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Chimeric Cytochrome P450 3A4 used for *in vitro* prediction of food-drug interactions

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Running title: Food-drug interactions in Chimeric P450 3A4 enzyme

Abstract

Inhibition of cytochrome P450-mediated drug metabolism by dietary substances is the main cause of drug-food interactions in humans. The present study reports on the *in vitro* inhibition assays of human cytochrome P450 3A4 genetically linked to the reductase domain of bacterial BM3 of *Bacillus* megaterium (BMR) resulting in the chimeric protein CYP3A4-BMR. The activity of this chimeric enzyme was initially measured colorimetrically with erythromycin as the substrate where K_M values similar to published data were determined. Subsequently, the inhibition assays with three different dietary products; grapefruit juice, curcumin and resveratrol, were carried out with the chimeric enzyme both in solution and immobilised on electrode surfaces. For the solution studies NADPH was added as the electron donor whereas the need for this cofactor was obviated in the immobilised enzyme as it was supplied by the electrode. Inhibition of the N-demethylation of erythromycin by CYP3A4-BMR chimera was measured at increasing concentrations of the different dietary compounds with calculated IC_{50} values of 0.5%, 31 μM and 250 μM for grapefruit juice, curcumin and resveratrol measured in solution compared to 0.7%, 24 μM and 208 μM measured electrochemically, respectively. These data demonstrate the feasibility of the use of both CYP3A4-BMR chimera as well as bioelectrochemistry for *in vitro* studies of not only drug-food interactions but also prediction of adverse drug reactions in this important P450 enzyme.

Keywords

Cytochrome 3A4, chimeric proteins, grapefruit, resveratrol, electrode, inhibition

Abbreviations

CYP: cytochrome P450; BMR: reductase domain of P450 BM3; FAD: flavin adenine dinucleotide; GC: glassy carbon; PDDA: poly(diallyldimethylammonium chloride.

1. Introduction

More than three decades have passed since the unintentional discovery that grapefruit juice interacts with certain drugs [1]. Since then it has been shown that the co-administration of some drugs with dietary food or drinks can markedly increase drug bioavailability and can therefore alter pharmacokinetic and pharmacodynamic parameters of the drug. The predominant mechanism for this interaction is the inhibition of cytochrome P450 (CYP) enzymes, resulting in a significant reduction of drug metabolism.

Drug-food interactions can reduce or increase the pharmacological effect, leading to failure of the therapy or an increase in drug toxicity. Since the real extent of the problem is not always known, it is necessary to develop strategies that identify and prevent the occurrence of such interactions. These can be divided into pharmacokinetic and pharmacodynamic interactions. The first are those that affect the absorption, distribution, metabolism or excretion of drugs (ADME) and are the most common, while there are few examples of pharmacodynamic interactions, in which a food affects the duration of action of the drug at the level of the receptor.

The most important drug-food pharmacokinetic interactions are caused by changes in drug absorption but these have little clinical relevance. On the other hand, the pharmacokinetic interactions that influence metabolism are mainly those involving CYPs and a great variety of such interactions have been reported including fruits, alcoholic drinks, herbs, spices and dietary supplements. These food-drug interactions lead to inhibition or induction of the activity of CYPs [2-4].

Of the many different CYPs, the 3A4 isoform appears to be the most important in drug-food interactions. The inhibition of CYP3A4 by some anti-bacterials, antidepressants, antifungals, antivirals and immunosuppressants is a well-known cause of drug-drug interactions [5-8]. However, little was known about food-drug interactions of CYP3A4 until grapefruit juice was reported as an inhibitor of this enzyme signalling a new and important mechanism of interaction between drugs and food components [1]. Following this initial observation, numerous cases have now been described of

drugs that respond in the same way to grapefruit juice including anti-allergics, antibiotics, anticoagulants, sedative-hypnotics, calcium channel blockers, β-blockers to name a few [9, 10].

Currently for *in vitro* drug metabolism and inhibition studies one can use human hepatocytes, liver microsomes or individually purified CYP proteins which are easier to manipulate. However, one major drawback in the use of purified CYP proteins is their need for the presence of their redox partner, cytochrome P450 reductase. To overcome this problem, the protein used in this work is a chimera obtained by the "molecular Lego" approach [11, 12] from the fusion of CYP3A4 and the reductase domain of the bacterial BM3 (BMR) [13]. This chimera, CYP3A4-BMR, was originally constructed based on the high sequence similarity between the BMR and the mammalian cytochrome P450 reductase and has been shown to support the activity of mammalian cytochrome P450s [14-18]. This self-sufficient chimera only requires the addition of the NADPH as the electron donor for its activity.

In vitro inhibition experiments were carried out with CYP3A4-BMR chimera and three different dietary food components namely; grapefruit juice, curcumin and resveratrol. In all cases, erythromycin was used as the substrate and the inhibition of its N-demethylation by CYP3A4-BMR was investigated in the presence of the three different food components. In addition, the *in vitro* experiments were carried out both in solution with the addition of NADPH (as the electron donor) as well as with the chimera immobilised on glassy carbon electrodes modified with PDDA (poly(diallyldimethylammonium chloride). In the latter electrochemical set up, the electrode was the source of electrons and therefore no NADPH addition was required.

As mentioned earlier, grapefruit juice is a known inhibitor of CYP3A4 [1] and was used as "proof-of-principle" for our experimental set up both for solution studies as well as the bioelectrochemical ones. Curcumin, the second food component to be tested, is the principal curcuminoid of turmeric (Curcuma longa), a member of the ginger family and found in curry powder. It is a diarylheptanoid, belonging to the group of curcuminoids, which are natural phenols responsible for turmeric's yellow colour. It is sold as an herbal supplement and food flavouring and colouring. The third food substance

tested is resveratrol (3,5,4'-trihydroxy-trans-stilbene), which is a natural phenol, and a phytoalexin produced by plants in defence to pathogens, such as bacteria or fungi. Sources of resveratrol in food include grapes (red wine), different berries and also peanuts.

Finally, the IC_{50} data calculated for the inhibition of the demethylation activity CYP3A4-BMR both in solution and immobilised on electrode surfaces, by grapefruit juice, curcumin and resveratrol were analysed and compared.

2. Methods and materials

2.1. Reagents

All chemicals and reagents including erythromycin, curcumin, resveratrol, NADPH and PPDA were purchased from Sigma-Aldrich (Italy) and used as received.

2.2. CYP3A4-BMR expression and activity

The CYP3A4-BMR chimera was expressed in *E. coli* DH5a and purified as previously described [13]. After growth the cells were harvested and stored at -20 ºC until purification. All purification steps were performed on ice or at 4 ºC. The harvested cells were sonicated and the membrane fractions solubilized by the addition of surfactant (octylphenoxypolyethoxyethanol) after clarification by ultracentrifugation. The protein was then purified by a two-step chromatography procedure including diethylaminoethyl (DEAE-Sepharose) followed by hydroxylapatite [13].

2.3. Preparation of enzyme electrodes

Direct electrochemistry of CYP3A4-BMR was achieved by immobilisation on glassy carbon (GC) electrodes (0.07 cm^2 - BASi, UK) polished with aqueous slurries of alumina and then by sonication. Subsequently the GC electrodes were modified with the polymer PDDA in a 1:1 mixture of PDDA: protein (15 μM in 100 mM potassium phosphate buffer pH 7.4) and allowed to set overnight at 4°C.

The protein film was rinsed with 100 mM potassium phosphate buffer pH 7.4 containing 100 mM potassium chloride prior to use it as previously reported [19, 20].

2.4. Kinetic Measurements

In the process of N-demethylation of drugs by CYP enzymes, formaldehyde is formed which can react with a particular reagent (NASH), leading to the formation of 3,5-diacyl-dihydrolutidine (DDL). The reaction is carried out at 50 ºC for 30 minutes, at the end of which the DDL can be detected spectrophotometrically based on the Hantzsch reaction, as it has a maximum absorption at 412 nm. Therefore, the N-demethylated product of erythromycin formed during chronoamperometric experiments with immobilised P450 CYP3A4-BMR was measured spectrophotometrically (HP 8452A diode array, Agilent Technologies, Italy) at 412 nm after incubation with NASH reagent [21]. To calculate the formaldehyde concentration that is formed during the reaction, a standard curve was constructed by incubating 250 μL standard solutions containing a range of formaldehyde concentrations (0–400 μM) with 250 μL Nash reagent for 30 min at 50 ºC and measuring the absorbance at 412 nm after the reaction had cooled [13].

In this work stocks were prepared with different percentages of grapefruit juice diluted in the reaction buffer, starting from one of the most common varieties of the fruit purchased (white grapefruit). Using a fixed concentration of 250 μM of substrate and increasing concentrations of grapefruit juice, the decrease in CYP3A4-BMR capacity of N-demethylation of erythromycin, and therefore the decrease in absorption at 412 nm, corresponding to the decrease in product formation was measured.

The second inhibitor tested with this system is curcumin. The experiment was conducted using an erythromycin concentration of 250 μM and increasing the inhibitor concentration in a range from 0 to 80 μM of curcumin.

The protein was used at a concentration of 0.5 μM in a final reaction volume of 300 μl, in the presence of erythromycin (250 μM) and increasing concentrations of resveratrol, from 0 to 1 mM. A pre-incubation of the inhibitor with the enzyme of 15 min in the presence of 1.5 mM NADPH was carried

out, following which the substrate and more NADPH were added. For each inhibitor concentration, the corresponding control was carried out in the absence of protein.

For the inhibition experiments, the amount of erythromycin substrate was kept constant (250 μM) whilst varying the concentration of the inhibitory food component: grapefruit juice (0- 5%), curcumin (0-300 μM), resveratrol (0-1000 μM).

The IC_{50} values were calculated by plotting the residual activity (%) as function of the logarithm of the substrate concentration added. Data were fitted to non-linear regression with SigmaPlot software (SYSTAT software, USA) using the sigmoidal equation to determine IC_{50} values [8]. All inhibition experiments were carried out in triplicates and the mean values were plotted with error bars indicative of the standard error of the mean of the three separate measurements.

2.5. Bioelectrochemistry

The electrochemical experiments were performed using an AUTOLAB PGSTAT 12 potentiostat (Eco Chemie -Netherlands). Measurements were carried out as described previously [8] with a three-electrode system with a platinum wire as the counter electrode, an Ag/AgCl (3M KCl) as the reference and the GC electrode as the working. For cyclic voltammetry (CV) experiments the potential range 0f -0.6 to +0.2 V (vs Ag/AgCl) was chosen. The measurements were performed in 100 mM potassium phosphate buffer pH 7.4 containing 100 mM potassium chloride.

Chronoamperometric measurements were carried out in fully oxygenated, constantly stirred electrochemical cell containing 100 mM potassium phosphate buffer pH 7.4 and 100 mM potassium chloride at 37°C for 30 min [8]. For catalysis experiments an excess of marker substrate, erythromycin (500 μM) was used and the reactions were conducted for 30 min with the bias potential of -650 mV (vs Ag/AgCl). Control experiments were carried out in the absence of the substrate. As for the catalysis described above, also in this case, at the end of the 30 minute reaction the solution containing the product was recovered from the electrochemical cell and incubated with the Nash reagent. The decrease in the erythromycin N-demethylation activity of immobilised CYP3A4-BMR, in response

to the presence of the tested inhibitor in the reaction mixture was then monitored. The inhibition studies were carried out with the reaction conditions applied to the experiments performed in solution, in terms of substrate and inhibitor concentrations, pre-incubation times with the inhibitor and reaction times.

3. Results and discussion

3.1. Activity of purified chimeric CYP3A4-BMR protein

The chimeric protein was expressed and purified as previously described [13] and its concentration measured using the Omura and Sato method [22]. As a result, the yield was calculated to be 30 mg per litre of culture, a value in line with the already published data [13]. Figure 1 shows the SDS-PAGE gel of the purified CYP3A4-BMR with the expected molecular weight.

To characterise the activity of the purified chimera, a colorimetric assay was performed, usually used for recombinant or microsomal isoforms of human CYP3A4 *in vitro* [23-25]. What is measured in this assay is the N-demethylation activity of the CYP3A4 marker substrate, the macrolide antibiotic erythromycin. The catalytic activity of CYP3A4-BMR chimera was performed in solution by simply adding NADPH since the chimera is a catalytically self-sufficient enzyme. As mentioned in the methods section, a peak absorbance was observed at 412 nm confirming the N-demethylation of erythromycin and hence the catalytic activity of the purified protein (Fig. 2). Using the standard curve, the efficiency of CYP3A4-BMR in catalysing the N-demethylation reaction at increasing concentrations of erythromycin was determined resulting in the Michaelis-Menten kinetic parameters with a K_M value of 95 ± 11 μM and a Vmax of 1.93 ± 0.07 μM. These values are within the range found in the literature [13, 26].

3.2. Inhibition of CYP3A4-BMR catalytic activity by different dietary substances

Similar to drug-drug interactions of CYP3A4, drug-food interactions can also represent a serious clinical problem, causing adverse reactions or toxicity in the humans. To this end, the self-sufficient CYP3A4-BMR chimera was used to evaluate these possible interactions in solution, using increasing

concentrations of the three food components under study with constant concentration of the substrate erythromycin.

As mentioned earlier, the most recognised dietary substance known to cause drug-food interactions was identified as grapefruit juice in late 80s when it was shown to increase the bioavailability of many drugs, including erythromycin, by inhibiting CYP3A4 [1, 9]. The inhibition of the N-demethylation activity of the CYP3A4-BMR chimera was therefore studied by addition of increasing concentrations of grapefruit juice as detailed in the methods section. The results obtained are shown in Figure 3A, where a strong inhibition of the CYP3A4-BMR activity is seen at very low percentages of grapefruit juice with total inhibition reached at 2% grapefruit juice. In addition, based on percentages of grapefruit juice added, the IC_{50} was also calculated from the sigmoidal curve shown in Figure 5A and was found to be 0.5%.

The results obtained cannot be directly compared to available literature data since the majority of *in vitro* data concerning grapefruit juice are carried out with single compounds extracted from grapefruit which are considered to be responsible for the inhibition [27]. A number of constituents have been proposed to be involved in the interaction between grapefruit juice and drugs including furanocoumarins and naringin.

In 2007 Girennavar and colleagues reported the percentage inhibition of CYP3A4 activity against luciferin by 8 grapefruit varieties [28]. In all cases, the concentration of grapefruit juice that caused the maximum inhibition was around 25%, and the activity was halved to around 1%.

The second dietary substance to be tested was curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl) -1,6-heptadiene-3,5-dione], a polyphenolic component of turmeric (Curcuma longa of the ginger family, the roots of which are used in cooking). As mentioned in the methods section, the inhibition experiments were conducted using increasing concentrations of curcumin in a range from 0 to 300 μM. The results are shown in Fig. 3B and confirm the strong inhibitory potential of this compound towards CYP3A4. Appiah-Opong and co-wokers have reported that curcumin competitively inhibits

the activity of CYP3A4 towards benzyloxyresorufin and dibenzylfluorescein with an IC_{50} values of 16.7 μM and 13.9 μM, respectively [29].

As in the case of grapefruit juice, the intake of certain quantities of curcumin can lead to an increase in the plasma concentration of many drugs. It appears, however, that there is no major interaction in the liver, as pharmacokinetic data have shown a low exposure of the liver to curcumin, even when administered in high doses. A concentration of curcumin in the blood of the order of nanomoles per litre was found [30], which suggests that the IC50 value found in this experiment is too high to have an important clinical significance, at liver level.

Curcumin, in addition to being widely used in cooking as a spice (especially in India, being the main ingredient of curry), for years has been known for its multiple antioxidant, anti-inflammatory, and anticancer properties [31]. To this end, many studies have been conducted on humans with high dose preparations of this compound up to 8g /day, as curcumin is considered as a safe product for human consumption [32]. Given the inhibitory effect of this compound on the activity of the most important CYP regarding drug metabolism, as shown in Figure 5B, further studies should be carried out to weigh out the beneficial vs toxic effects which this dietary food product might have on different drug substrates of CYP enzymes.

The last compound to be tested was resveratrol (3,5,4 '- trihydroxystylbene), a non-flavonoid phenol. It is a natural molecule belonging to the class of phytoalexins, defence molecules produced by plants against pathogenic microorganisms such as fungi. The main source of resveratrol is red wine (Vitis vinifera, which can contain 0.6 to 0.8 mg/ml), but it can also be found in peanuts (0.02–1.79 mg/g) and berries. A glass of wine can contain up to 600–700 mg of resveratrol, the concentration depends on the fermentation time and is higher in red wine than in white. A number of studies have attributed the beneficial effects of moderate wine consumption to the presence of resveratrol. Many studies suggest that moderate alcohol consumption can reduce the incidence of coronary heart disease and cancer [33, 34].

Red wine, like grapefruit juice, has been previously shown to inactivate CYP3A4 in a time- and NADPH-dependent manner [35]. The magnitude of effect of red wine on the pharmacokinetics of CYP3A4 substrates may depend on both the amount and type of red wine consumed. Differentiating the effects of ethanol and wine components also poses a challenge. The red wine components resveratrol [36] has been shown to inhibit CYP3A4 in vitro in a mechanism-based manner.

Mechanism-based inhibition of CYP3A4 requires a substrate that forms a reactive intermediate, leading to enzymatic inactivation by modification of haem [37]. An unsaturated electron-rich molecule such as resveratrol, with two aromatic rings linked by an ethylene bridge, is a possible substrate for CYP3A4 which can inactivate the enzyme during catalysis. The hydroxylation and epoxidation of resveratrol are possible reactions that can occur at the enzyme active site. In support of the latter mechanism, inhibition of testosterone 6-β-hydroxylation activity of CYP3A4 by resveratrol has been reported where a pre-incubation of 20 minutes leads to around 59.4% inactivation [38].

Similar to the inhibition studies with grapefruit juice and curcumin, experiments were carried out with CYP3A4-BMR chimera this time using resveratrol but with a preincubation of 15 min before this inhibitor was added. The result obtained are shown in Figure 3C. As can be seen in the figure, resveratrol does not seem to be a strong inhibitor of N-demethylation activity of CYP3A4-BMR enzyme with a calculated IC_{50} value of 218.7 μM (Figure 5C). IC_{50} values in a range of 4 to 150 μM have been reported showing a certain variability also depending on the enzyme system used (human liver microsomes or supersomes) [36]. Even higher IC_{50} values have been reported by other CYPs isoforms, such as 1.2 mM for CYP1A2 [39].

3.3. Inhibition studies of the immobilized chimeric CYP3A4-BMR

Having determined that the CYP3A4-BMR chimera can be used for inhibition studies, another set of experiments were carried out this time replacing the need for NADPH addition by using an electrode to provide the required electrons. Our group has successfully demonstrated the use of immobilised

monooxygenases for exactly this purpose i.e. bypassing the need for NADPH [40, 41]. In order to immobilise the CYP3A4-BMR on glassy carbon electrodes, they were modified by PDDA as detailed in the methods section. We have also used this same surfactant for studying the drug-drug interactions of CYP3A4 [8].

Initially, cyclic voltammetry of the immobilised CYP3A4-BMR was performed in the presence and absence of erythromycin as shown in Figure 4. As can be seen in the figure the presence of oxygen and substrate results in a catalytic current when the cell is scanned from 0 to -0.6 V (Figure 4B). Subsequently, the ability of the immobilized CYP3A4-BMR to catalyse the erythromycin N-demethylation reaction was followed by chronoamperometry. A potential bias of -650 mV (vs Ag/AgCl) was applied to the electrochemical cell. After 30 minutes of reaction in the presence of erythromycin (500 μM), the N-demethylated product was measured using the same colorimetric assay which were performed for the previous studies with the protein in solution.

Subsequently, inhibition studies were carried out with the same set up as solution studies with the three dietary compounds. The data are shown in Figure 5 (right panel).

Finally, the IC_{50} values calculated were 0.5%, 31 μM and 250 μM for grapefruit juice, curcumin and resveratrol measured in solution compared to 0.7%, 24 μM and 208 μM measured electrochemically, respectively. It is also evident that the inhibition of CYP3A4-BMR by the three food components is dose-dependent, in each case there is a decrease in the activity of the chimera proportional to the increase in the amount of the inhibitor.

4. Conclusions

Food-drug interactions using the self-sufficient chimera, CYP3A4-BMR, were investigated by *in vitro* inhibition assays both in solution and while the chimera was immobilised on electrode surfaces. In the solution studies, the cofactor NADPH was added as the source of the electrons for the catalytic activity of the enzyme to proceed. Using the immobilised enzyme, the requirement for the expensive NADPH cofactor was removed since the electrons were provided directly by the electrode. In

addition, inhibition studies of the CYP3A4-BMR chimera were carried out and compared with the two different approaches using the three different selected dietary substances.

In all three cases using the two different *in vitro* approaches it was found that the CYP3A4-BMR is indeed inhibited by the food components to various degrees. In general, the data obtained are within the range of published values (where available) and demonstrate the potential of using *in vitro* approaches instead of the microsomal systems which are more difficult to manipulate.

Finally, although the clinical implications of food-drug interactions are recognised worldwide by regulatory agencies, these interactions remain a relatively understudied area and the *in vitro* methodologies outlined in this work could help provide new approaches for studying these interactions.

Notes

The authors declare no competing financial interest.

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Figure captions

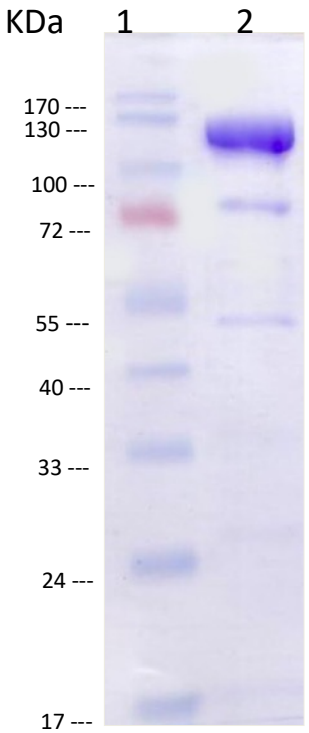
Figure 1: 7.5% SDS-PAGE gel of the purified CYP3A4-BMR. Lane 1: the molecular weight marker and Lane 2: purified CYP3A4-BMR chimera with a molecular weight of around 120 kDa.

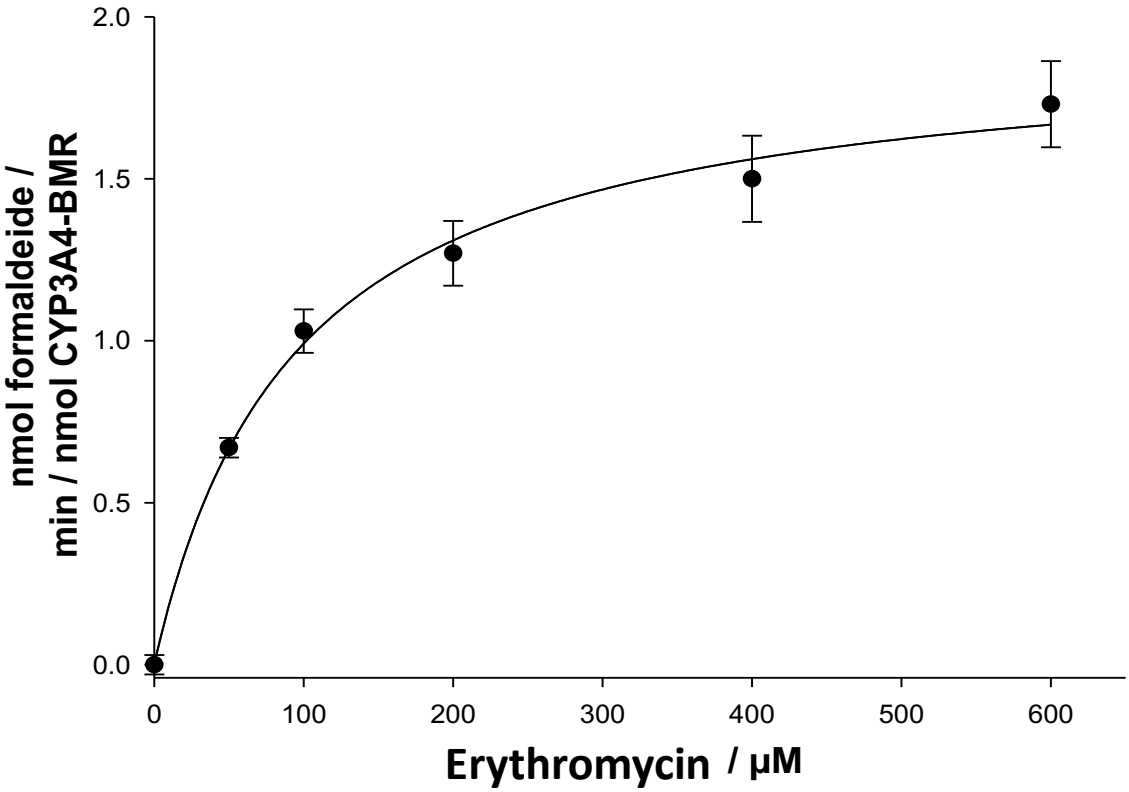
Figure 2: The effect of varying erythromycin concentration on the erythromycin N-demethylase activity of purified CYP3A4-BMR chimera. The error bars correspond to the standard deviation calculated from the mean of three different experiments.

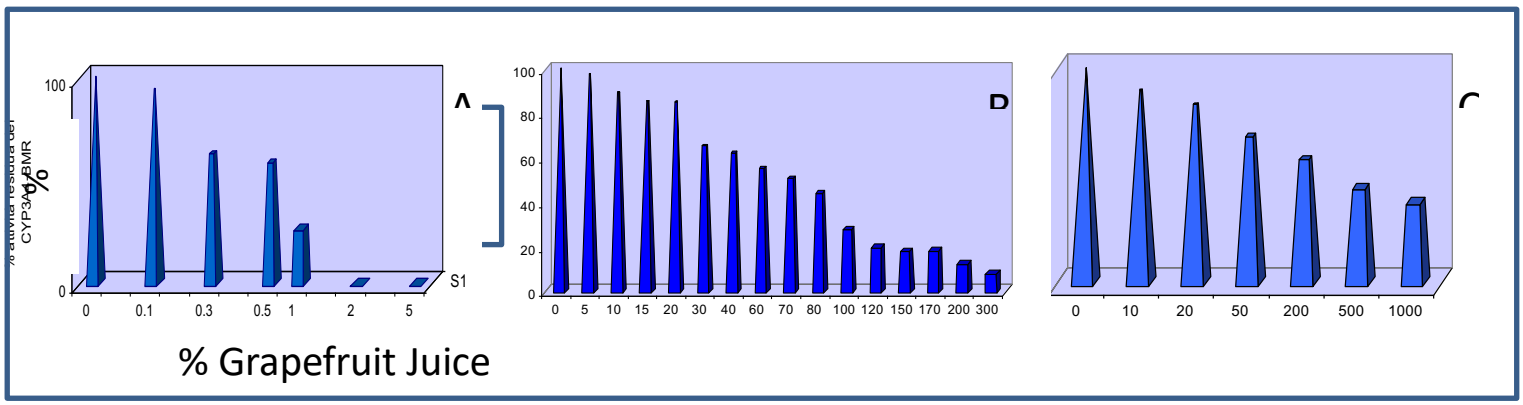
Figure 3: Percentage residual N-demethylase activity of purified CYP3A4-BMR chimera with increasing concentrations of the three dietary food substances: Grapefruit juice (A), curcumin (B) and resveratrol (C).

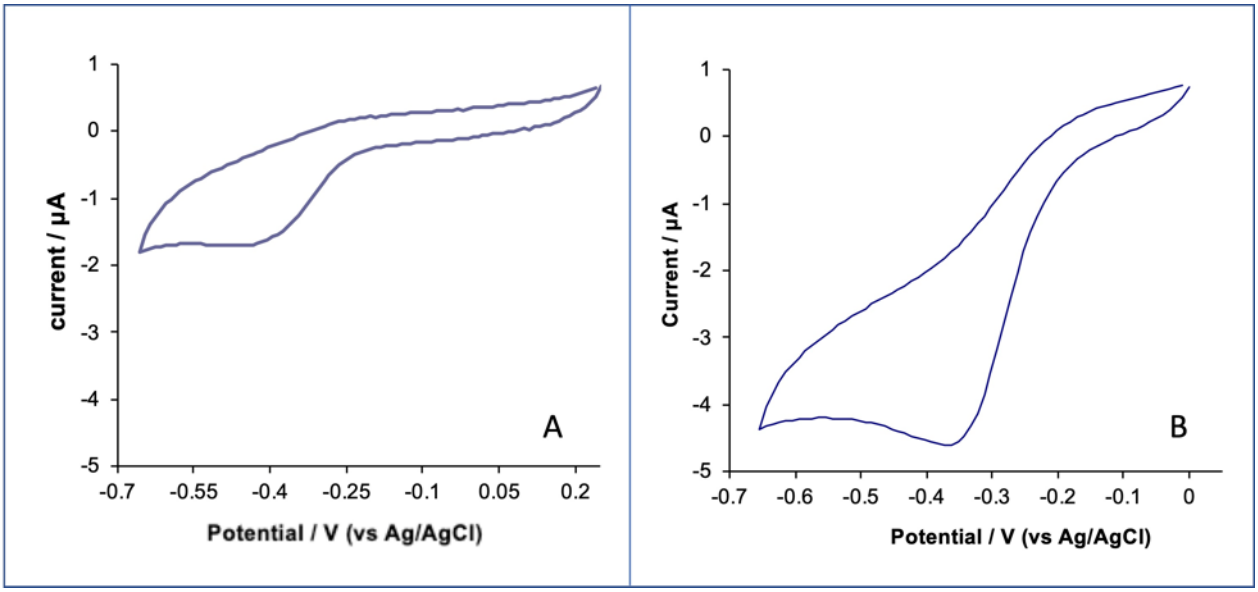
Figure 4: Cyclic voltammograms of CYP3A4-BMR on GC electrode modified with PDDA in the absence (A) and the catalytic current in the presence of erythromycin and oxygenated buffer (B). Scan rate 50 mVs^{-1} in 100 mM potassium phosphate buffer with 100 mM KCl, pH 7.4 at 25ºC.

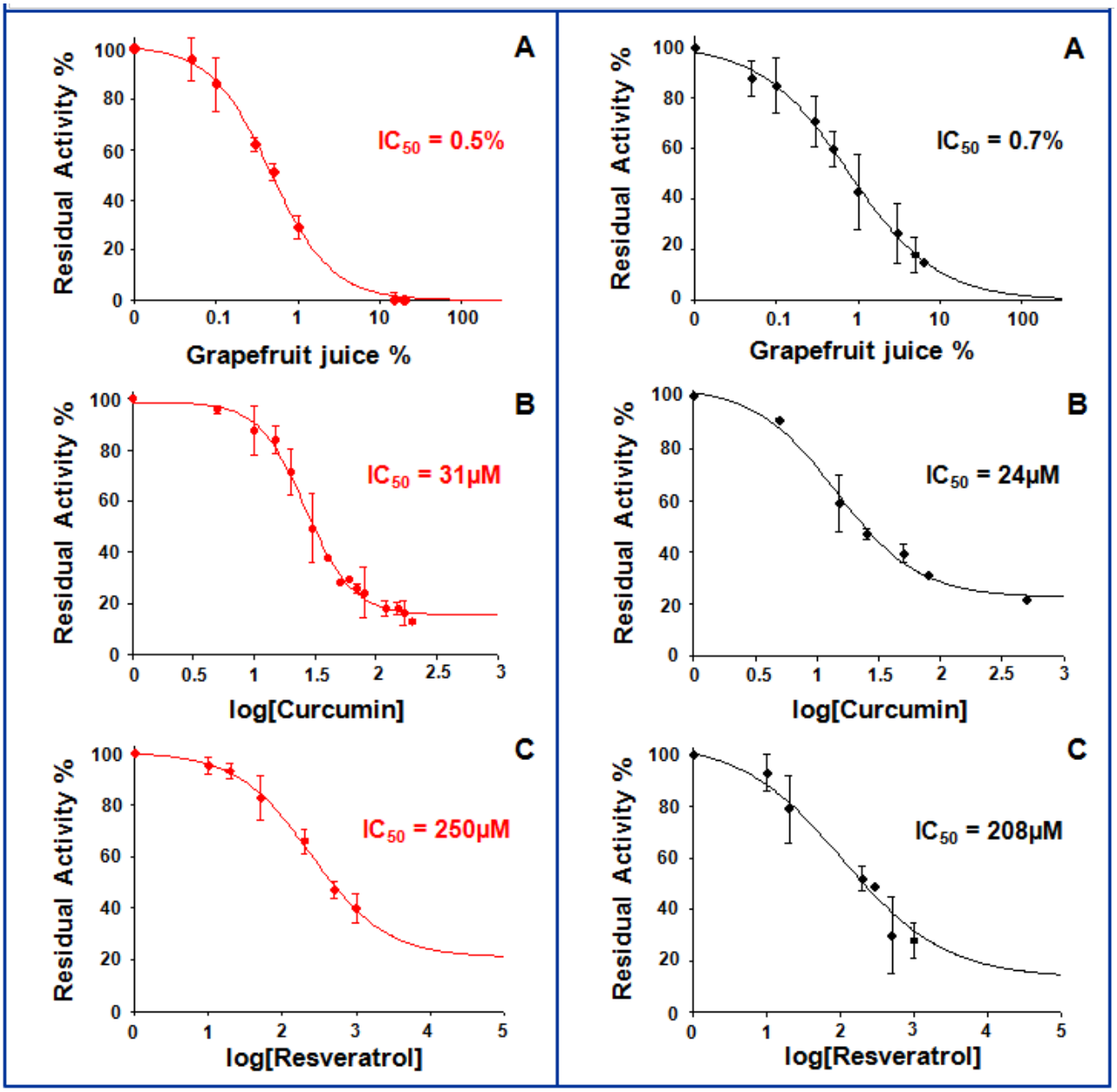
Figure 5: Concentration-dependent inhibition of erythromycin N-demethylation of purified CYP3A4-BMR chimera in solution (Left panel) and immobilised on glassy carbon electrode (Right panel) by: Grapefruit juice (A), curcumin (B) and resveratrol (C) and the calculated IC_{50} values. Error bars indicate the standard error of the mean of three separate measurements.


KDa
1
2
170 ---
130 ---
100 ---
72 ---
55 ---
40 ---
33 ---
24 ---
17 ---


2.0
1.5
1.0
0.5
0.0
nmol formaldeide / min / nmol CYP3A4-BMR
0
100
200
300
400
500
600
Erythromycin / µM


% activity residual del CYP3A4 BMR
%
100
0
0 0.1 0.3 0.5 1 2 5
S1
A
100 80 60 40 20 0
0 5 10 15 20 30 40 60 70 80 100 120 150 170 200 300
B
0 10 20 50 200 500 1000
C
% Grapefruit Juice


current / µA
1
0
-1
-2
-3
-4
-5
-0.7
-0.55
-0.4
-0.25
-0.1
0.05
0.2
Potential / V (vs Ag/AgCl)
A
Current / µA
1
0
-1
-2
-3
-4
-5
-0.7
-0.6
-0.5
-0.4
-0.3
-0.2
-0.1
0
Potential / V (vs Ag/AgCl)
B


A
Residual Activity %
IC50 = 0.5%
Grapefruit juice %
B
IC50 = 31μM
log[Curcumin]
C
IC50 = 250μM
log[Resveratrol]
A
IC50 = 0.7%
B
IC50 = 24μM
C
IC50 = 208μM