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A predominate role of CYP1A2 for the metabolism of nabumetone to the active metabolite, 6-methoxy-2-naphthylacetic acid (6-MNA), in human liver microsomes

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Abbreviations: 6-MNA, 6-methoxy-2-naphthylacetic acid; ACN, acetonitrile; C_{max}, maximum concentration in plasma; CYP, Cytochrome P450; DDI, drug-drug interaction; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; HLMs, human liver microsomes; ISTD, internal standard: K_i, inhibition constant ; K_m, Michaelis-Menten constant; LC-MS, liquid chromatography mass spectrometry; MeOH, methanol; *m/z*, mass-to-charge ratio; NSAID, nonsteroidal anti-inflammatory drug; SIM, selective ion monitoring; V_{max}, maximal reaction velocity

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ABSTRACT

Nabumetone, a widely used NSAID, requires biotransformation into 6-methoxy-2-naphthylacetic acid (6-MNA), a close structural analogue to naproxen, in order to achieve its analgesic and anti-inflammatory effects. Despite its wide use, the enzymes involved in metabolism have not been identified. In the present study, several *in vitro* approaches were used to identify the cytochrome P450 (CYP) enzyme(s) responsible for 6-MNA formation. In human liver microsomes 6-MNA formation displayed monophasic Michaelis-Menten kinetics with apparent K_m and V_{max} values (mean ± SD) of 75.1 ± 15.3 μM and 1304 ± 226 pmol/min/mg of protein, respectively, and formation rate of 6-MNA varied approximately 5.5-fold (179 to 983 pmol/min/mg of protein). 6-MNA activity correlated strongly with both CYP1A2-mediated phenacetin *O*-deethylation activity and CYP1A2 protein content (r =0.85 and 0.74, respectively; p <.0001 for both). Additional correlations were found with model activities of CYP2C19 and CYP3A4. Of 11 *c*DNA-expressed recombinant CYPs employed, rCYP1A2 was the major form catalyzing the 6-MNA formation with an apparent K_m of 45 μM and V_{max} of 8.7 pmol/min/pmol P450. Minor fractions were catalysed by rCYPs 1A1, 2B6, 2C19, 2D6, and 2E1. Experiments with CYP-selective chemical inhibitors and monoclonal anti-P450 antibodies showed that furafylline, a mechanism-based inhibitor CYP1A2, and anti-CYP1A2 antibody markedly inhibited 6-MNA formation, whereas inhibitors for other CYPs did not show significant inhibitory effects. Taken together, these studies indicate that the formation of the active metabolite of nabumetone, 6-MNA is predominately catalyzed by CYP1A2 in human liver microsomes with only minor contribution of other CYPs.

INTRODUCTION

Nabumetone is a nonsteroidal anti-inflammatory drug (NSAID) used mainly for the management of pain and inflammation in patients with osteoarthritis or rheumatoid arthritis (Dahl, 1993; Hedner et al., 2004). Due to its non-acidic nature and pro-drug formulation, nabumetone has been considered to offer distinct advantages over other NSAIDs especially with regard to better gastrointestinal tolerability and the once-daily dosing scheme (Dahl, 1993; Rothstein, 1998). Thus nabumetone has established its position among widely prescribed medicines and is listed under the top 200 prescribed medicines in the United States (the RxList data; http://www.rxlist.com).

Pharmacokinetic studies have shown that nabumetone is subject to extensive hepatic biotransformation resulting in several metabolites to be excreted into urine and feces and negligible amounts of unchanged drug in the plasma. In the major metabolic pathway approximately 35% of a standard oral dose is converted to the pharmacologically active metabolite, 6-methoxy-2-naphthylacetic acid (6-MNA) (Haddock et al., 1984; Hyneck, 1992; Davies, 1997). 6-MNA is a close structural analogue of naproxen (Figure 1) and effects more potent and more selective inhibition of cyclooxygenase 2 compared to the parent drug (Dahl, 1993; Davies, 1997). Besides formation of 6-MNA, other identified, minor metabolic pathways of nabumetone include *O*-demethylation and reduction of the ketone group. 6-MNA undergoes further hepatic metabolism, resulting in pharmacologically inactive metabolites with 6-hydroxy-2-naphthylacetic acid (6-HNA) as the principal component in humans. Approximately 75% of a radiolabelled dose of nabumetone was recovered within 48 hours in urine, mainly consisting of 6-MNA and 6-HNA in both free and conjugated forms (Haddock et al. 1984).

Recent studies have suggested that elimination of nabumetone relies mostly on hepatic Phase I metabolism, probably mediated *via* cytochrome P450 (CYP) enzymes (Kumpulainen et al., 2006). However, no studies identifying the enzymes responsible for the conversion of nabumetone to 6-MNA have been published. Considering the clinical importance of nabumetone and the pro-drug nature of this drug, our aim was to characterize the CYP isoenzymes responsible for metabolizing nabumetone to 6-MNA *in vitro*. Various approaches utilizing human liver microsomes with a panel of chemical inhibitors and specific monoclonal antibodies, as well as correlation analysis with model CYP-activities and recombinant expressed CYP enzymes were employed. In addition, the potential of *in vivo* drug interactions caused by certain potentially co-administrated drugs was investigated.

MATERIALS AND METHODS

Chemicals

Nabumetone (4-(6-methoxy-2-naphthyl)-2-butanone) and 6-MNA (6-methoxy-2-naphthylacetic acid) were purchased from Fluka (Buchs, Switzerland) and naproxen ((*S*)-(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid) from Sigma-Aldrich (St. Louis, MO). S-mephenytoin was a kind gift from Prof. Urs Meyer (Biozentrum, Basel, Switzerland) and diclofenac and 4'-hydroxydiclofenac were donated from Novartis AG (Basel, Swizerland). Propafenone, verapamil, and their metabolites were obtained from Knoll (Ludwigshafen, Germany). Bupropion and metabolites were synthesized as described by Richter et al. (2004). Glucose-6-phosphate was purchased from Roche Diagnostics GmbH (Mannheim, Germany) and glucose-6-phosphate-dehydrogenase from Calbiochem (Schwalbach, Germany). (+)-N-3-benzyl-nirvanol and the metabolite standards and internal standards d4-acetaminophen, N-deethylamodiaquine, d5-N-deethylamodiaquine, d4-4-hydroxydiclofenac, 6-hydroxychlorzoxazone, and 6-hydroxychlorzoxazone-C136 were obtained from Toronto Research Chemicals (North York, Canada) and phenacetin from Fluka (Buchs, Switzerland). All other chemicals used were from Sigma Aldrich (St. Louis, MO) and were of the highest purity available. Water was in-house freshly prepared with a Milli-Q (Millipore, Molsheim, France) purification system and was UP grade (ultra pure, 18.2 MW).

Human liver samples

Human liver tissue was obtained as non-tumorous tissue surrounding surgically removed liver tumors or metastases or material surgically resected for other reasons at the Department of Surgery, Charité, Campus Virchow-Clinic, Humboldt University, Berlin, Germany. Clinical patient documentation for all samples included age, sex, medical diagnosis, presurgical

medication including known CYP-inducers and inhibitors, alcohol intake, and smoking habits. Samples from patients with hepatitis, cirrhosis or chronic alcohol use were excluded. The use of liver samples was approved by the ethics committee of the Medical Faculties of the Charité, Humboldt-University Berlin and the written informed consent from each patient was obtained prior to surgery. Liver microsomes were prepared with differential ultracentrifugation described in Lang et al. (2001). A weight-balanced pool of 150 individual microsomal samples with known demographics was employed for primary screening.

Incubations using Human Liver Microsomes

Incubation mixtures contained 0.30 mg microsomal protein/ml, 0.1 M potassium phosphate buffer (pH 7.4), NADPH-generating system (5 mM $MgCl_2$, 4 mM glucose 6-phosphate, 0.5 mM $NADP^+$, and 4.0 U/ml glucose 6-phosphate dehydrogenase), and 10 to 1000 μM nabumetone dissolved in acetonitrile. The final amount of acetonitrile in the incubation mixture was 0.5% (v/v), which is expected to a have minimal effect on the studied CYP activities (Chauret et al., 1998). Reaction mixture, in a final volume of 200 μl, was preincubated for 2 min at 37 ºC in a water bath before reaction was initiated by addition of the NADPH-generating system. After incubation period of 30 min, each reaction was terminated by adding 100 μl of ice-cold acetonitrile containing naproxen (20μM) as an internal standard (ISTD). Samples were then subsequently cooled in an ice bath to precipitate the proteins. The mixture was vortex mixed and spun at 10,000 x g for 15 minutes. 6-MNA formation was linear with respect to incubation time and protein content under assay conditions described above.

The method for 6-MNA determination was applied with only minor modifications from Nobilis and coworkers (Nobilis et al., 2003). Shortly, the separation and detection of 6-MNA and naproxen (20 μM, ISTD) was performed by HPLC-UV (HP 1100, Agilent Technologies,

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Waldbronn, Germany) equipped with a LiChrospher 100 RP-18 column (100 x 3 mm, 5 µm particle size; Merck, Darmstadt, Germany). An isocratic elution of acetonitrile/0.1% acetic acid in water (45/55% v/v) was carried out at the eluent flow rate of 0.5 ml/min. The UV-detection was performed at λ = 265 nm.

Correlation analysis

For correlation analysis, microsomes from 25 livers were employed with respect to activities of 6-MNA formation (50 µM of nabumetone) and index reactions for CYP1A2 (phenacetin *O*-deethylation), CYP2A6 (coumarin 6-hydroxylation), CYP2B6 (bupropion hydroxylation), CYP2C8 (amodiaquine *N*-deethylation), CYP2C9 (diclofenac 4'-hydroxylation), CYP2C19 (*S*-mephenytoin 4'-hydroxylation), CYP2D6 (propafenone 5-hydroxylation), CYP2E1 (chlorzoxazone 6-hydroxylation), and verapamil *N*-demethylation (CYP3A4). Determinations of activities for CYP2A6 and CYP3A4 are described in detail in Wolbold et al. (2003), for CYP2B6 in Richter et al. (2004), and for CYP2D6 in Toscano et al. (2006).

CYP1A2 activity was determined with 25 µM phenacetin (dissolved in ACN, 20 min incubation period), CYP2C8 with 2 µM amodiaquine (water, 15 min), CYP2C9 with 4 µM diclofenac (dissolved in MeOH, 20 min), and CYP2E1 activity with 75 µM chlorzoxazone (MeOH, 20 min). The final amount of substrate solvents in incubation mixtures was 0.5% (*v/v*) and each incubation mixture contained 0.1 mg/ml of microsomal protein. Otherwise the incubations were carried out as described above. Formation of all metabolites was verified to be linear with respect to both incubation time and protein content under these assay conditions.

A HP Series 1100 LC-MSD system (Agilent, Waldbronn, Germany) with binary pump, degasser, autosampler and mass selective detector equipped with an electrospray ion source was used in all model activity analyses. Chromatography for phenacetin and diclofenac assays

was performed on a Phenomenex LUNA C8 column (2.0 x 150 mm, 5 µm particle size; Phenomenex, Torrance, CA), and for amodiaquine and chlorzoxazone assays a Waters SymmetryShield C18 column (2.0 x 150 mm, 5 µm particle size; Waters Corporation, Milford, MA) was employed. A flow rate of 0.5 ml/min for the mobile phase was used for all assays. Nitrogen was used as drying gas at a flow rate of 10 l/min and as nebulizer gas at a pressure of 40 psig, drying temperature was 350 °C. The details of other analytical parameters are given in the Table 1.

Standardisation of the analytical assays was performed with calibration samples prepared directly from the working solutions, in the concentration ranges given in Table 1. Calibration curves based on internal standard calibration were obtained by weighted (1/x2) linear regression for the peak-area ratio of the analyte to the respective internal standard against the amount of the analyte. The concentration of the analytes in unknown samples was obtained from the regression line. Assay accuracy and precision were determined by analyzing quality controls that were prepared in the same way as the calibration samples in water as matrix.

Analysis for correlations and statistical significances were carried out using GraphPad Software Prism 3.03 (GraphPad Software Inc. San Diego, CA). Bivariate linear Spearman correlation coefficients (r) were calculated between 6-MNA formation and model activities in 25 individual liver samples and subjected to Student's t-test. The limit of statistical significance was set at $p < .05$.

Kinetic analysis

Estimates of apparent K_m and V_{max} values were determined by nonlinear least square regression using GraphPad Software Prism 3.03 (GraphPad Software Inc. San Diego, CA)

and the Michaelis-Menten equation. The data were also analyzed by Eadie-Hofstee plot to confirm a single-enzyme model.

Incubations using cDNA Expressed Human P450s

For measuring the formation of 6-MNA in human recombinant expressed CYP enzymes (CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) co-expressing human P450 reductase (SUPERSOMES™, BD Biosciences Discovery Labware, Bedford, MA), the standard incubation mixture (200 μl) contained 0.1 M potassium phosphate buffer (pH 7.4), NADPH-generating system described above, 50 μM nabumetone and recombinant expressed CYP enzymes (50 pmol CYP/ml). Incubations were carried out according to manufacturer's instructions. Shortly, the reaction was started by adding recombinant enzymes into the preincubated reaction mixture (2 min at +37 °C), mixed gently and incubated for 30 min at +37 °C in a water bath without agitating the reaction. Otherwise the incubation protocol was similar to microsomal incubations.

Inhibition of 6-MNA formation with chemical inhibitors and immunoinhibition studies

Inhibition studies with two concentrations of CYP-selective reference inhibitors were carried out at a final nabumetone concentration of 50 μM. Inhibitor concentrations were selected on the basis of previous experience of selectivity (lower concentration) and potency (higher concentration) (Suzuki et al., 2002; Turpeinen et al., 2005; Pelkonen et al, 2008). Reference inhibitors furafylline (1 and 10 μM; CYP1A2), tranylcypromine (0.5 and 5 μM; CYP2A6), ticlopidine (0.1 and 1 μM; CYP2B6), montelukast (0.1 and 1 μM; CYP2C8), sulfaphenazole (1 and 10 μM; CYP2C9), (+)-N-3-benzyl-nirvanol (1 and 10 μM; CYP2C19), quinidine (1 and 10 μM; CYP2D6), pyridine (5 and 50 μM; CYP2E1), and ketoconazole (0.5 and 5 μM; CYP3A4) were added into the incubation mixture in a small volume (0.5% *v/v*) of an

appropriate solvent (water, methanol, ACN or DMSO) and incubations were carried out as described under the section '*Incubations using Human Liver Microsomes*'. Due to the mechanism-based inhibition nature of furafylline (Kunze and Trager, 1993) and ticlopidine (Richter et al., 2004, Walsky and Obach, 2007), experiments with these two inhibitors were performed otherwise similarly, but with 15 min preincubation time and initiating the reaction with addition of nabumetone. The enzyme activities in the presence of inhibitors were compared with control incubations (incubations with the same amount of inhibitor solvent but without an inhibitor). Triplicate incubates were carried out and all data points represent the mean.

For determining the K_i values of fluvoxamine for 6-MNA formation, three concentrations of nabumetone (50, 100, and 200 μM, corresponding approximately to $K_m/2$, K_m, and 2 x K_m) were incubated with a range of inhibitor concentrations in the presence of human liver microsomal pool or human recombinant expressed CYP1A2, as described above. Graphical analysis of data was performed according to Lineweaver-Burk, Dixon, Eadie-Hofstee, and Hanes plots.

Experiments with a panel of CYP isoenzyme specific monoclonal antibodies raised against CYP1A2, 2A6, 2B6, 2C8, 2C19, 2D6, 2E1, and 3A4 (BD Biosciences Discovery Labware, Bedford, MA) were performed according to the manufacturer's instructions. Shortly, distinct amounts of antibody (or comparable amount of 25 mM Tris buffer, pH 7.5, for vehicle controls) was added to microsomes and incubated for 15 min (20 min for anti-CYP1A2) on ice. After that, 0.1 M potassium phosphate buffer (pH 7.4) and 50 μM nabumetone were added and incubations were carried out as described above.

Immunoblotting

For Western blot analysis of CYP1A2 microsomal samples were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The immunodetection was performed with human CYP1A2-selective monoclonal antibody mAb1A2 (BD Biosciences Discovery Labware, Bedford, MA) and IRD800 labelled secondary anti mouse antibody (Li-cor, Biosciences GmbH, Bad Homburg, Germany). CYP1A2 apoprotein was quantified using cDNA expressed CYP1A2 (BD Biosciences Discovery Labware, Bedford, MA) as standard protein on each blot (range 0.25-4 pmol). The data analysis was performed using the infrared imaging system Odyssey (Li-cor, Biosciences GmbH, Bad Homburg, Germany) and Odyssey software version 2.1beta. All samples were measured at least in duplicate and mean values were calculated and expressed in pmol/mg of microsomal protein.

RESULTS

In the presence of NADPH, human liver microsomes (HLMs) metabolized nabumetone to 6-MNA. No 6-MNA formation was observed when NADPH was omitted from the incubation mixtures (data not shown). In 25 individual liver samples, the formation rate of 6-MNA varied approximately 5.5-fold, ranging from 179 to 983 pmol/min/mg of protein, with a mean value of 561 pmol/min mg of protein (Figure 2). A monophasic Michaelis-Menten kinetics for 6-MNA activity was observed in four individual liver microsomal preparations and in a microsomal pool of 150 individuals. The apparent K_m and V_{max} values for 6-MNA formation were 75.1 ± 15.3 μM and 1304 ± 226 pmol/min/mg of protein, respectively (Figure 3, Table 2).

In characterized microsomal preparations from 25 livers, velocity of 6-MNA formation was found to be strongly correlated both with CYP1A2-associated phenacetin *O*-deethylation activity (r = 0.85, p <.0001) and with immunochemically determined CYP1A2 protein content (r = 0.74, p <.0001) (Figure 2, Table 3). As CYP1A2 is known to be induced in smokers (Gunes and Dahl, 2008) we analyzed the data in relation to smoking status of the liver donors. As evident from figure 2, regular smokers had significantly increased levels of CYP1A2 protein (120 vs 53 pmol/mg microsomal protein; p = 0.04), phenacetin *O*-deethylation activity (3137 vs 1119 pmol/min/mg protein; p = 0.005), and 6-MNA formation (744 vs 489 pmol/min/mg protein; p=0.005). Statistically significant positive correlations with model activities of CYP2C19 (r = 0.59, p = 0.002) and CYP3A4 (r = 0.64, p = 0.0005) were also observed (Table 3). The correlation between CYP2C8 model activity amodiaquine *N*-deethylation also achieved the level statistical significance (r = 0.41, p= 0.042), but was

considered to be weak, since no clear trend was observed and the data distribution was found to be very sparse.

From the panel of chemical inhibitors studied, only furafylline, a mechanism-based inhibitor of CYP1A2, was able to reduce the 6-MNA formation to 75%, 37%, and 23% of control at 1 μM, 10 μM, and 100 μM concentration levels, respectively. The effect of other inhibitors was less than 15% to the 6-MNA activity (Figure 4). In immunoinhibition experiments with diverse anti-CYP antibodies, only antibody against CYP1A2 substantially inhibited 6-MNA formation yielding up to 65% inhibition at the highest antibody concentration. The effect of other antibodies was found to be weak to negligible (Figure 5). Combination of another selected chemical inhibitor with furafylline or another antibody with anti-CYP1A2 antibody did not increase their inhibitory effect on 6-MNA foramation activity (Figure 6). Fluvoxamine, a potent inhibitor of CYP1A2, was found to inhibit 6-MNA formation in a competitive manner with K_i values of 7.2 and 4.1 μM in HLMs and recombinant CYP1A2, respectively (Figure 7).

A screen of 11 human recombinant CYPs showed that all other rCYPs tested except CYP2A6, CYP2C8, CYP2C9, CYP3A4, and CYP3A5 were able to catalyze the formation of 6-MNA (Figure 8). Taken into account the average human hepatic CYP content, it could be estimated that the majority of 6-MNA formation was attributable to CYP1A2 (53%) and CYP2B6 (19%), whereas each of the other CYPs contributed only approximately 10% of the total activity (Table 4). The kinetics of 6-MNA formation was further assessed in rCYP1A2 and was found to follow a first-order Michaelis-Menten kinetics with a with apparent K_m and V_{max} values of 45.1 μM and 8.7 pmol/min/pmol P450, respectively (data not shown).

DISCUSSION

Although nabumetone has been in clinical use for decades, knowledge about its metabolic characteristics is very fragmentary. Many reports have shown nabumetone to rely on hepatic bioactivation processes in its clearance, but no studies identifying the metabolic pathways to the pharmacologically active substance, 6-MNA, have been published. Therefore in this study we characterised the enzymes responsible for 6-MNA formation in the presence of human liver microsomes and recombinant CYP enzymes. On the basis of the present findings, nabumetone was found to be metabolized to 6-MNA predominately by CYP1A2.

At the enzyme activity level, the strongest correlation with 6-MNA formation was seen with CYP1A2-associated phenacetin *O*-deethylation activity, although model activities of CYP2C19 and CYP3A4 also showed positive correlations. Phenacetin and *S*-mephenytoin are generally regarded as selective probes for measuring activities of CYP1A2 (Tassaneeyakul et al., 1993) and CYP2C19 (Lasker et al., 1998), respectively, although the rate of *S*-mephenytoin conversion in microsomes is relatively slow (Walsky and Obach, 2004). Regarding the correlation between 6-MNA formation and verapamil N-demethylation, we do not believe that this is due to a significant contribution of CYP3A4 to nabumetone metabolism, which was convincingly excluded by the findings that ketoconazole did not have any effect and recombinantly expressed CYP3A4 was completely inactive in 6-MNA formation. The correlation seen may rather be based on covariation, due to common regulation mechanisms and cofactors (e.g. cytochrome P450 reductase). In addition Verapamil *N*-demethylation to norverapamil is catalysed mainly by CYP3A4, but CYP1A2 is known to play a minor role in verapamil *N*-demethylation (Kroemer et al., 1993).

Furafylline, a specific and potent inhibitor of CYP1A2, was seen to have a clear effect on the 6-MNA formation, which further supports the assumption that CYP1A2 has a central role in the process. In addition, out of the panel of monoclonal inhibitory antibodies, only anti-CYP1A2 was found to inhibit 6-MNA formation. It is also of note that fluvoxamine, a selective serotonin reuptake inhibitor with known inhibition potency towards CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP2B6 (Pelkonen et al., 2008), was found to inhibit 6-MNA formation competitively with the K_i values of 7.2 μM and 4.1 μM in HLMs and recombinant CYP1A2, respectively. Usually higher IC_{50} and/or K_i values in HLMs compared to recombinant enzymes suggest the contribution of multiple CYPs in the metabolic reaction. Also in a situation where multiple enzymes participate to the biotransformation process, diagnostic inhibitors tend to loose their power in identifying the particular CYPs involved. Taking into account the slight discrepancy observed in fluvoxamine inhibition and the fact that also recombinant CYPs 1A1, 2B6, 2C19, 2D6, and 2E1 were able to catalyse 6-MNA formation, it is likely that also other CYPs besides CYP1A2 may play a minor role in the metabolism of nabumetone to 6-MNA. Inhibition of CYP1A2 was found to result approximately 60% decrease in 6-MNA formation. Based to the combinatory experiments with chemical inhibitors and anti-CYP antibodies, it is likely that the remaining 6-MNA activity is distributed to more than one minor CYP form.

Inhibition of CYP enzymes is the most common cause of drug-drug interactions (DDI) and has led to the removal of several drugs from the market during the past years (Lasser et al., 2002; Issa et al., 2007). CYP-inhibition can lead to increased bioavailability of the parent compound normally subject to extensive first-pass elimination or to decreased elimination of compounds dependent on metabolism for systemic clearance. Especially with prodrugs, inhibition may result in a decrease in the amount of the active drug form, further leading to

the reduced efficacy of a drug. The identification of enzymes involved in the metabolism of nabumetone allows a prediction of potential DDI with nabumetone metabolism. Based on our results, clinically inhibition of CYP1A2 could result in decreased plasma concentrations of 6-MNA and consequently, to an attenuation of analgesic effects of nabumetone. Besides fluvoxamine, potent inhibitors of CYP1A2 are known to include fluoroquinolone antibiotic ciprofloxacin, oral hormone replacement therapy and oral contraceptives (Pelkonen et al., 2008). However, clinical CYP-interaction studies concerning nabumetone are basically non-existent. In the context of the efficacy of nabumetone it should also be kept in mind that plasma levels of 6-MNA are the result of the balance between the formation and elimination of 6-MNA. Thus, besides inhibition/induction of CYP1A2, alterations in the activities of elimination routes could also play an important role in nabumetone efficacy. In addition, although CYP1A2 is the major but not the only CYP involved in the 6-MNA formation, induction of minor CYPs could potentially result in distinct outcomes in the 6-MNA formation. This might especially be relevant regarding CYP2B6, which is susceptible to a wide range of inducers and known to display a significant genetic variability (Turpeinen, et al. 2006; Zanger, et al. 2007).

Besides the aspects of drug interactions, characterisation of the enzymes responsible for nabumetone metabolism allows an estimation of the variability in 6-MNA formation in a given population. In the present study, the formation rate of 6-MNA was found to vary 5.5-fold in a relatively small study set of 25 livers. In previous studies, the interindividual variability of CYP1A2 at the level of mRNA and enzyme activity has been shown to be 40-fold and up to 130-fold, respectively (Schweikl, et al. 1993; Eaton et al., 1995; Gunes and Dahl, 2008). The difference between individuals has been traditionally considered to be mainly due to the impact of environmental factors known to affect CYP1A2-activity,

although the role of genetics has not been completely ruled out (Gunes and Dahl, 2008; Zanger et al., 2008). The most well established example of exogenous factors attributable to variation in CYP1A2 is smoking, which is a very potent inducer of CYP1A2 through polycyclic aromatic hydrocarbons present in tobacco smoke (Gunes and Dahl 2008). In the present study, smokers had on average higher CYP1A2 expression/activity and 6-MNA-activity, except for one individual, which remained unexplained. CYP1A2 is one of the major hepatic drug-metabolizing enzymes contributing to the metabolism of a wide range of clinically important substances including caffeine and theophylline, several antipsychotics, antidepressants, and also to the endogenous substrates like melatonin and estradiol (Pelkonen et al., 2008; Zanger et al., 2008). Indeed, for example in the case of antipsychotic drug clozapine, several studies have reported a notable variation between individuals in the clearance of clozapine and a close relationship between CYP1A2 activity and the amount of effective clozapine doses (Ozdemir et al., 2001; Dailly et al., 2002; Doude van Troostwijk et al., 2003). Thus, one might assume the metabolism of nabumetone to 6-MNA to be highly variable at the population level and further to have an impact on the therapeutic effect of nabumetone.

Despite a few exceptions, the substrate and inhibitor specificity of CYP1A2 seems to be highly similar to CYP1A1, albeit their catalytic capabilities towards same substrate may differ and their tissue expression patterns are highly distinct (Tassaneeyakul et al., 1993; Ding and Kaminsky 2003). Also with nabumetone, both rCYP1A1 and 1A2 were active in 6-MNA formation, but the difference in catalytic activity was notable. Regarding other extrahepatic CYPs, a very recent congress abstract (Lee et al., 2008) showed that nabumetone is a substrate of CYP2J2. However, the metabolites formed through CYP2J2 do not include 6-MNA. Because only major hepatic CYPs and CYP1A1 were included in our present study,

the observation of the contribution of CYP2J2 stays unverified by us. Taking into account the importance of hepatic clearance to the metabolism of nabumetone (Hyneck, 1992; Kumpulainen et al., 2006) and the abundance of different CYPs in the liver, it is likely that the role of CYP2J2 – and CYP1A1 - to the overall metabolism of nabumetone remains relatively small. CYP1A1 may, however, possess some role in the first pass of nabumetone, since it is known to be relatively abundant in the gut wall of some individuals (Paine et al., 2006)

In conclusion, several approaches were adopted to identify the CYP enzymes involved in the metabolism of nabumetone to the pharmacologically active compound, 6-MNA, in the present study. The data strongly suggests a central role of CYP1A2 in the nabumetone bioactivation. In addition, other CYPs including CYP2B6, 2C19, 2D6, and 2E1 might act as minor metabolic pathways in 6-MNA formation. Concomitant use of nabumetone with inhibitors of CYP1A2 or exogenous factors inducing CYP1A2 may modify the metabolic fate and therapeutic response for nabumetone. However, clinical drug interaction studies are required to ascertain the importance of these anticipated drug interactions.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. The chemical structures of nabumetone, 6-MNA and naproxen (ISTD).

Figure 2. Formation of 6-MNA in human liver microsomes from 25 different individuals (50 μM nabumetone). Correlation with immunoquantified CYP1A2 protein content (a) and phenacetin *O*-deethylation activity (b). For a reference, correlation of phenacetin *O*-deethylation activity and CYP1A2 protein also showed (c). Closed circles = non-smokers, open circles = smokers.

Figure 3. Representative kinetics of 6-MNA formation in human liver microsomes. The inset depicts the same data on Eadie-Hofstee plot. Kinetic parameters are collected in Table 1.

Figure 4. Inhibition of 6-MNA formation by chemical CYP-inhibitors in a pool of human liver microsomes (50 μM nabumetone). Each bar represents a mean ± SD of triplicate samples.

Figure 5. Effects of CYP isoenzyme specific monoclonal antibodies (MABs) on the formation of 6-MNA in a pool of human liver microsomes (50 μM nabumetone).

Figure 6. Effects of 10 μM furafylline (FUR) in combination with selected chemical CYP-inhibitors (white bars) and anti-CYP1A2 antibody in combination with selected CYP antibodies (grey bars) into 6-MNA formation in HLMs (50 μM of nabumetone, 5 μl of each antibody *per* 100 μg of HLM). TIC = ticlopidine, 3-BN = (+)-N-3-benzyl-nirvanol, PYR = pyridine.

Figure 7. Dixon plots for the inhibition of 6-MNA formation by fluvoxamine in a pool of human liver microsomes (a) and in *c*DNA-expressed human CYP1A2 (b). Nabumetone concentrations are selected to correspond to the mean $K_m/2$, K_m, and 2 x K_m for 6-MNA formation. Each point represents the mean of duplicate measurements.

Figure 8. Formation of 6-MNA in a panel of *c*DNA-expressed human CYPs. Each bar represents a mean ± SD of triplicate samples (50 μM nabumetone).

Table 1. Analytical parameters for CYP1A2, CYP2C8, CYP2C9, and CYP2E1 assays.

Assay	Analyte, concentration range and SIM[1] mass	Internal standard, concentration and SIM[1] mass	Ionization mode	Capillary voltage (kV)	Fragmentor voltage (V)	Mobile phase	Gradient, %B(min)
CYP1A2	Acetaminophen (0.25 – 20 μM) *m/z* 152	Acetaminophen-D4 (1 μM) *m/z* 156	ESI+	2.5	60	0.1% (v/v) formic acid (A) and 0.1% (v/v) formic acid in acetonitrile (B)	2(0) → 30(5) → 80(7)
CYP2C8	*N*-deethylamodiaquine (0.1 – 20 μM) *m/z* 328	*N*-deethylamodiaquine-D5 (0.5 μM) *m/z* 333	ESI+	1.5	80	5 mM ammonium acetate pH 3.0 (A) and acetonitrile (B)	30(0) → 55(9)
CYP2C9	4-hydroxydiclofenac (0.05 – 10 μM) *m/z* 310	4-hydroxydiclofenac-D4 (1 μM) *m/z* 314	ESI-	3.5	50	5 mM ammonium acetate pH 3.0 (A) and acetonitrile (B)	5(0) → 35(5)
CYP2E1	6-hydroxychlorzoxazone (0.1 – 10 μM) *m/z* 184	6-hydroxychlorzoxazone-$^{13}C_6$ (2 μM) *m/z* 190	ESI-	3.5	80	water (A) and acetonitrile (B)	70 isocratic

[1]SIM = selective ion monitoring

Table 2. Apparent kinetic parameters of 6-MNA formation in microsomes from four individual human liver microsomal preparations and from the pool of 150 liver preparations.

Liver sample	V_{max} (pmol/min/mg of protein)	K_m (μM)	V_{max}/K_m [a]
IKP148-58	896	93.5	9.6
IKP148-79	1442	55.8	25.8
IKP148-98	1552	63.3	24.5
IKP148-282	1254	70.3	17.8
IKP-Pool	1378	92.4	14.9
Mean ± SD	1304 ± 226	75.1 ± 15.3	18.5 ± 6.0

[a] V_{max}/K_m = intrinsic clearance, in units of μl/min/mg of protein

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Table 3. Correlation of 6-MNA formation with CYP model activities in human liver microsomes from 25 individuals.

CYP	Model activity	*r*
1A2	Phenacetin *O*-deethylation	0.85***
2A6	Coumarin 7-hydroxylation	0.16
2B6	Bupropion hydroxylation	0.33
2C8	Amodiaquine *N*-deethylation	0.41*
2C9	Diclofenac 4-hydroxylation	0.26
2C19	*S*-Mephenytoin 4'-hydroxylation	0.59**
2D6	Propafenone 5-hydroxylation	-0.24
2E1	Chlorzoxazone 6-hydroxylation	0.36
3A4	Verapamil *N*-demethylation	0.64***

*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

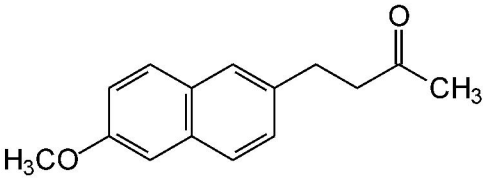
Table 4. 6-MNA formation in *c*DNA Expressed Human CYPs.

rCYP	6-MNA formation rate [a] *(pmol/min/pmol P450)*	Mean CYP content in the liver[b] *(nmol/mg protein)*	Normalized 6-MNA formation rate *(nmol/min/mg protein)*	Percentage of total activity *(%)*
1A2	6.25	0.045	0.281	53
2B6	2.57	0.039	0.100	19
2C19	2.41	0.019	0.046	9
2D6	4.62	0.010	0.046	9
2E1	1.09	0.049	0.053	10
Total			*0.526*	*100.0*

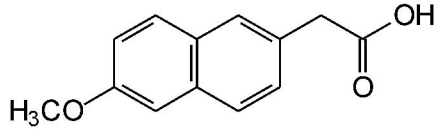
[a] 6-MNA formation rate is a mean of triplicate incubates.

[b] Data from Rodrigues (1999)

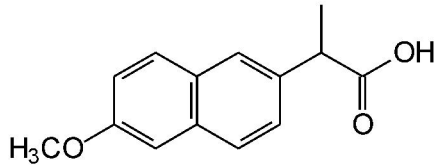
Figure 1.



Nabumetone



6-MNA



Naproxen (ISTD)

Figure 2

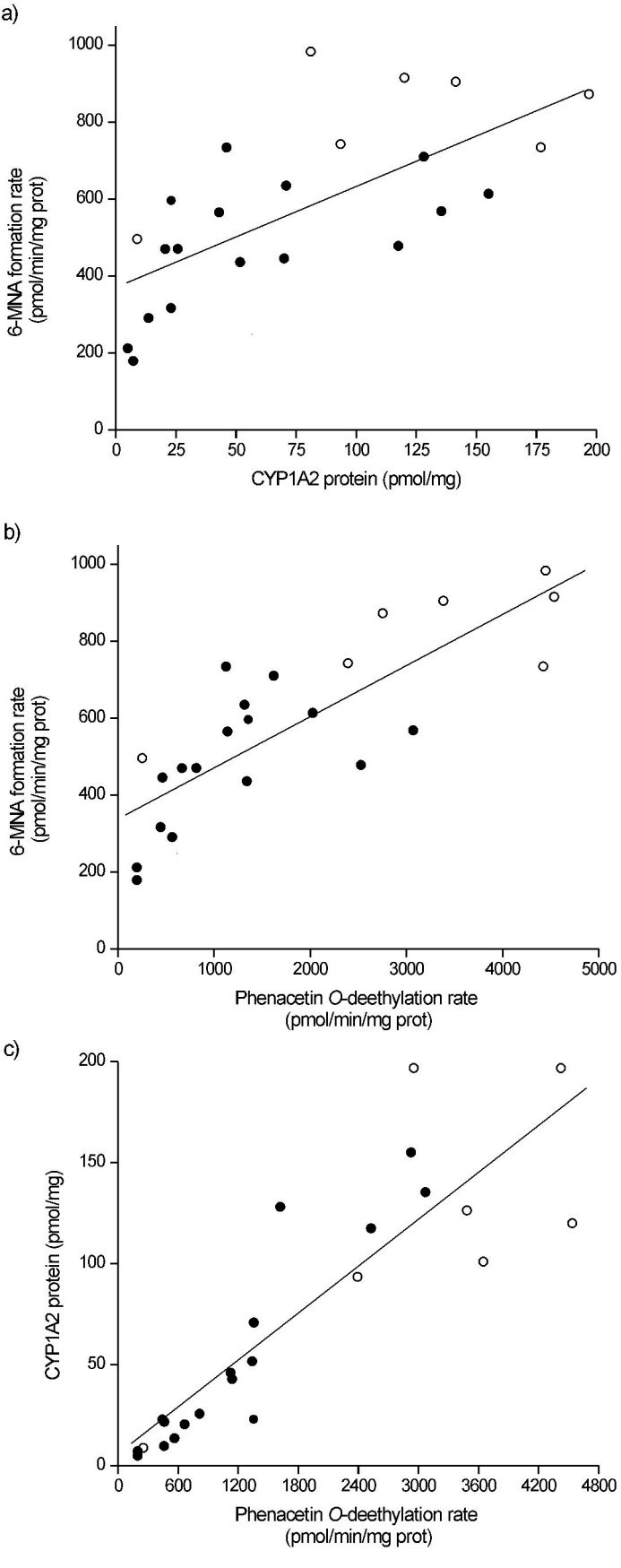

a)
6-MNA formation rate (pmol/min/mg prot)
CYP1A2 protein (pmol/mg)
b)
6-MNA formation rate (pmol/min/mg prot)
Phenacetin O-deethylation rate (pmol/min/mg prot)
c)
CYP1A2 protein (pmol/mg)
Phenacetin O-deethylation rate (pmol/min/mg prot)

Figure 3.

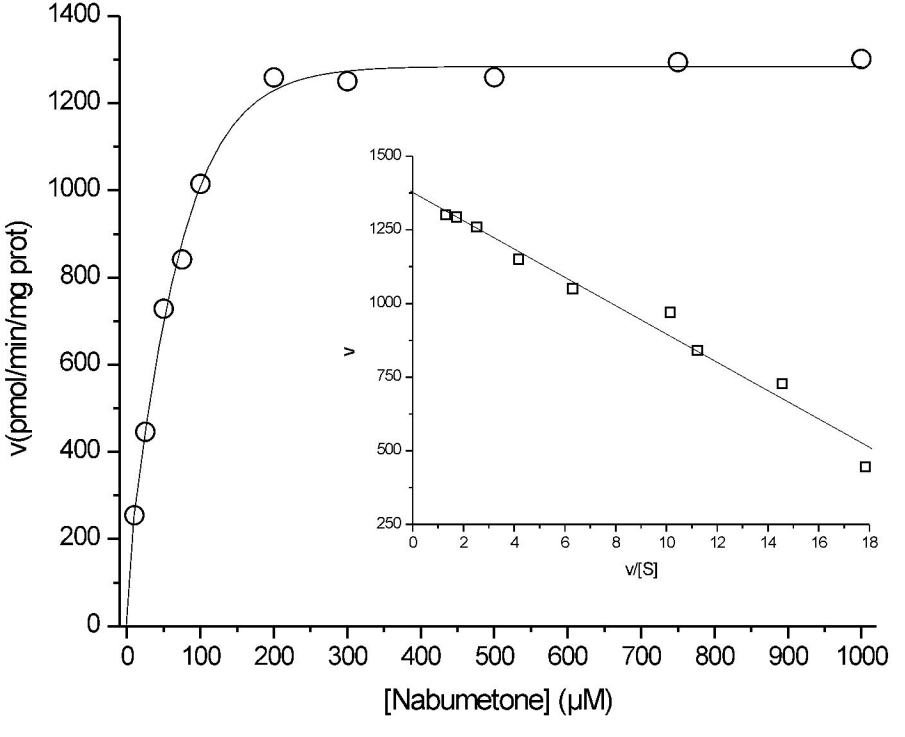


Figure 4.

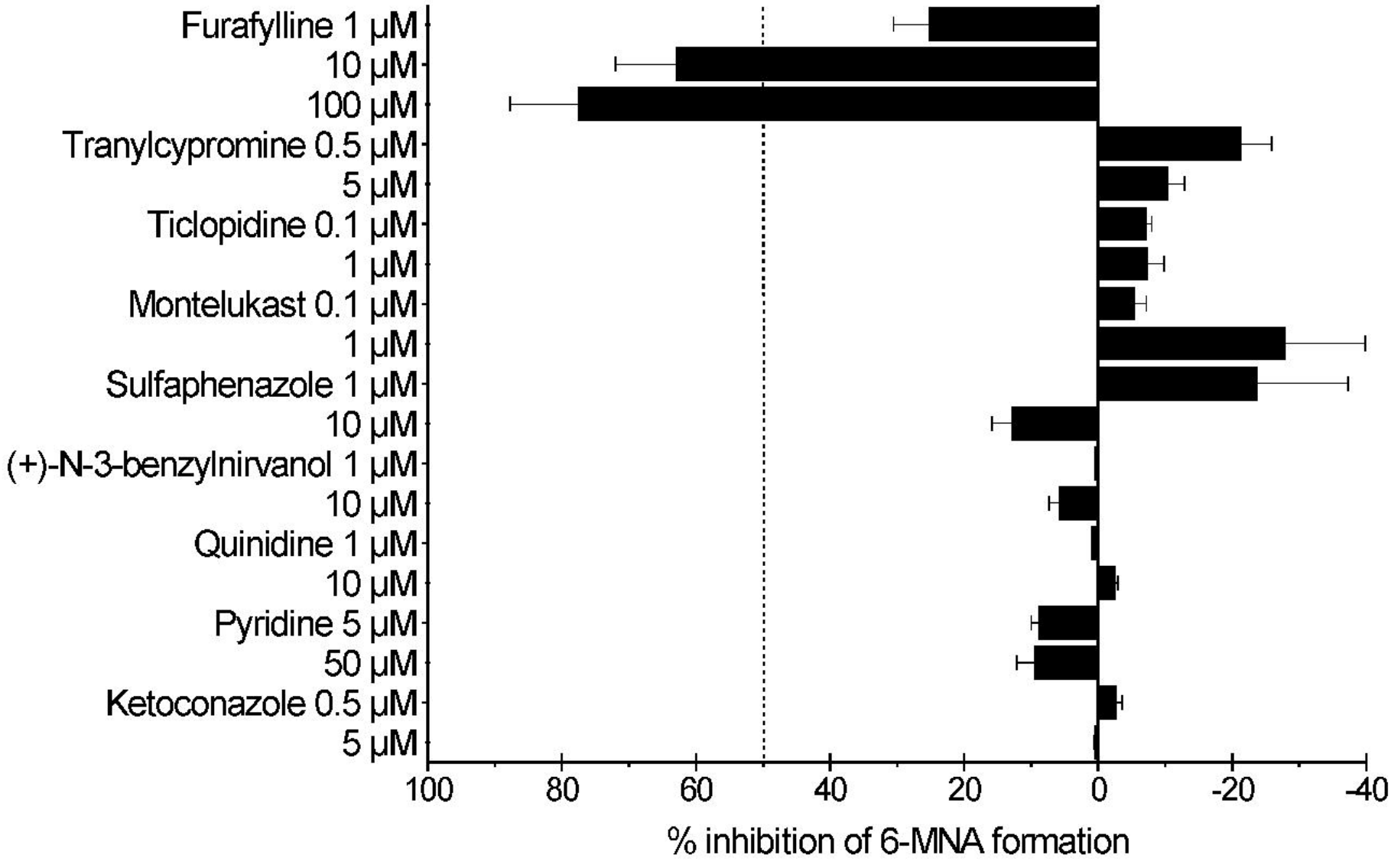

Furafylline 1 μM
10 μM
100 μM
Tranylcypromine 0.5 μM
5 μM
Ticlopidine 0.1 μM
1 μM
Montelukast 0.1 μM
1 μM
Sulfaphenazole 1 μM
10 μM
(+)-N-3-benzylnirvanol 1 μM
10 μM
Quinidine 1 μM
10 μM
Pyridine 5 μM
50 μM
Ketoconazole 0.5 μM
5 μM
100
80
60
40
20
0
-20
-40
% inhibition of 6-MNA formation

Figure 5.

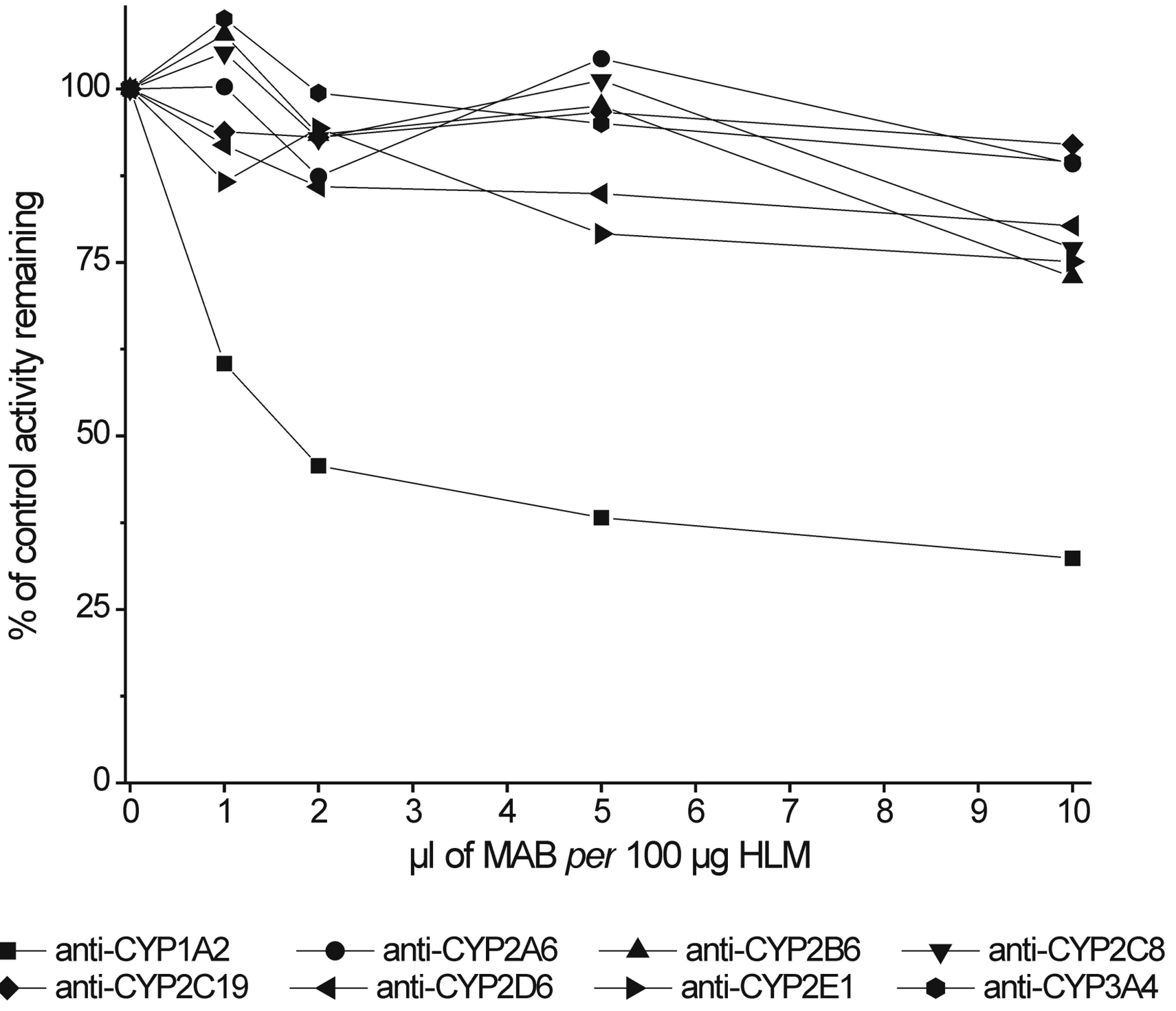

% of control activity remaining
100
75
50
25
0
0
1
2
3
4
5
6
7
8
9
10
µl of MAB *per* 100 µg HLM
anti-CYP1A2
anti-CYP2A6
anti-CYP2B6
anti-CYP2C8
anti-CYP2C19
anti-CYP2D6
anti-CYP2E1
anti-CYP3A4

Figure 6.

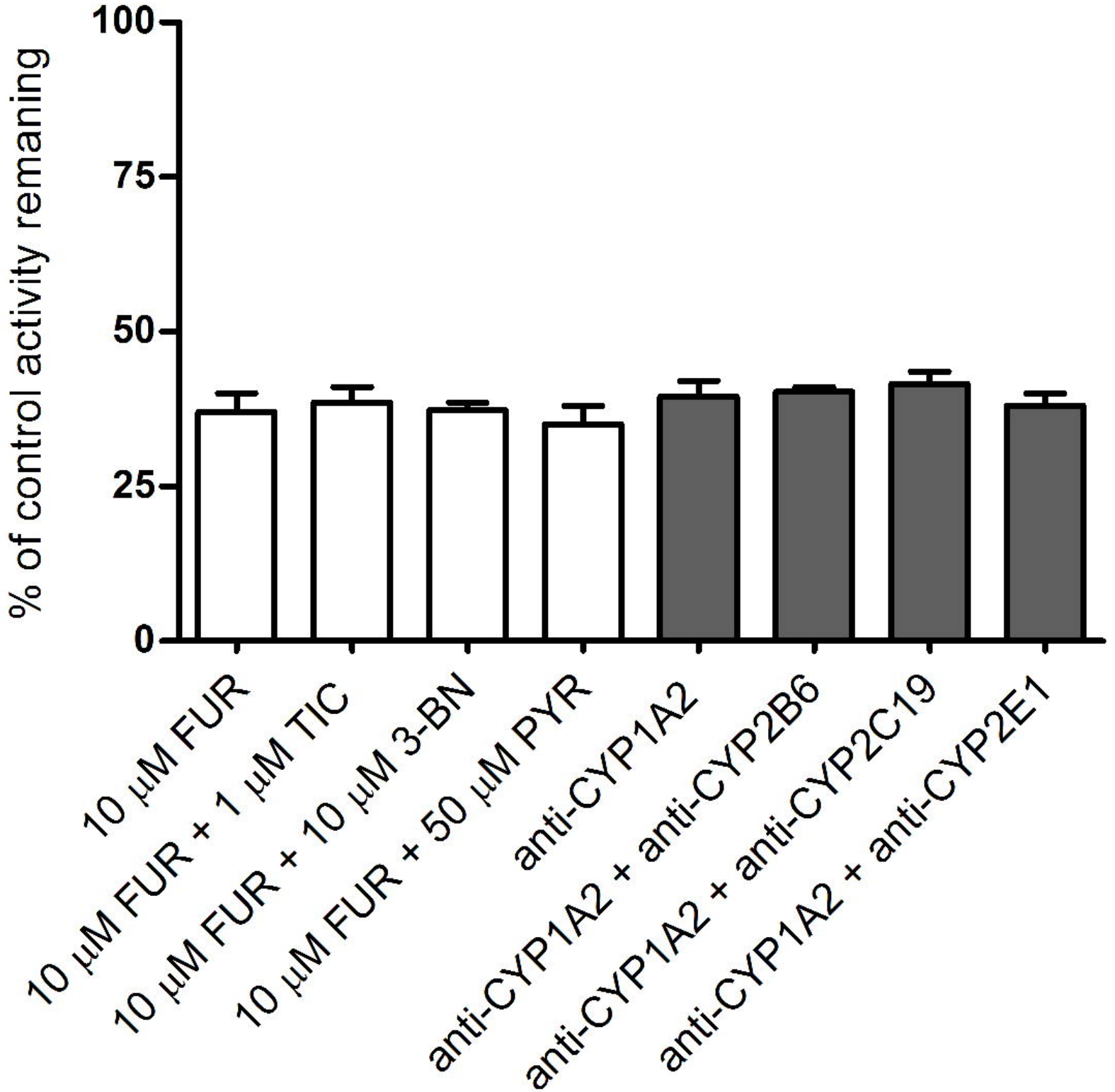

% of control activity remaning
100
75
50
25
0
10 μM FUR
10 μM FUR + 1 μM TIC
10 μM FUR + 10 μM 3-BN
10 μM FUR + 50 μM PYR
anti-CYP1A2
anti-CYP1A2 + anti-CYP2B6
anti-CYP1A2 + anti-CYP2C19
anti-CYP1A2 + anti-CYP2E1

Figure 7.

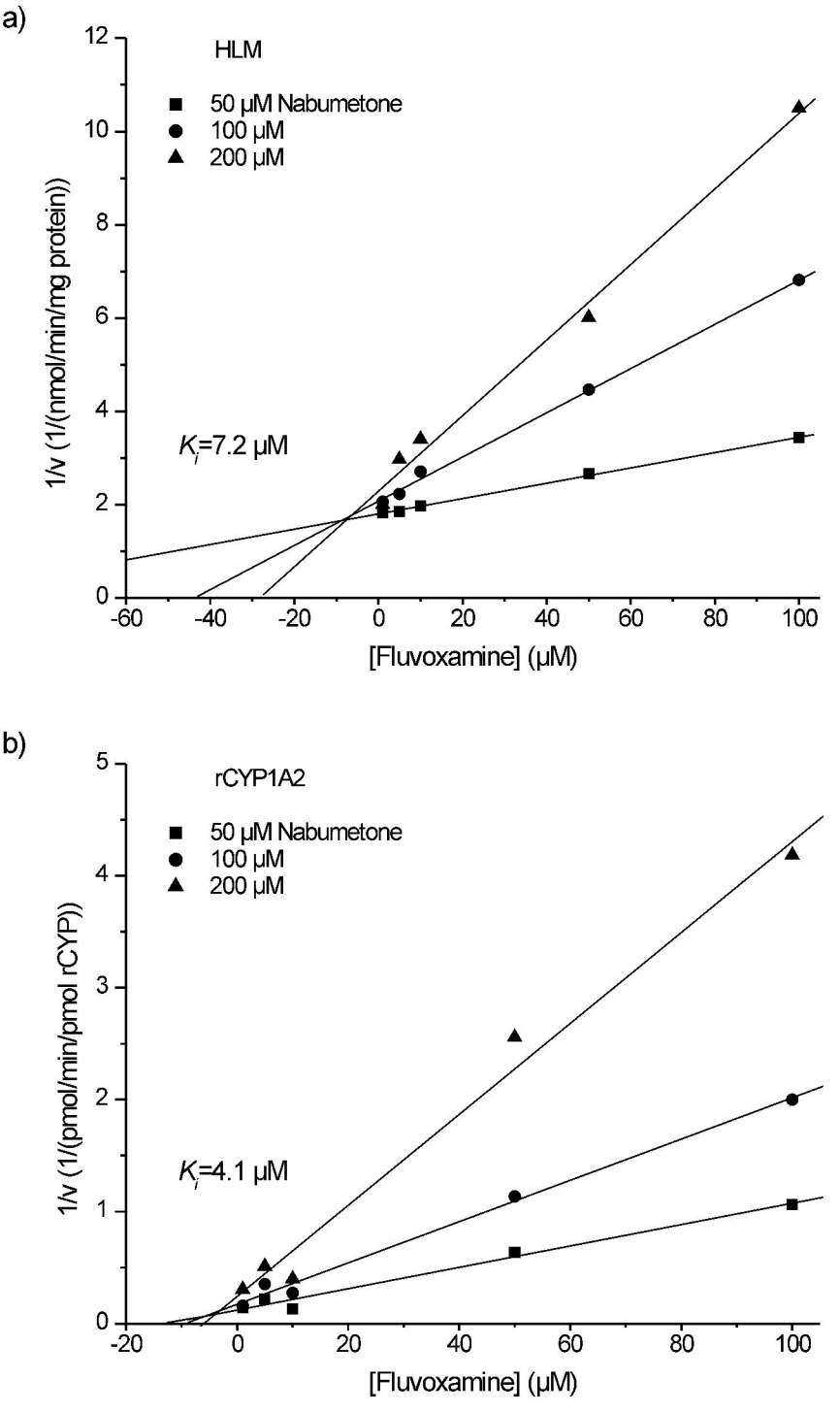
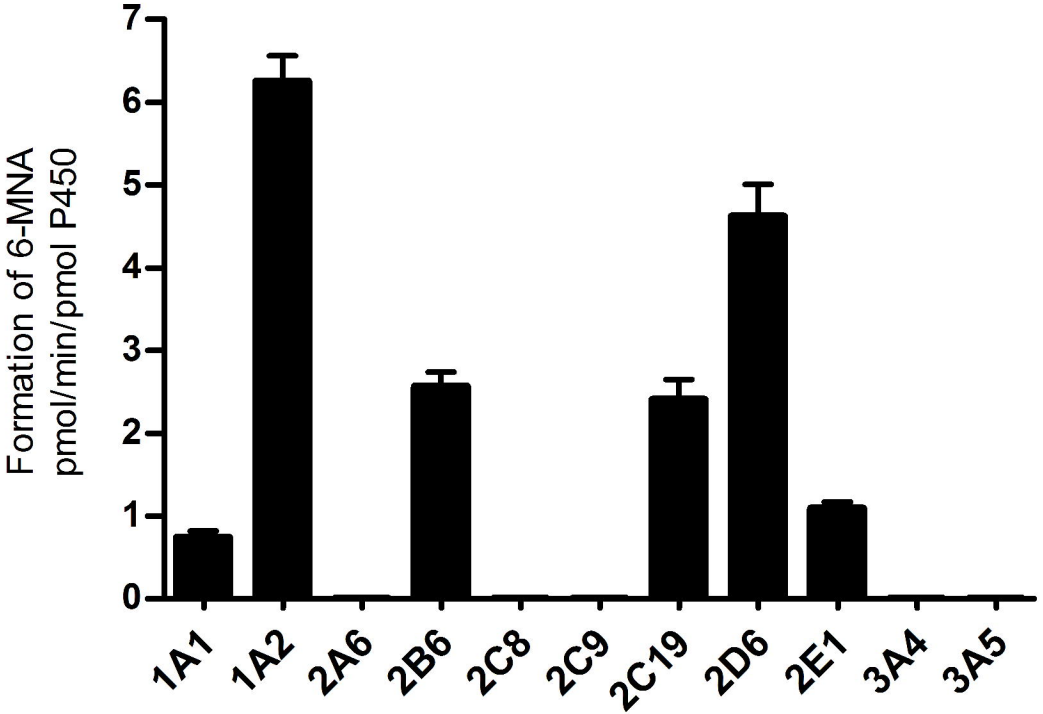

a)
HLM
50 µM Nabumetone
100 µM
200 µM
K_i=7.2 µM
1/v (1/(nmol/min/mg protein))
[Fluvoxamine] (µM)
b)
rCYP1A2
50 µM Nabumetone
100 µM
200 µM
K_i=4.1 µM
1/v (1/(pmol/min/pmol rCYP))
[Fluvoxamine] (µM)

Figure 8.


Formation of 6-MNA
pmol/min/pmol P450
0
1
2
3
4
5
6
7
1A1
1A2
2A6
2B6
2C8
2C9
2C19
2D6
2E1
3A4
3A5