not previously reported for the human enzyme, were calculated. The fact that a high K_m value of 314 μM was measured is in line with the low level of inhibition observed. The exact opposite was observed with clomiphene, with a 17 folds lower K_m (18 μM) which was shown to strongly compete with methimazole exhibiting the highest percentage of inhibition amongst the drugs tested i.e. 60%.

Finally, the data presented demonstrate how this colorimetric assay, which is both rapid and easy to perform, can lead to the identification of new substrates or inhibitors of hFMO3.

ACKNOWLEDGEMENTS

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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TABLES

Table 1. Competitive inhibition data of hFMO3 using the colorimetric assay including its kinetic parameters with five selected drugs.

	Selegiline	Tozasertib	Benzydamine	Tamoxifen	Clomiphene
%Inhibition	21	41	47	55	60
K_m (μM)	314	23 ^a	56 ^b	5 ^c	18
k_{cat} (min⁻¹)	1.4	9.3 ^a	9.0 ^b	16.4 ^c	0.1

^a ref [10]

^b ref [9]

^c ref [6]

Figure legends

Figure 1. Scheme depicting the reactions involved in the colorimetric inhibition assay. Human FMO3 converts methimazole to its S-oxide which in turn oxidizes TNB (yellow) to generate DTNB (colorless). If a second drug competes with methimazole for binding to hFMO3, TNB oxidation is inhibited and therefore the reaction is not bleached.

Figure 2. Competitive inhibition of methimazole turnover by benzydamine. Histograms represent the average of 3 replicate reactions of inhibition obtained after 30 (blue) or 60 (red) minutes at concentration of 0.1, 0.5 and 1 mM benzydamine.

Figure 3. The colorimetric inhibition assay. Histograms represent the average of 3 replicate reactions of inhibition obtained after 30 minutes with two previously known substrates of hFMO3; Tamoxifen (a) and Tozasertib (b).

Figure 4. The colorimetric inhibition assay extended to the screening of two new drugs of hFMO3. Histograms represent the average of 3 replicate of inhibition reactions obtained after 30 minutes with two previously unknown substrates of hFMO3; Selegiline (a) and Clomiphene (b).

Figure 5. High resolution MS/MS spectra of Selegiline-N-oxide protonated ions. The corresponding fragmentation pattern of these molecular ions is reported on the right.

Figure 6. High resolution MS/MS spectra of Clomiphene-N-oxide protonated ions. The corresponding fragmentation pattern of these molecular ions is reported on the right.

Figure 1

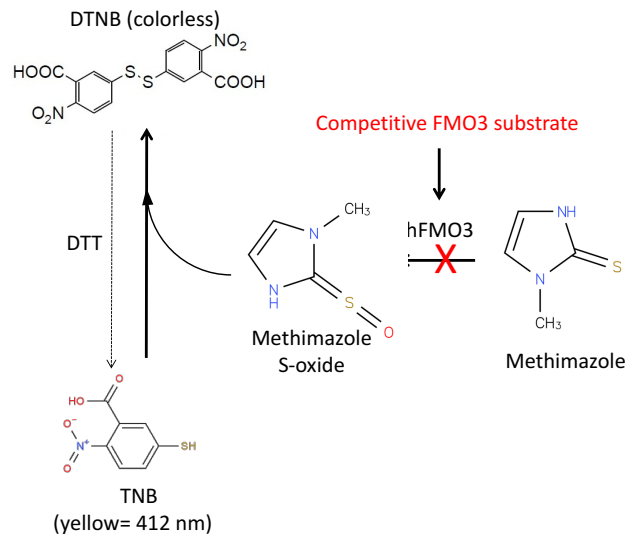


Figure 2

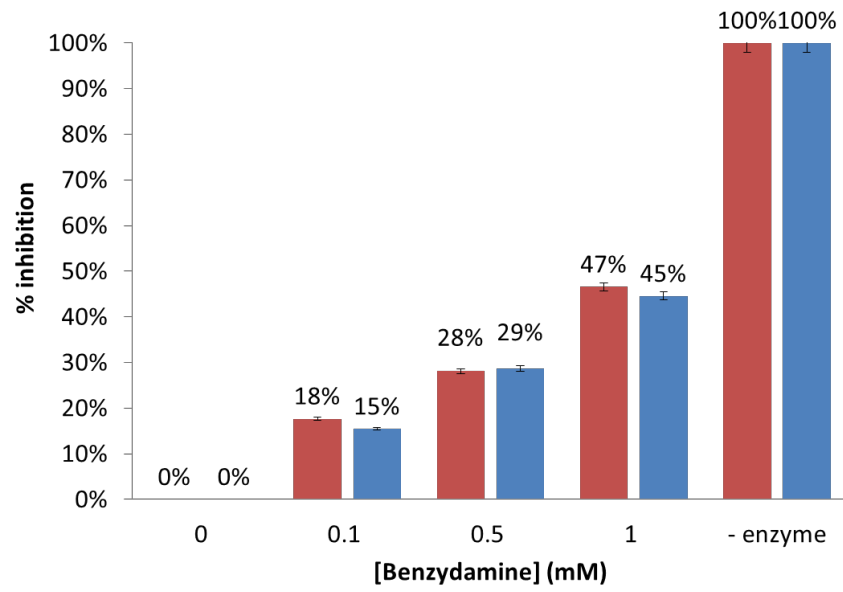


Figure 3

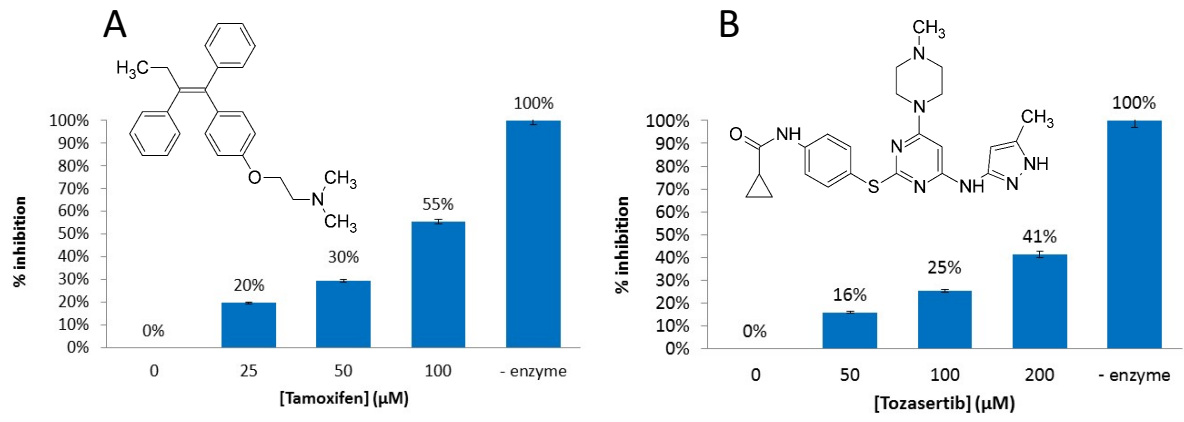


Figure 4

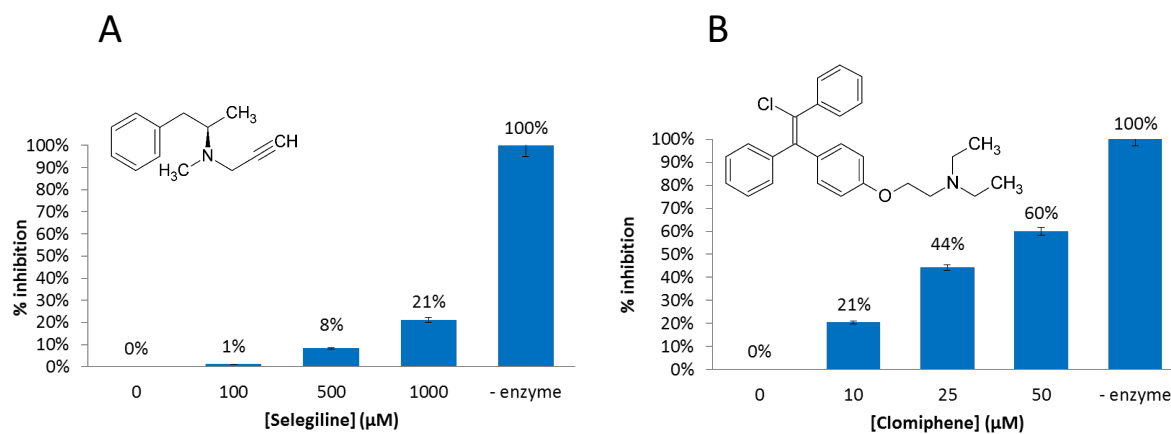


Figure 5

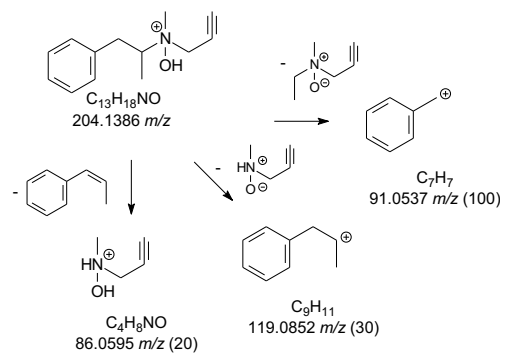
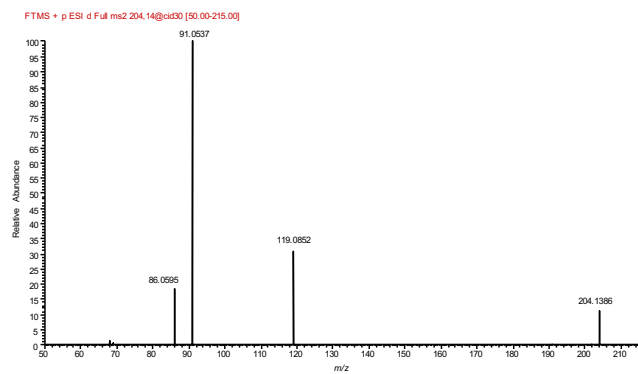


Figure 6

