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EVALUATION OF 3-*O*-METHYLFLUORESCEIN AS A SELECTIVE FLUOROMETRIC SUBSTRATE FOR CYP2C19 IN HUMAN LIVER MICROSOMES

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Running title page

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A list of non-standard abbreviations used in the paper:

HLM, human liver microsomes; $NADP^+$, β-Nicotinamide adenine dinucleotide phosphate; OMF, 3-*O*-methylfluorescein; DDI, drug-drug interactions; CYP, cytochrome P450; FL, fluorescein; CEC, 3-cyano-7-ethoxycoumarin; AMMC, 3-[2-(*N*,*N*-diethyl-*N*-methylammonium)-ethyl]-7-methoxy-4-methylcoumarin; MAMC, 7-methoxy-4-(aminomethyl)-coumarin; MAA, 9-*N*-(methylamino)acridine; DFB, 3-[(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(5H)-one ; DBF, dibenzylfluorescein

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Abstract:

Cytochrome P450 (CYP) fluorometric high throughput inhibition assays have been widely used for drug-drug interaction (DDI) screening particularly at the pre-clinical drug discovery stages. Many fluorometric substrates have been investigated for their selectivity, but most are found to be catalyzed by multiple CYP isozymes, limiting their utility. In this study 3-*O*-methylfluorescein (OMF) was examined as a selective fluorescence substrate for CYP2C19 in human liver microsomes (HLM). The kinetic studies of OMF *O*-demethylation in HLM using a LC/MS method exhibited two-enzyme kinetics with apparent K_m and V_{max} values of 1.14 ± 0.90 μM and 11.3 ± 4.6 pmol/mg/min respectively for the high affinity component(s), and 57.0 ± 6.4 μM and 258 ± 6 pmol/mg/min respectively for the low affinity component(s). Studies utilizing cDNA-expressed individual CYP isoforms and CYP-selective chemical inhibitors demonstrated that OMF *O*-demethylation to fluorescein (FL) was selective for CYP2C19 at substrate concentrations $\leq$ 1 μM. At substrate concentrations $\geq$ 10 μM, other CYP isozymes were found to catalyze OMF *O*-demethylation. In HLM, analysis of the two-enzyme kinetics in the presence of CYP isozyme-selective chemical inhibitors (ticlopidine for CYP2C19, sulfaphenazole for CYP2C9, and furafylline for CYP1A2) indicated that CYP2C19 was the high affinity component and CYP2C9 the low affinity component. On the basis of these findings, a fluorometric assay was developed using 1 μM OMF and 2 μM sulfaphenazole for probing CYP2C19-mediated inhibition in HLM. The IC_{50} data of thirteen substrates obtained from the fluorometric assay developed in this study correlated well with that reported in the literature using non-fluorescence assays.

Introduction

Understanding of the potential for drug-drug interactions (DDI) has emerged as an important safety consideration during drug discovery and development [Somogyi and Muirhead, 1987; Jurima-Romet et al., 1994; Michalets, 1998; Ito et al., 1998; Lin and Lu, 1998; Tucker et al., 2001; Yu et al., 2001]. Drug-drug interactions can occur through gastrointestinal absorption, plasma and/or tissue protein binding, distribution, excretion, and metabolism. One major route of metabolism-based DDI is through cytochrome P450 enzymes (CYP), which can be inhibited, activated, or induced by concomitant drug treatment. Many *in vitro* assays have been developed to evaluate the potential of DDI via CYP inhibition using a variety of CYP systems [Moltke et al., 1994; Crespi et al., 1997; Stresser et al., 2000; Bapiro et al., 2001; Dierk et al., 2001; Chauret et al., 2001; Yan and Caldwell, 2001; Stresser et al., 2002; Wienkers, 2002; Cohen et al., 2003; Weaver et al., 2003; Yao et al., 2003; Andersson et al., 2004; Yamamoto et al., 2003; Walsky and Obach, 2004; Atkinson et al., 2005]. Fluorescence-based CYP inhibition assays have been adopted by many laboratories during drug discovery to evaluate CYP-mediated DDI potential because of their high throughput capacities and simplicity. Many of the fluorescence substrates have been characterized in cDNA expressed CYP systems [Crespi et al., 1997; Stresser et al., 2000; Stresser et al., 2002]; however, their utility in the human liver microsomes (HLM) is limited due to the lack of specificity toward specific CYPs.

The substrate typically used in a fluorometric inhibition assay for CYP2C19 is 3-cyano-7-ethoxycoumarin (CEC). However, CEC also exhibits catalytic activity toward CYP1A1, 1A2, 1B1, 2B6, and 2E1 [Stresser et al., 2002; Ghosal et al., 2003], and is therefore unsuitable as a selective substrate for CYP2C19. The purpose of this study was to characterize the enzyme kinetics and the specificity of *O*-demethylation reaction of 3-*O*-methylfluorescein (OMF) in both

c-DNA expressed CYP isozyme systems and HLM. On the basis of these findings, a fluorometric assay using OMF was developed as a selective substrate for probing CYP2C19 mediated DDI.

Materials and Methods

Materials. OMF, (+)-*N*-3-benzyl-nirvanol, cDNA-expressed human CYPs: 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5 were purchased from BD Biosciences (Woburn, MA). Pooled HLM (n=20 donors of mixed gender) at 20 mg/mL protein concentration were purchased from Xenotech LLC (Lenexa, KS). Acetonitrile (HPLC grade) was obtained from EMD Chemicals Inc. (Gibbstown, NJ). Magnesium chloride hexahydrate was obtained from JT Baker (Phillipsburg, NJ). Amitriptyline, felbamate, fluvoxamine maleate, fluorescein (FL) sodium salt, fluoxetine, furafylline, glucose 6-phosphate, β-Nicotinamide adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate dehydrogenase, imipramine, ketoconazole, labetalol hydrochloride, lansoprazole, omeprazole, progesterone, quinidine, sulfaphenazole, ticlopidine, tranylcypromine, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Linearity and Enzyme Kinetics of OMF *O*-demethylation by CYP2C19 in HLM. The HLM incubations were carried out in a total volume of 200 µL. The reaction mixtures containing NADPH-regenerating system (1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride) in 50 mM potassium phosphate buffer (pH 7.4) were pre-incubated at 37 ºC for 10 min. In the enzyme kinetic experiments, 2 µL of OMF in acetonitrile stock solutions (50 – 12000 µM) was added into the reaction mixtures. The reactions were initiated by the addition of HLM (0.5 mg/mL final protein concentration) followed by incubation at 37 ºC. Before running the enzyme kinetics for the formation of FL (OMF *O*-demethylation metabolite), the linearity of the FL formation was determined by incubating 0.5 µM OMF with varying amounts of microsomal protein (0.3-2.35 mg/mL). The time-dependency of FL formation was also investigated by incubating 0.5 µM

OMF with 0.5 mg/mL microsomal protein followed by sample drawing at 5, 10, 15, 30, 45 and 60 min. The optimal protein concentration and incubation time were chosen to be 0.5 mg/mL and 30 min respectively for the enzyme kinetic experiments. The reactions were terminated by the addition of 600 µL of ice-cold 50% v/v acetonitrile/water containing 0.4 µM labetalol as an internal standard for LC/MS analysis. The reaction mixtures were then centrifuged in Heraeus Biofuge Pico (Kendro Laboratory Products, Newtown, CT) at 14443 *g* for 5 min and the resultant supernatant (20 µL) analyzed by LC/MS analysis. The kinetic parameters were calculated from untransformed data by non-linear regression using Prism® software (GraphPad Software Inc., San Diego, CA).

Incubation with cDNA-expressed Human CYP Isozymes. CYP2C19 selectivity for the OMF substrate was demonstrated by performing incubations with eleven cDNA-expressed human CYP isozymes namely 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5. The reaction mixtures were prepared as previously described in the HLM incubation assay except that the OMF concentrations employed were 1 µM and 40 µM and the HLM were substituted with each of the eleven cDNA-expressed CYP isozymes. For the CYP isozyme whose activity was derived from the same marker substrate used by the two suppliers (cDNA-expressed CYP isozymes from BD Biosciences vs. HLM from Xenotech LLC), the concentration of cDNA-expressed CYP isozyme in the incubations was chosen to have approximate activity as HLM incubations (Table 1). All the other isozyme concentrations were chosen to reflect the relative abundance of CYPs present in human liver (Williams, 2002) (Table 2).

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Inhibition of CYP Isozymes by Selective Chemical Inhibitors. The involvement of CYP2C9, CYP2C19 and CYP1A2 in OMF *O*-demethylation was evaluated using CYP2C19, CYP2C9 and CYP1A2 selective inhibitors. The incubations were performed by pre-incubating HLM with the NADPH-regenerating system in 50 mM potassium phosphate buffer (pH 7.4) and 2 μM sulfaphenazole (CYP2C9 competitive selective inhibitor) or 10 μM furafylline (CYP1A2 mechanism-based selective inhibitor) or 5 μM (+)-*N*-3-benzyl-nirvanol (CYP2C19 competitive selective inhibitor) at 37 ºC for 10 min. In order to remove the activity of CYP2C19, 100 μM ticlopidine was pre-incubated with the HLM and NADPH regenerating system for 30 min at 37 ºC. All reactions were initiated by the addition of OMF in acetonitrile stock solutions to give final concentrations ranging from 0.5 to 120 μM. In addition to the concentration ranges of OMF, incubations of OMF at 1 and 10 μM in the presence or absence of CYP2C19, CYP2C9 and CYP1A2 selective inhibitors, either alone or in combination, were conducted to further identify the enzymes involved in the *O*-demethylation reaction. The reactions were terminated as previously described in the HLM incubation experiment and analyzed by LC/MS.

LC/MS Analysis. The LC/MS system was comprised of an Agilent 1100 series binary pump (Agilent Technologies Inc., Santa Clara, CA), a CTC Leap PAL autosampler (Leap Technologies, Carrboro, NC), and either an API3000 or an API4000 triple quadrupole mass spectrometer (Applied Biosystems/Sciex, Foster City, CA) equipped with a Valco® valve (VICI Valco Instruments, Houston, TX), and an electrospray ionization (ESI) interface operated in positive ion detection mode. The LC/MS system was controlled by Analyst® software version 1.4.1(Applied Biosystems/Sciex). The HPLC column used was a YMC ODS-AQ (3 μm, 120 Å, 4.6 mm x 50 mm, Waters Corporation, Milford, MA). The linear gradient elution was performed

using the following mobile phase systems at a flow rate of 0.5 mL/min; solvent A contained 5 mM ammonium acetate buffer (pH 4.5) and solvent B was acetonitrile. The gradient program initiated at 30% B ramping to 90% B over 4 min and then held for 2 min before returning to the initial starting conditions. The LC eluent for the first 1.5 min was diverted to waste. The samples were analyzed for FL formation by LC/MS. The mass spectrometer was operated in Q1 multiple ion mode at 332.90 m/z for FL and at 328.90 m/z for labetalol (internal standard). The standard curve was prepared by spiking 10 µL of FL solution at concentrations ranging from 0.39 µM to 100 µM into 190 µL of blank incubation mixture without the NADPH regenerating system components and then immediately quenching the reaction by addition of 600 µL ice-cold 50% v/v acetonitrile/water containing 0.4 µM labetalol as internal standard. The standard solutions were then treated as previously described in the HLM incubation assay and analyzed by LC/MS.

IC_{50} Determination by Fluorometric Assay. The FL fluorescence detection sensitivity was evaluated by spiking FL into the blank incubation mixture at a final concentration range of 0.32 nM – 2 µM. The blank incubation mixture contained pre-incubated microsomal protein with NADPH-regenerating system at 37 ^{o}C for 30 min. The incubation was terminated by the addition of 75 µL of ice-cold 2N sodium hydroxide in water followed by the addition of 2 µL of OMF in acetonitrile stock solution to give 1 µM final concentration. Fluorescent signals from the blank incubation mixture without FL were then acquired and subtracted from the signal of the blank incubation mixture spiked with FL. The IC_{50} fluorometric assay was conducted in black Costar 96-well plates (Corning Incorporated, Corning, NY) at a total incubation volume of 200 µL. The 96-well reaction plate layout is shown in Fig. 1. The reaction mixtures in the wells from column 3 to 12 consisted of NADPH-regenerating system in 50 mM potassium phosphate buffer (pH

7.4), 0.5 mg/mL microsomal protein, 2 μM sulfaphenazole, and thirteen substrates (0.75 μL from acetonitrile stock solution) serially diluted 3-fold with the final concentration range of 0.045 -100 μM for ketoconazole and quinidine, 0.041 - 90 μM for tranylcypromine, 0.036 – 80 μM for omeprazole, 0.022 – 50 μM for probenecid, 0.013 – 30 μM for lansoprazole, 0.0022 – 5 μM for fluvoxamine, 0.17 – 125 μM for felbamate, 0.069 to 150 μM for imipramine, 0.006 to 12.5 μM for amitriptyline, (+)-3-*N*-benzyl-nirvanol and progesterone, and 0.0011 to 2.5 μM for fluoxetine. The blank incubations in wells A1-D1 contained 0.5 mg/mL microsomal protein in 50 mM potassium phosphate buffer (pH 7.4), NADPH-regenerating system, CYP2C9 selective chemical inhibitor (i.e., 2 μM sulfaphenazole) and CYP2C19 chemical selective inhibitor (i.e., 100 μM ticlopidine). The control incubations without inhibitors in wells E1-F1 contained only NADPH-regenerating system and 0.5 mg/mL microsomal protein in 50 mM potassium phosphate buffer (pH 7.4). The control incubations without OMF in wells G1-H1 contained the same components as the control incubations without inhibitors except for the addition of OMF substrate post-reaction termination. The control incubations in the presence of 2 μM sulfaphenazole were conducted in wells A2-H2. The 96-well plate was pre-incubated at 37 °C for 30 min. The reactions were initiated by the addition of 1 μM OMF and incubated at 37 °C for 30 min. For the IC_{50} experiments with imipramine, amitriptyline, (+)-3-*N*-benzyl-nirvanol, progesterone, fluvoxamine, and fluoxetine, the substrates were pre-incubated along with ticlopidine. The rest substrates were added with OMF together to initiate the reaction. The reactions were terminated by the addition of 75 μL of ice-cold 2N sodium hydroxide in water. The fluorescence signals were measured at 30 min post-reaction termination to obtain sufficient signal above background. The fluorescence signal was measured using CytoFluor® 4000 TC Fluorescence Multi-Well Plate Reader (Applied Biosystems, Foster City, CA) with excitation

filter at 485 nm (bandwidth 20 nm) and emission filter at 530 nm (bandwidth 25 nm). The IC_{50} values were calculated as described by Eq. 1:

$$Y = Bottom + \frac{(Top - Bottom)}{(1+10^{((X - LogIC_{50}) \bullet n)})} \qquad \text{(Eq. 1)}$$

where X = logarithm concentration of a substrate, Y = fluorescence intensity, n = Hill slope, Top = fluorescence intensity of the sample obtained after the reaction is terminated in the absence of CYP2C19 inhibitor, and Bottom = fluorescence intensity of the sample obtained after the reaction is terminated in the presence of CYP2C19 inhibitor and CYP2C9 inhibitor.

Data Analysis. The kinetic parameters were determined from untransformed data by non-linear regression using Prism® software (GraphPad Software Inc., San Diego, CA). Data were fitted to the Michaelis-Menten equations for single- and two-enzyme models. The choice of the best-fit enzyme model was based on the examination of Michaelis-Menten plots and Eadie-Hofstee plots. When necessary, a statistical analysis (F test) was performed to determine whether there was a significant difference between the sums of squares between the two models (Motulsky and Ransnas, 1987). The equations for single- (Eq. 2) and two-enzyme (Eq. 3) models were:

$$V = \frac{V_{\max} \times S}{(K_m + S)} \qquad \text{(Eq. 2)}$$

$$V = \frac{V_{\max 1} \times S}{(K_{m1} + S)} + \frac{V_{\max 2} \times S}{(K_{m2} + S)} \qquad \text{(Eq. 3)}$$

where S = concentration of the substrate, V = rate of the product formation, K_m = Michaelis-Menten constant expressing the substrate concentration at half of V_{max}, V_{max1} = maximum rate of the product formation of the reaction catalyzed by enzyme 1, V_{max2} = maximum rate of the product formation of the reaction catalyzed by enzyme 2, K_{m1} = Michaelis-Menten constant

expressing the substrate concentration at half of V_{max1}, and K_{m2} = Michaelis-Menten constant expressing the substrate concentration at half of V_{max2}.

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Results

Kinetics Determination of OMF *O*-Demethylation in HLM. From Fig. 2, the kinetics for the formation of FL was optimized with respect to incubation time at 30 min and HLM protein concentration at 0.5 mg/mL. In the substrate-velocity analysis, the Eadie-Hofstee plot exhibited biphasic kinetic characteristic (Fig. 3 insert) over the OMF concentration range 0.5 – 120 μM, suggesting more than one enzyme was involved in OMF *O*-demethylation [Tracy and Hummel, 2004]. A comparison between non-linear data fitting for Michaelis-Menten single-enzyme and two-enzyme plots was performed and the F-test ($P<0.05$) indicated a significantly better fit with the two-enzyme model (Eq. 3). Using this equation, the kinetic parameters determined for OMF *O*-demethylation by HLM were K_{m1} = 1.14 ± 0.90 μM and V_{max1} = 11.3 ± 4.6 pmol/mg/min for the high-affinity enzyme(s) and K_{m2} = 57.0 ± 6.4 μM and V_{max2} = 258 ± 6 pmol/mg/min for the low-affinity enzyme(s).

OMF *O*-Demethylation by Recombinant Human CYP Isoforms. OMF concentrations of 1 μM and 40 μM were selected for the recombinant human CYP isoform experiment as these concentrations approximated the apparent K_{m1} and K_{m2} respectively obtained from the HLM kinetic study. At 1 μM OMF (Fig. 4 A), the FL formation was observed to be highly selective for CYP2C19. The reaction efficiency for CYP2C19 when normalized by the amount of CYP was about 3-fold higher than CYP1A1 and 9-fold or higher than the other CYP isozymes. However at 40 μM OMF, CYP1A2, CYP2C8, CYP2C9 and CYP2C19 all contributed to OMF *O*-demethylation (Fig. 4 B).

CYP Isozyme Selective Chemical Inhibition Studies. The effect of CYP isozyme- selective chemical inhibitors, ticlopidine and (+)-*N*-3-benzyl-nirvanol (for CYP2C19), sulfaphenazole (for CYP2C9) and furafylline (for CYP1A2) toward *O*-demethylation of OMF by HLM over the concentration range 0.5 – 120 μM OMF in HLM are shown in Figs. 5, 6 and 7 respectively and their associated kinetic parameters are summarized in Table 3. When (*S*)-mephenytoin was added to pre-incubated HLM, NADPH-regenerating system, and ticlopidine reaction mixture, >95% inhibition was observed for the formation of (*S*)-4'-hydroxy-mephenytoin, a reaction catalyzed by CYP2C19 (data not shown). Therefore the HLM pre-incubation with ticlopidine effectively removed CYP2C19 activity. When ticlopidine was added to inhibit the contribution of CYP2C19, the Eadie-Hofstee plot for OMF *O*-demethylation exhibited single-like enzyme characteristics over the concentration range 0.5 – 120 μM OMF (Fig. 5A). The apparent kinetic parameters obtained from the Michaelis-Menten single enzyme model were K_m = 103.9 ± 32.6 μM and V_{max} = 143 ± 25 pmol/mg/min. Similarly, when the CYP2C19 competitive selective chemical inhibitor, (+)-*N*-3-benzyl-nirvanol was added to the incubation, single-like enzyme characteristics were observed (Fig. 5B). The apparent kinetic parameters obtained from the Michaelis-Menten single enzyme model were K_m = 92.26 ± 8.09 μM and V_{max} = 442 ± 20 pmol/mg/min. In the presence of sulfaphenazole, the Eadie-Hofstee plot exhibited single-like enzyme characteristics over the OMF concentration range 0.5 – 10 μM with apparent K_m of 3.97 ± 0.28 μM and V_{max} of 22.8 ± 0.7 pmol/mg/min (Fig. 6). The apparent kinetic parameters obtained for OMF *O*-demethylation in the presence of furafylline were K_{m1} = 1.74 ± 1.79 μM and V_{max1} = 8.1 ± 4.0 pmol/mg/min for high affinity enzyme(s) and K_{m2} = 98.9 ± 43.4 μM and V_{max2} = 284 ± 41 pmol/mg/min for low affinity enzyme(s) (Fig. 7).

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The HLM incubations were also performed at 1 and 10 μM OMF using ticlopidine (100 μM), sulfaphenazole (2 μM), and furafylline (10 μM), alone or in combination. At 1 μM OMF, ticlopidine inhibited ~ 80% of OMF *O*-demethylation and sulfaphenazole contributed an additional ~ 6% when co-incubated with ticlopidine (Fig 8). At 10 μM OMF, ticlopidine inhibited ~ 65% of OMF *O*-demethylation, and sulfaphenazole contributed an additional ~ 8% inhibitory effect when co-incubated. Sulfaphenazole incubated alone resulted in ~ 44% and ~ 52% relative inhibitory effect toward OMF *O*-demethylation at 1 μM and 10 μM OMF respectively. Only ~ 10% relative inhibition effect was observed toward OMF *O*-demethylation when furafylline was added alone at 1 μM or 10 μM OMF. When furafylline was co-incubated with either ticlopidine, or sulfaphenazole, or both, there was no apparent additional inhibitory effect toward OMF *O*-demethylation at either 1 μM or 10 μM OMF.

IC_{50} Determination for CYP2C19 in HLM. The FL standard curve constructed with blank incubation mixture spiked with standard solutions of FL was linear over the concentration range (0.32 nM – 2 μM) with r^2 = 0.9987. Thirteen substrates were selected to evaluate OMF *O*-demethylation for use in IC_{50} determinations in HLM. The substrates were added to HLM incubation mixtures containing 1 μM OMF. The IC_{50} values of the substrates were determined and compared to literature values obtained by LC/MS method using (*S*)-mephenytoin as a substrate in HLM. The results are summarized in Table 4.

Discussion

At 1 μM OMF, greater selectivity of CYP2C19 toward *O*-demethylation of OMF was observed in a panel of eleven c-DNA expressed human CYP isozymes (see Fig. 4 A). These results agreed well with informal reports [Stresser, personal communication] that CYP2C19, CYP1A1, and to a lesser degree, CYP1A2 and CYP2C9 possessed OMF *O*-demethylase activities at similar experimental conditions. Since CYP1A1 is detected primarily in extra-hepatic tissues under induced conditions and not present in a significant amounts in HLM [Williams, 2002], it should not have contributed to OMF *O*-demethylase activity in the HLM experiments. When OMF concentration was increased to 40 μM, multiple enzymes significantly contributed to the *O*-demethylation of OMF (Fig. 4B). As observed in Fig. 4B, the magnitude of this increased activity differed between individual CYP isozymes: 1.7-fold for CYP2C19, 10-fold for CYP2C9, 36-fold for CYP1A2, and 41-fold for CYP2C8. On the basis of the Michaelis-Menten enzyme kinetics and the observed substrate concentration-isozyme activity relationship, CYP2C19 appeared to be the high affinity component, whereas CYP2C9, 1A2, and 2C8 could be considered the low affinity components in HLM.

In HLM incubations, a multiple-enzyme kinetics profile was observed as demonstrated by the atypical biphasic in the Eadie-Hofstee plot (Fig. 3). The finding of multiple enzyme involvement in the *O*-demethylation reaction of OMF in HLM was consistent with the data from the c-DNA expressed human CYP isozyme incubations. Studies with CYP isozyme selective chemical inhibitors in HLM demonstrated that CYP2C19 and CYP2C9 were the two major enzymes catalyzing the *O*-demethylation reaction, contributing to ~ 86% activity at 1 μM and ~ 73% activity at 10 μM OMF (Fig. 8). The percent activity inhibition by sulfaphenazole was greater at higher OMF concentrations, indicating CYP2C9 contributed more activity at higher

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OMF levels. These findings suggest CYP2C9 is the low affinity or the high K_m component of the characterized two-enzyme kinetics. When furafylline was added to HLM, the non-linearity in Eadie-Hofstee plot (Fig. 7) and the enzyme kinetics (Table 3) remained almost the same as without furafylline. Only ~ 10% inhibitory effect was observed when furafylline was added alone to 1 μM or 10 μM OMF and no significant additional inhibitory effect when furafylline was co-incubated with either ticlopidine, sulfaphenazole, or both. These data indicate that CYP2C9, rather than CYP1A2, was the low affinity component of the multiple-enzyme kinetics in catalyzing OMF *O*-demethylation in HLM incubations

Kinetic parameters for OMF *O*-demethylation in the absence and presence of isozyme selective chemical inhibitors for CYP2C19, 2C9 and 1A2 in HLM revealed K_m of the high affinity CYP (i.e., CYP2C19) was approximately 1 μM. The K_{m1} and V_{max1} for the high affinity enzyme obtained from the sulfaphenazole experiment was higher than the parameters obtained from the experiments performed in the absence of chemical inhibitors (Table 3). The use of a different lot of HLM in the sulfaphenazole experiment may have attributed to this discrepancy. The V_{max2} for the low affinity enzyme obtained from the ticlopidine experiment was lower than V_{max2} obtained from those experiments in the absence or presence of other chemical inhibitors (Table 3). The decrease of V_{max2} was more likely due to ticlopidine, a mechanism-based CYP2C19 chemical inhibitor [Ha-Duong et al., 2001]. As the mixture had to be pre-incubated for 30 minutes prior to initiating OMF *O*-demethylation reaction, some enzyme activity could be lost during the longer incubation time. Another reason, probably more likely, was due to the selectivity of ticlopidine. Ko et al. [2000] found that at 100 μM, ticlopidine also inhibited both CYP1A2 and CYP2C9 activities in HLM. Since CYP2C9 was involved in the OMF *O*-demethylation in HLM incubations, it was expected that V_{max} would decrease. When (+)-*N*-3-

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benzyl-nirvanol, a more selective competitive inhibitor of CYP2C19, was used in the inhibition study as shown in Table 3, V_{max2} did not decrease.

OMF was employed at 1 μM in the developed fluorometric assay as this concentration was selective toward OMF *O*-demethylation and also similar to the K_m of CYP2C19. Sulfaphenazole (2 μM) was added to the incubation mixture to minimize the effect of CYP2C9 activity. For thirteen substrates in which the CYP2C19 inhibition profile was characterized [Table 4], the IC_{50} data obtained from the fluorometric assay reported in this study correlated well with those reported in the literature using LC/MS with (*S*)-mephenytoin as a chemical substrate. Though the HLM incubation mixture in the chemical substrate approach did not contain sulfaphenazole (a CYP2C9 inhibitor), similar IC_{50} values were obtained from this fluorometric assay to those obtained from the chemical substrate approach. Ko et al. [1998] conducted experiments to investigate (*S*)-mephenytoin *N*-demethylation by CYP2C9 in HLM. They found that CYP2C9 was one of two enzyme components with apparent K_m of 174 μM and V_{max} of 170.5 pmol/min/mg protein toward (*S*)-mephenytoin *N*-demethylation, a minor route of (*S*)-mephenytoin metabolism in HLM compared with 4'-hydroxylation of (*S*)-mephenytoin. Since most of the data shown in Table 4 were conducted at lower concentration of (*S*)-mephenytoin (i.e., ~ 30 μM, K_m of CYP2C19 for 4'-hydroxylation of (*S*)-mephenytoin), the contribution of (*S*)-mephenytoin by CYP2C9 (a non-target enzyme) would be a minor component. The IC_{50} values determined for the thirteen substrates ranged from 0.47 μM to 108.7 μM demonstrating the wide dynamic range of the assay. There are a number of reports on the application of fluorescence assays for CYP-mediated drug inhibition studies in HLM: coumarin for CYP2A6 [Donato, et al., 2004], AMMC and MAMC for CYP2D6 [Chauret et al., 2001; Onderwater et al., 1999; Venhorst et al., 2000 (a); Venhorst et al., 2000(b); Yamamoto et al.,

2003;], MAA for CYP1A1 and CYP2D6 [Mayer et al., 2007]; DFB for CYP3A4 [Chauret et al., 1999; Nicoll-Griffith et al., 2004], and DBF for CYP3A4 [Ghosal et al., 2003]. Interestingly, the use of MAMC as a selective substrate for probing CYP2D6 inhibition in HLM involved the addition furafylline (selective CYP1A2 inhibitor) in the incubation [Venhorst et al., 2000 (a)], similar to our approach which required the addition of sulfapenazole (selective CYP2C9 inhibitor) in the incubation. Venhorst [2000(a)] reported MAMC *O*-methylation was catalyzed mainly by CYP2D6 and to a small extent by CYP1A2. Addition of furafylline completely eliminated the contribution of CYP1A2 toward MAMC *O*-methylation. To our knowledge this study represents the first reported utility of OMF in evaluating CYP2C19 mediated drug inhibition in HLM.

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Figure legends

Figure 1. 96-well plate layout for IC_{50} determination toward CYP2C19 using OMF as substrate in HLM incubations.

Figure 2. Effect of time (A) and effect of HLM protein concentration (B) to the rate of formation of OMF *O*-demethylation product, FL, in HLM incubations.

Figure 3. Kinetic analysis of OMF *O*-demethylation: Michaelis-Menten plot with non-linear regression for a two-enzyme system; and Eadie-Hofstee plot of transformed data from FL formation (insert).

Figure 4. The OMF *O*-demethylation activities of expressed CYP isozymes at OMF concentrations of 1 μM (A) and 40 μM (B).

Figure 5. Michaelis-Menten plots and Eadie-Hofstee plots (insert) for FL formation in the presence of CYP2C19 inhibitors: (A) 100 μM ticlopidine (a mechanism-based inhibitor) and (B) 5 μM (+)-*N*-3-benzyl-nirvanol (a competitive inhibitor) in HLM incubations.

Figure 6. Michaelis-Menten plots and Eadie-Hofstee plots (insert) for FL formation in the presence of 2 μM sulfaphenazole (CYP2C9 inhibitor) in HLM incubations.

Figure 7. Michaelis-Menten plots and Eadie-Hofstee plots (insert) for FL formation in the presence of 10 μM furafylline (CYP1A2 inhibitor) in HLM incubations.

Figure 8. The effect of ticlopidine, sulfaphenazole, and furafylline on the *O*-demethylation of OMF at 1 μM and 10 μM OMF in HLM incubations.

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TABLE 1

The marker substrate reaction from human liver microsome and cDNA expressed CYP

Enzyme	Marker substrate reaction	Enzyme activity		Amount of cDNA expressed CYP per incubation* (pmol)
		Human liver microsome (pmol/pmol total CYP/min)	cDNA expressed CYP (pmol/pmol CYP/min)	
CYP2A6	Coumarin 7-hydroxylation	2.47	7.2	14.0
CYP2C8	Paclitaxel 6α-hydroxylation	0.56	7.6	2.8
CYP2C9	Diclofenac 4'-hydroxylation	4.12	22.0	7.6
CYP2C19	S-mephenytoin 4'-hydroxylation	0.24	25.0	0.4
CYP3A4	Testosterone 6β-hydroxylation	8.87	170.0	2.0
CYP3A5	Testosterone 6β-hydroxylation	8.87	14.0	25.0

*The amount of cDNA expressed CYP used per incubation had approximate equivalent CYP isozyme activity to that used in HLM incubations which contained 40 pmol CYP and 0.1 mg microsomal proteins.

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TABLE 2

Relative abundance of CYP present in human liver

Enzyme	Relative abundance of hepatic CYP	Amount of cDNA expressed CYP per incubation (pmol)
CYP1A1	< 1%	0.40
CYP1A2	13%	5.20
CYP2B6	1%	0.40
CYP2D6	3%	1.00
CYP2E1	7%	2.80

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TABLE 3

Kinetic parameters for OMF *O*-demethylation in the absence and presence of CYP isozyme selective chemical inhibitors in human liver microsomes

	K_{m1} (μM)	V_{max1} (pmol/mg/min)	K_{m2} (μM)	V_{max2} (pmol/mg/min)
Without Inhibitor	1.14 ± 0.90	11.3 ± 4.6	57.0 ± 6.4	258 ± 6
With furafylline	1.75 ± 1.79	8.0 ± 4.0	98.9 ± 43.4	284 ± 41
With ticlopidine	-	-	103.9 ± 32.6	143 ± 25
With (+)-*N*-3-benzyl-nirvanol	-	-	92.26 ± 8.09	442 ± 20
With sulfaphenazole	3.97 ± 0.28	22.8 ± 0.7	-	-

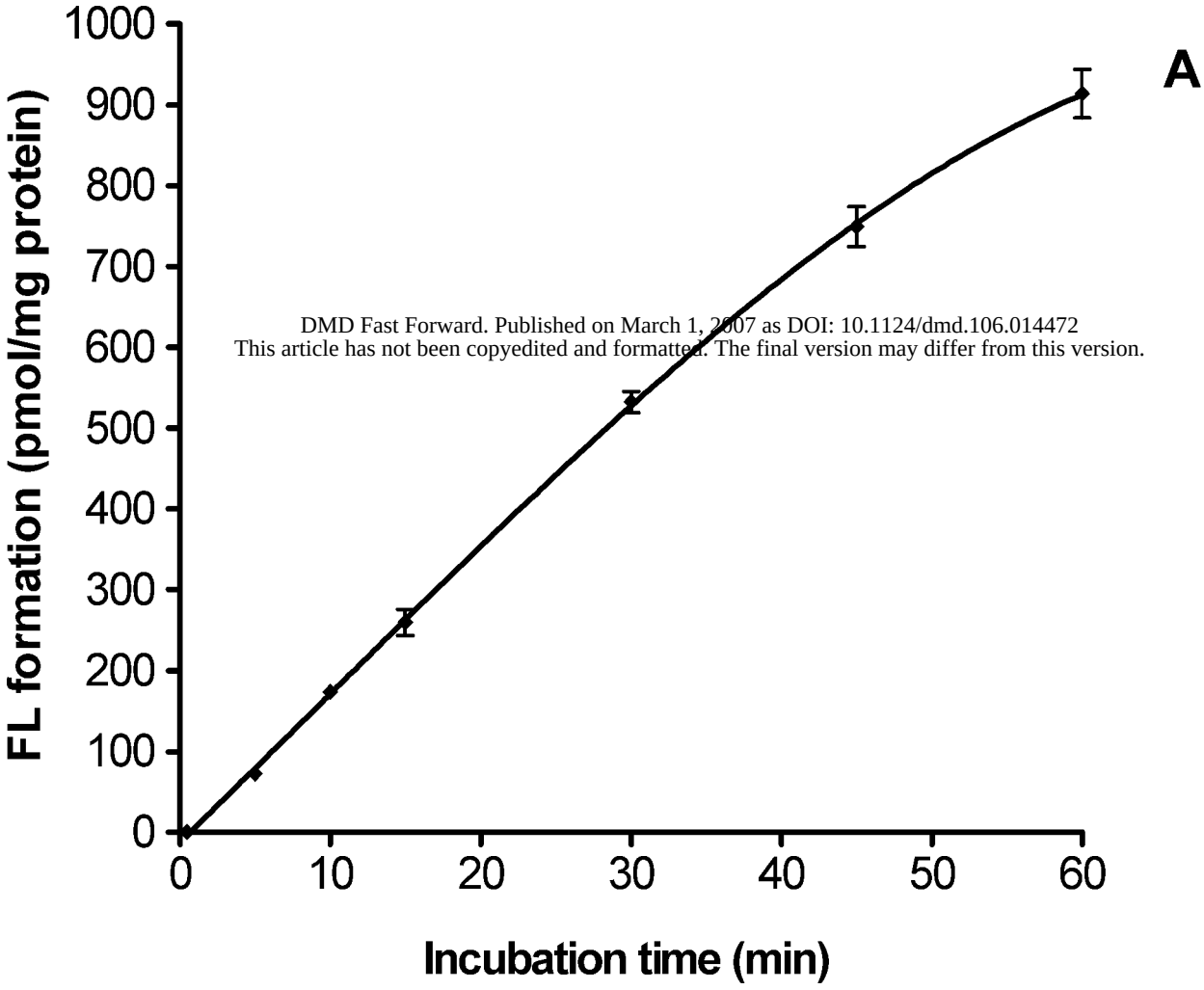
TABLE 4

Comparison of CYP2C19 IC_{50} values obtained using fluorometric assay with OMF as a substrate and the literature IC_{50} values using LC/MS with (*S*)-mephenytoin as a substrate

CYP Inhibitors	Experimental IC_{50} (μM)	Literature IC_{50} (μM)	References
Amitriptyline	42.34 ± 12.18	37.7*	Shin et al., 2002
Felbamate	>125	357*	Glue et al., 1997
Fluoxetine	8.54 ± 3.10	5.2*	Kobayashi et al., 1995
Fluvoxamine	0.58 ± 0.15	0.235*	Yao et al., 2002
Imipramine	41.48 ± 9.42	56.8*	Shin et al., 2002
Ketoconazole	18.5 ± 4.7	27	Dierks et al., 2001
Lansoprazole	0.742 ± 0.21	0.4 - 1.5*	Li et al., 2004
(+)-*N*-3-benzyl-nirvanol	0.47 ± 0.06	0.24 ± 0.04	Suzuki et al., 2002
Omeprazole	21.3 ± 6.3	30.2	Ko et al., 1997
Progesterone	4.92 ± 1.57	9.0*	Yamazaki and Shimada, 1997
Quinidine	108.7 ± 63.0	>50	Dierks et al., 2001
Ticlopidine	3.99 ± 1.82	3.7 ± 0.2*	Donahue et al., 1997
Tranylcypromine	13.6 ± 2.9	9	Dierks et al., 2001

*IC_{50} values were calculated with the competitive inhibition model at substrate concentration, (*S*)-mephenytoin, equal to K_m.

Figure 1



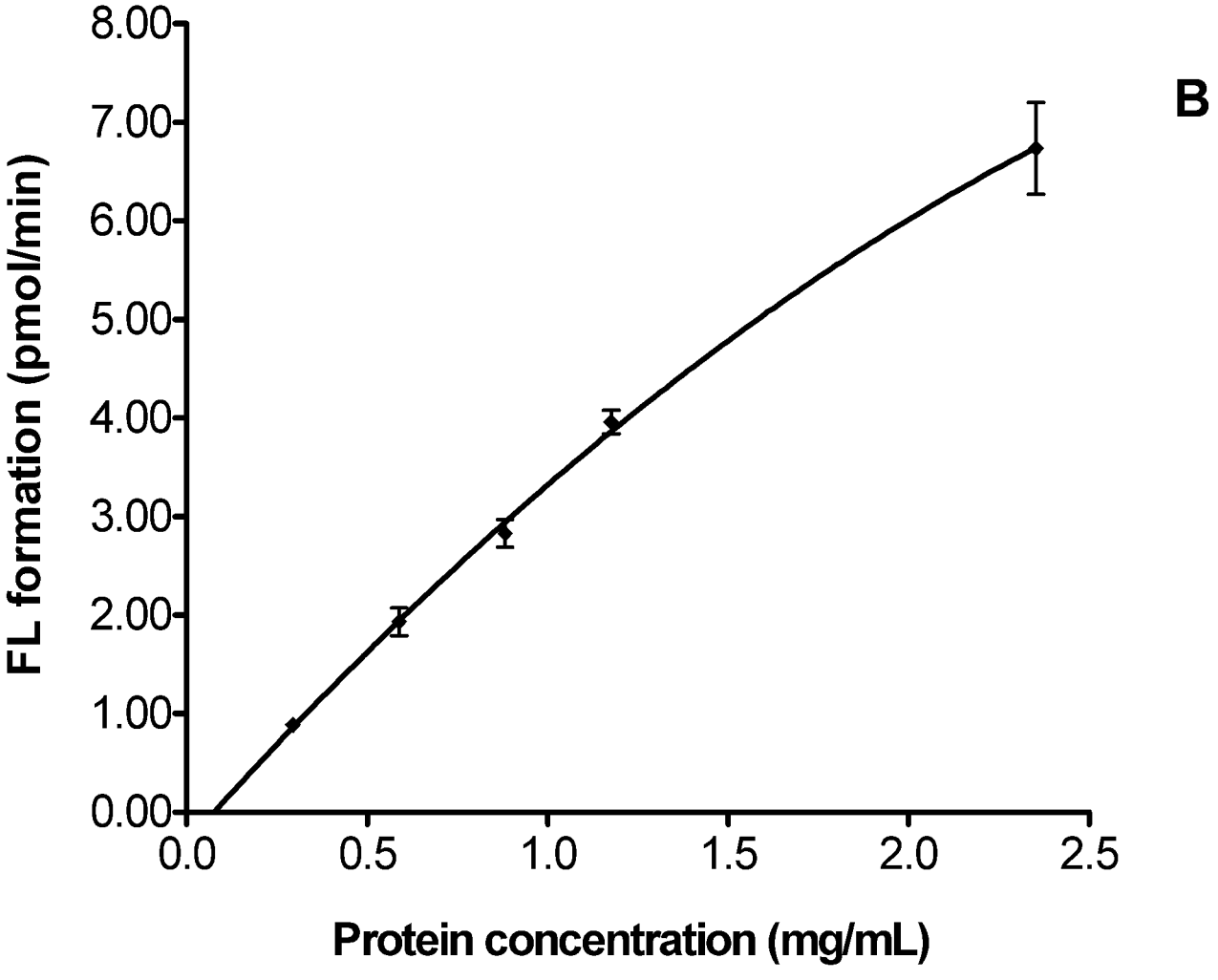
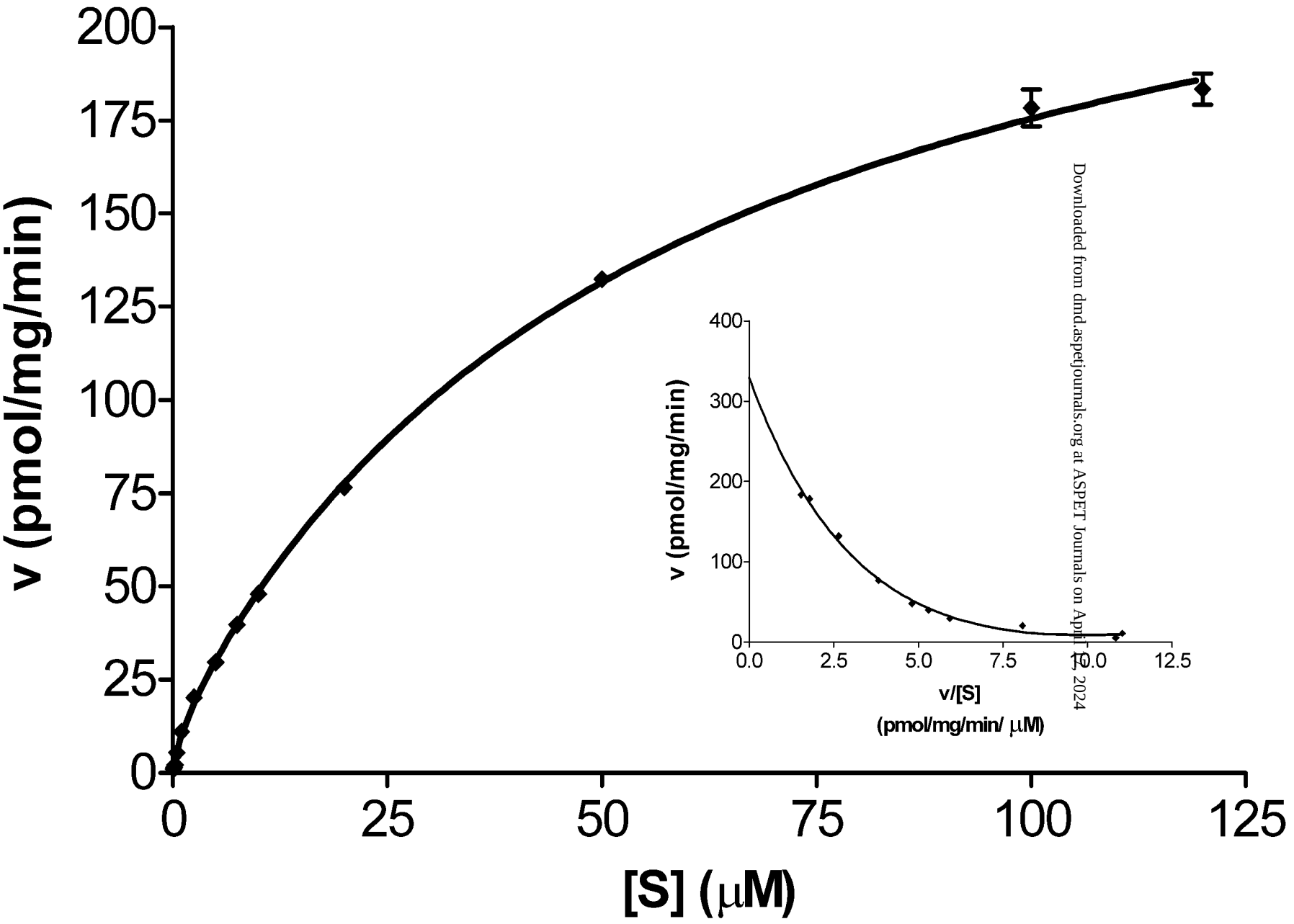


Figure 2



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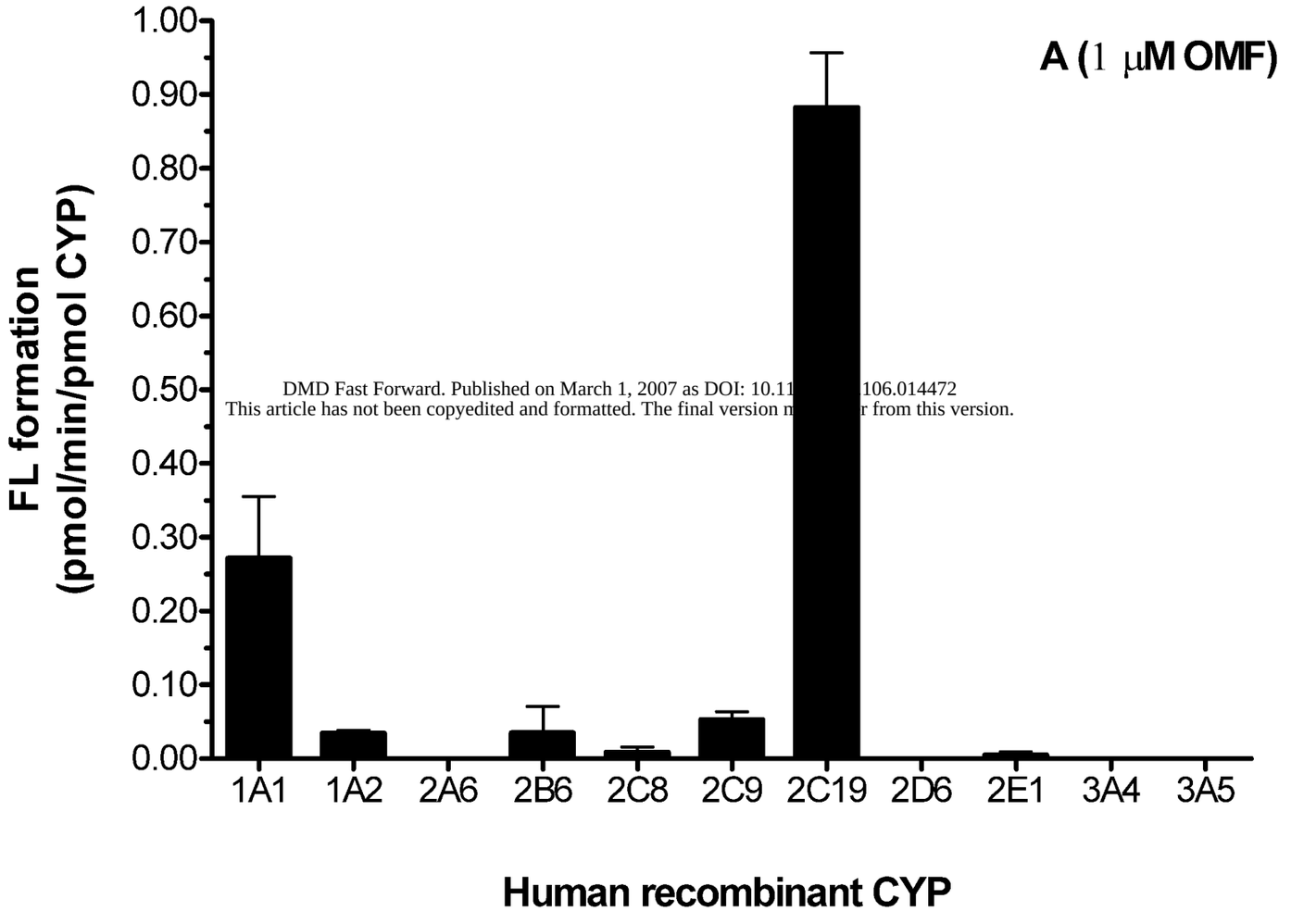

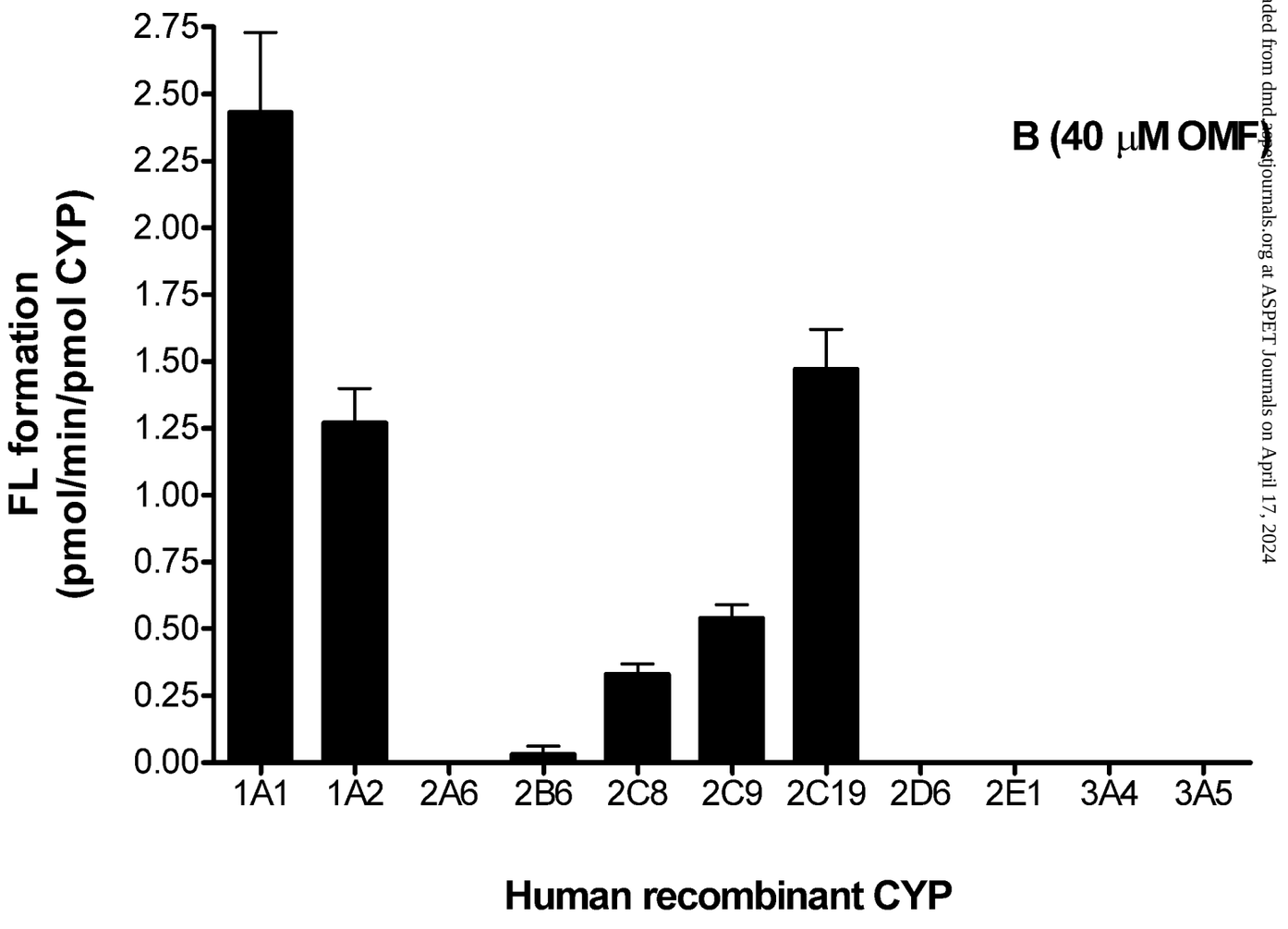

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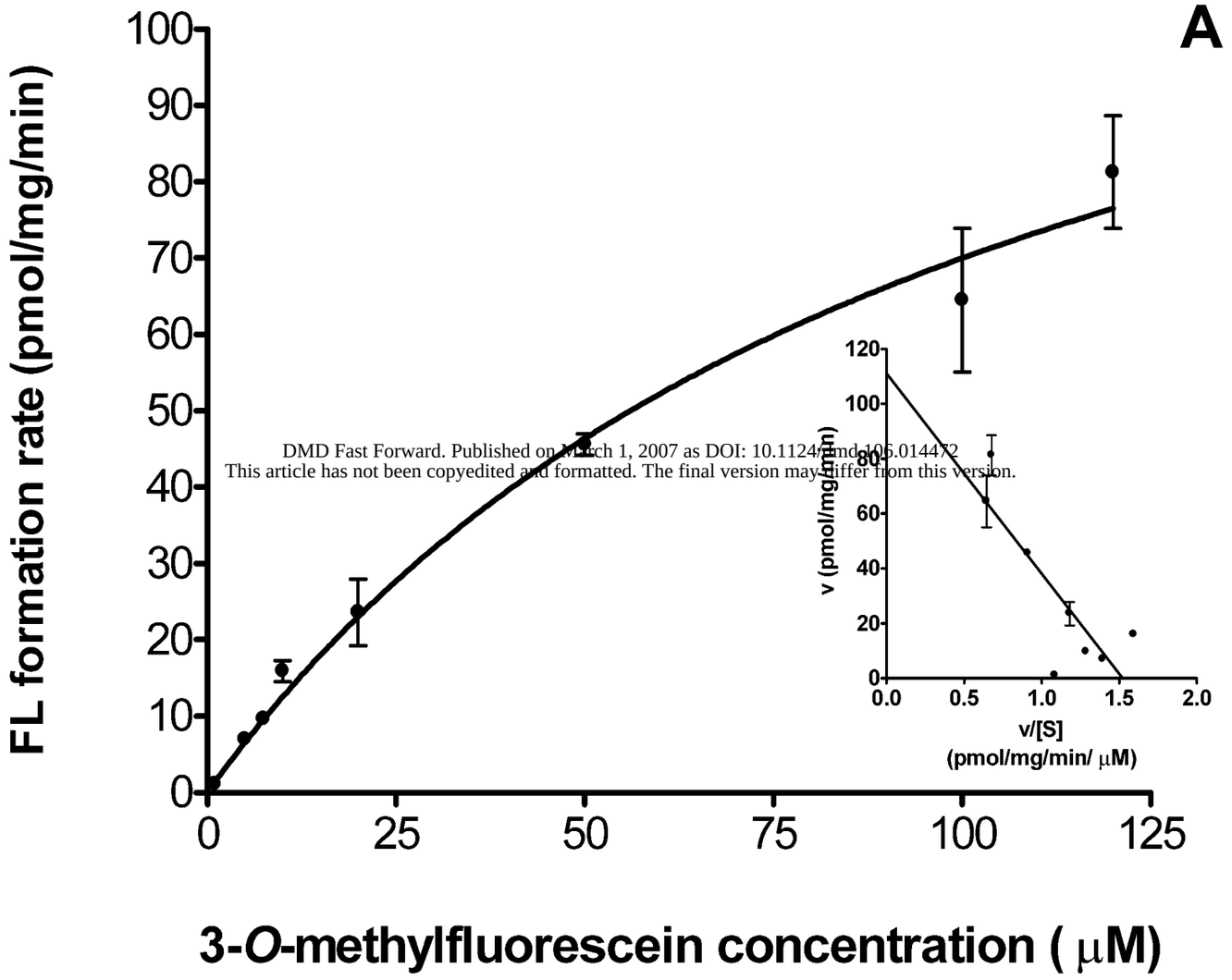

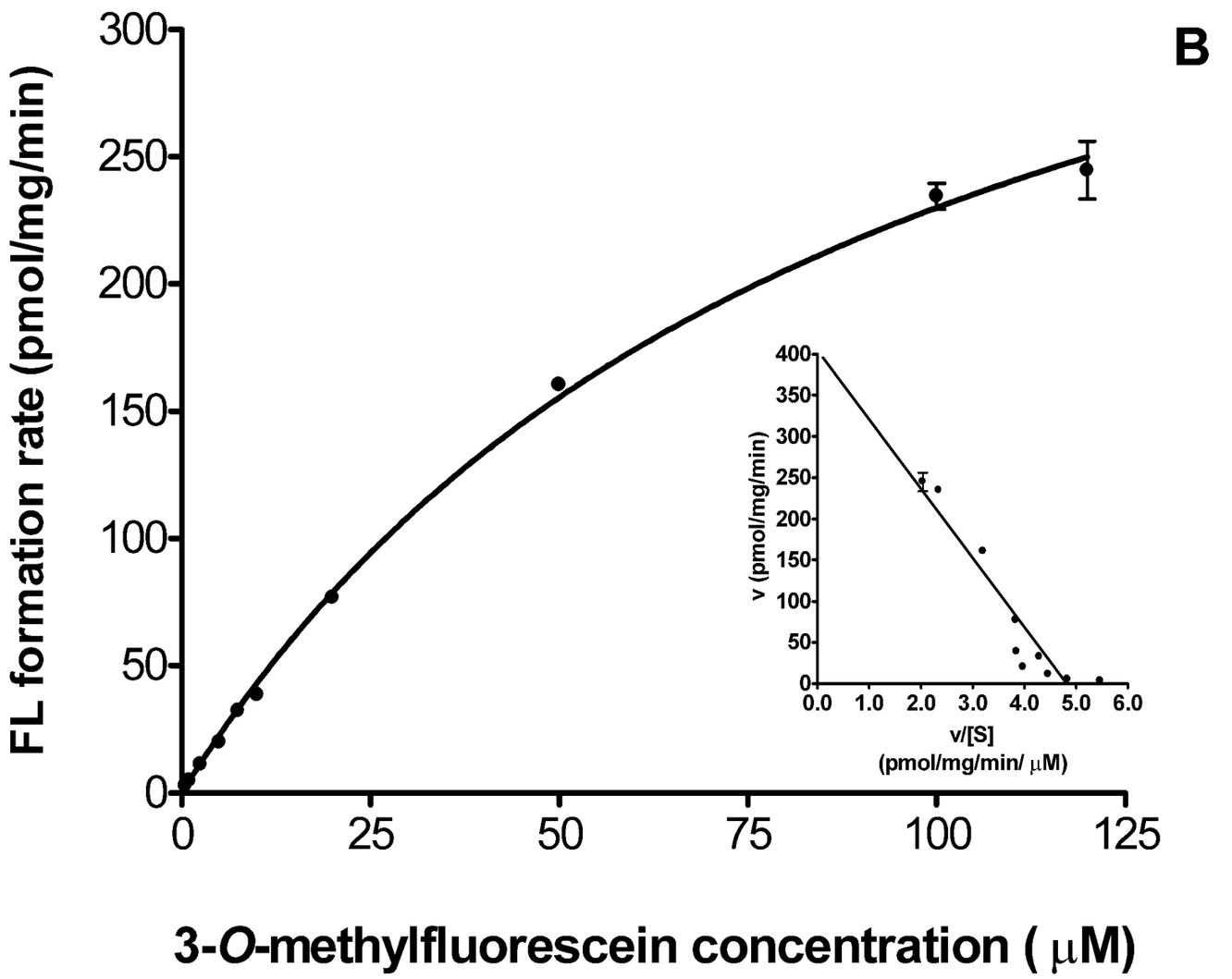

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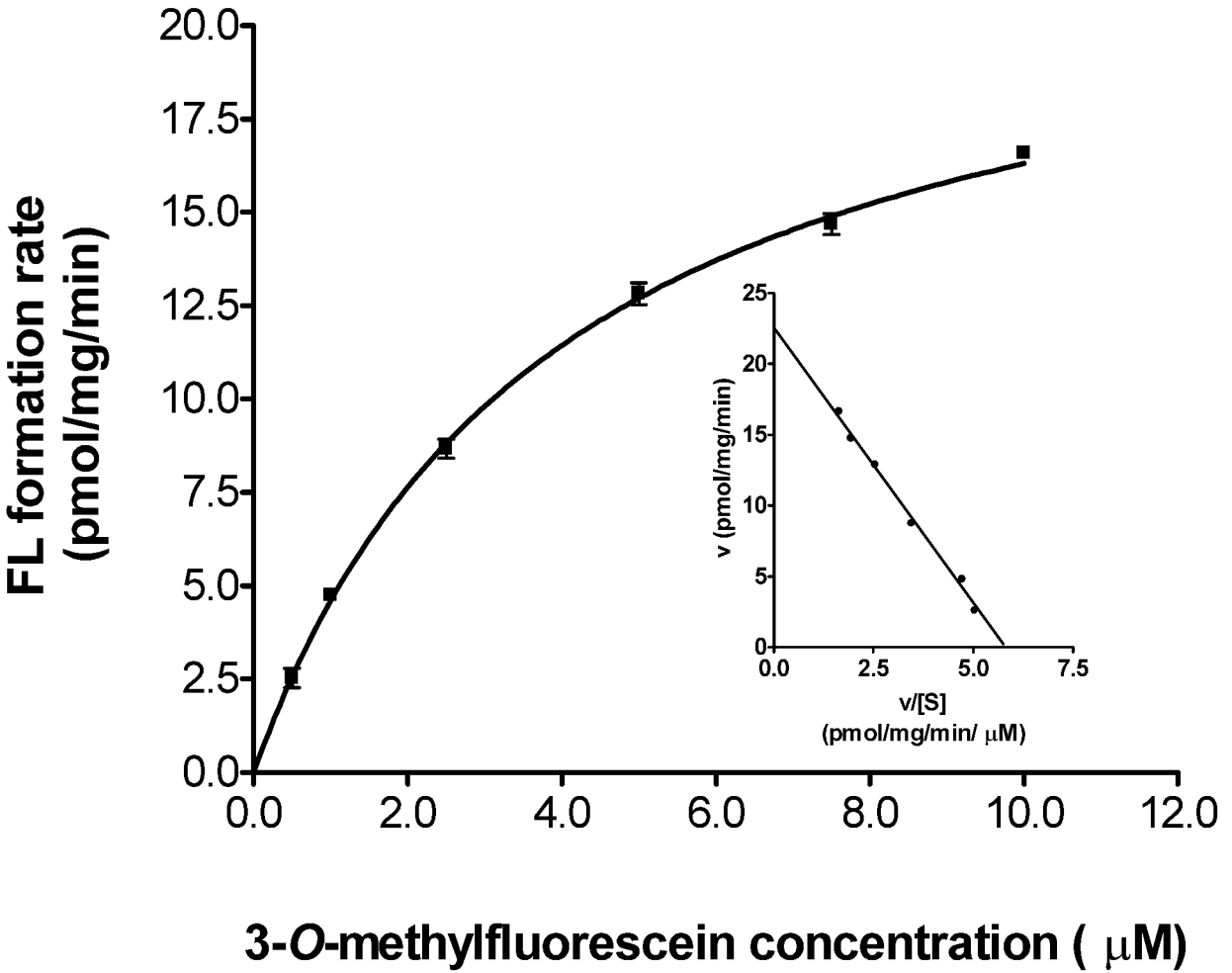
FL formation rate
(pmol/mg/min)
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0.0
2.0
4.0
6.0
8.0
10.0
12.0
3-O-methylfluorescein concentration (μM)
v (pmol/mg/min)
25
20
15
10
5
0
0.0
2.5
5.0
7.5
v/[S]
(pmol/mg/min/ μM)

Figure 6

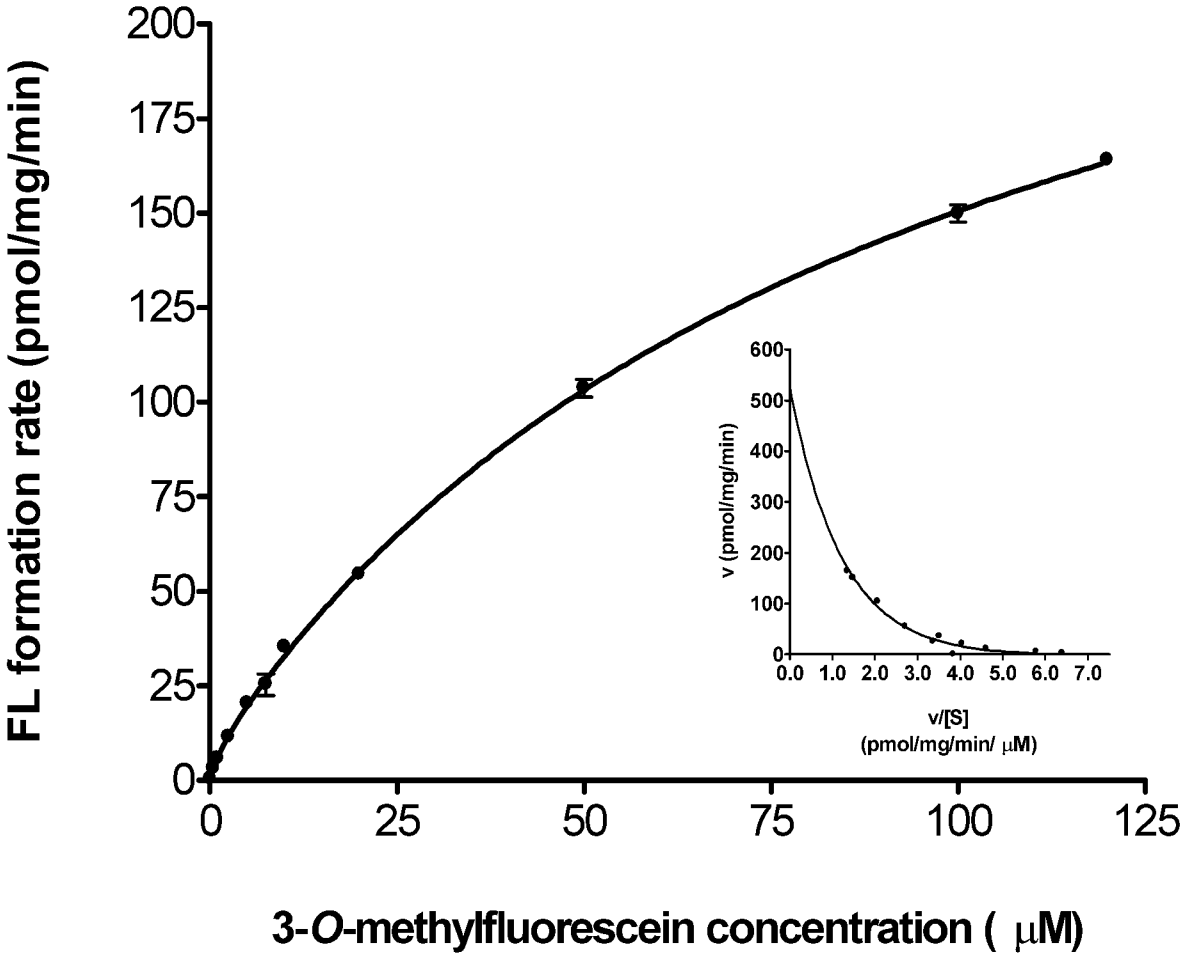
FL formation rate (pmol/mg/min)
200
175
150
125
100
75
50
25
0
0
25
50
75
100
125
3-O-methylfluorescein concentration (μM)
v (pmol/mg/min)
600
500
400
300
200
100
0
0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0
v/[S]
(pmol/mg/min/ μM)

Figure 7

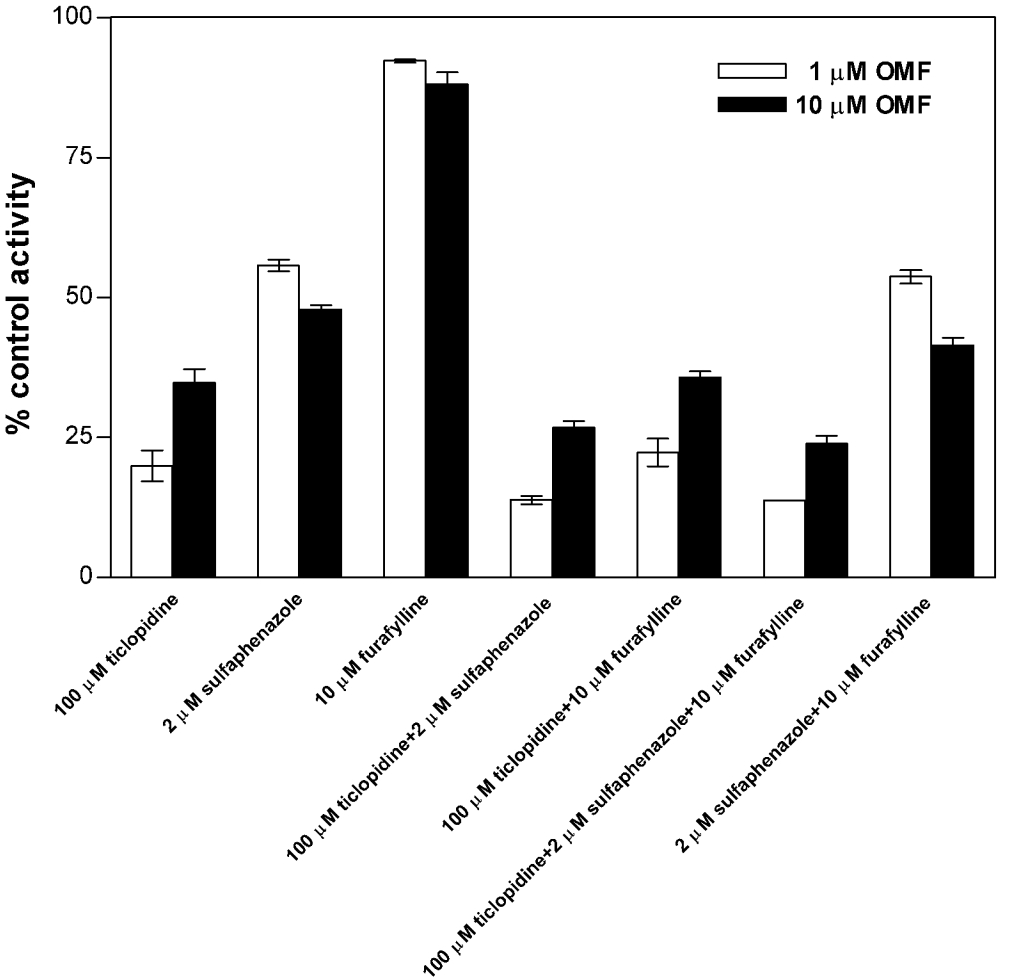

Figure 8