A Predominant Role of CYP1A2 for the Metabolism of Nabumetone to the Active Metabolite, 6-Methoxy-2-naphthylacetic Acid, in Human Liver Microsomes

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ABSTRACT:

Nabumetone, a widely used nonsteroidal anti-inflammatory drug, requires biotransformation into 6-methoxy-2-naphthylacetic acid (6-MNA), a close structural analog to naproxen, 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 (P450) enzyme(s) responsible for 6-MNA formation. In human liver microsomes (HLMs) 6-MNA formation displayed monophasic Michaelis-Menten kinetics with apparent $K_m$ and $V_{max}$ values (mean ± S.D.) of 75.1 ± 15.3 μM and 1304 ± 226 pmol/min/mg protein, respectively, and formation rate of 6-MNA varied approximately 5.5-fold (179–983 pmol/min/mg protein, respectively, and formation rate of 6-MNA varied approximately 5.5-fold (179–983 pmol/min/mg protein) with only minor contribution of other P450s. Additional correlations were found with model activities of CYP2C19 and CYP3A4. Of 11 cDNA-expressed recombinant P450s used, recombinant CYP1A2 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 catalyzed by recombinant P450s CYP1A1, CYP2B6, CYP2C19, CYP2D6, and CYP2E1. Experiments with P450-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 P450s did not show significant inhibitory effects. Taken together, these studies indicate that the formation of the active metabolite of nabumetone, 6-MNA, is predominantly catalyzed by CYP1A2 in HLMs with only minor contribution of other P450s.

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Nabumetone is a nonsteroidal anti-inflammatory drug used mainly for the management of pain and inflammation in patients with osteoarthritis or rheumatoid arthritis (Dahl, 1993; Hedner et al., 2004). Because of its nonacidic nature and prodrug formulation, nabumetone has been considered to offer distinct advantages over other nonsteroidal anti-inflammatory drugs 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-naphthylactic acid (6-MNA) (Haddock et al., 1984; Hyneck, 1992; Davies, 1997). 6-MNA is a close structural analog of naproxen (Fig. 1) and effects more potent and more selective inhibition of cyclooxygenase-2 compared with 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-naphthylactic acid as the principal component in humans. Approximately 75% of a radiolabeled dose of nabumetone was recovered within 48 h in urine, mainly consisting of 6-MNA and 6-hydroxy-2-naphthylactic acid 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 (P450) 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

ABBREVIATIONS: 6-MNA, 6-methoxy-2-naphthylacetic acid; P450, cytochrome P450; HLM, human liver microsome; ISTD, internal standard; ACN, acetonitrile.
clinical importance of nabumetone and the prodrug nature of this drug, our aim was to characterize the P450 isoenzymes responsible for metabolizing nabumetone to 6-MNA in vitro. Various approaches using human liver microsomes (HLMs) with a panel of chemical inhibitors and specific monoclonal antibodies, as well as correlation analysis with model P450 activities and recombinant expressed P450 enzymes, were used. In addition, the potential of in vivo drug interactions caused by certain potentially coadministered drugs was investigated.

Materials and Methods

Chemicals. Nabumetone [4-(6-methoxy-2-naphthyl)-2-butane] and 6-MNA were purchased from Fluka (Buchs, Switzerland), and naproxen [5S-(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid] was from Sigma-Aldrich (St. Louis, MO). S-Mephénytoin was a gift from Professor Urs Meyer (Biozentrum, Basel, Switzerland), and diclofenac and 4’-hydroxyclofenac were donated from Novartis AG (Basel, Switzerland). Propafenone, verapamil, and prostaglandin E1 were from Toronto Research Chemicals (North York, ON, Canada), and phenacetin was from Sigma-Aldrich and was used from Sigma-Aldrich and was used from Fluka. All the other chemicals were used from Sigma-Aldrich and were from the highest purity available. Water was freshly prepared in-house with a Milli-Q (Millipore, Molsheim, France) purification system and was ultrapure grade.

Human Liver Samples. Human liver tissue was obtained as non-tumorous tissue surrounding 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 the samples included age, gender, medical diagnosis, presurgical medication including known P450 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, and written informed consent from each patient was obtained before 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 used for primary screening.

Incubations Using HLMs. Incubation mixtures contained 0.30 mg of microsomal protein/ml, 0.1 M potassium phosphate buffer, pH 7.4, NADPH-generating system (5 mM MgCl2, 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 (ACN). The final amount of ACN in the incubation mixture was 0.5% (v/v), which is expected to have a minimal effect on the studied P450 activities (Chauvet et al., 1998). Reaction mixture, in a final volume of 200 µl, was preincubated for 2 min at 37°C 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 ACN containing naproxen (20 µM) as an ISTD. Samples were then subsequently cooled in an ice bath to precipitate the proteins. The mixture was vortex-mixed and spun at 10,000 g at 15 min. 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 et al. (2003). Shortly, the separation and detection of 6-MNA and naproxen (20 µM, ISTD) was performed by high-performance liquid chromatography/UV (HP 1100; Agilent Technologies, Waldbronn, Germany) equipped with a LiChrospher 100 RP-18 column (100 × 3 mm, 5-µm particle size; Merck, Darmstadt, Germany). An isocratic elution of ACN/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 used with respect to activities of 6-MNA formation (50 µM 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-mephénytoin 4’-hydroxylation), CYP2D6 (propafenone 5-hydroxylation), CYP2E1 (chlorozoxazone 6-hydroxylation), and verapamil N-deethylation (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 methanol, 20 min), and CYP2E1 activity with 75 µM chlorozoxazone (methanol, 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 microsomal protein. Otherwise, the incubations were carried out as described above. Formation of all the metabolites was verified to be linear with respect to both incubation time and protein content under these assay conditions.

An HP Series 1100 LC-MSD system (Agilent Technologies) with binary pump, degasser, autosampler, and mass selective detector equipped with an electrospray ion source was used in all the model activity analyses. Chromatography for phenacetin and diclofenac assays was performed on a Phenomenex (Torrance, CA) Luna C8 column (2.0 × 150 mm, 5-µm particle size), and for amodiaquine and chlorozoxazone assay a Waters (Milford, MA) SymmetryShield C18 column (2.0 × 150 mm, 5-µm particle size) was used. A flow rate of 0.5 ml/min for the mobile phase was used for all the 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 Table 1.

Standardization 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 ISTD calibration were obtained by weighted (1/²) linear regression for the peak-area ratio of the
analyte to the respective ISTD 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.

Analyses for correlations and statistical significances were carried out using GraphPad Prism 3.03 software (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 < 0.05.

**Kinetic Analysis**. Estimates of apparent $K_m$ and $V_{max}$ values were determined by nonlinear least square regression using GraphPad Prism 3.03 software (GraphPad Software Inc.) and the Michaelis-Menten equation. The data were also analyzed by Edie-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 P450 enzymes (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5) coexpressing 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 P450 enzymes (50 pmol of P450 ml⁻¹). Incubations were carried out according to manufacturer’s instructions. In brief, 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 Immuno-inhibition Studies**. Inhibition studies with two concentrations of P450-selective reference inhibitors were carried out at a final nabumetone concentration of 50 μM. Inhibitor concentrations were selected based on 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), ticloidipine (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 dimethyl sulfoxide), and incubations were carried out as described under *Incubations Using HLMs*. Because of the mechanism-based inhibition nature of furafylline (Kunze and Trager, 1993) and ticloidipine (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 the 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 $K_i/2$, $K_{max}$, and $2 \times K_{max}$) 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 P450 isoenzyme-specific monoclonal antibodies raised against CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 (BD Biosciences Discovery Labware) were performed according to the manufacturer’s instructions. In brief, distinct amounts of antibody (or comparable amount of 25 nM Tris buffer, pH 7.5, for vehicle controls) were added to microsomes and incubated for 15 min (20 min for anti-CYP2A6) 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) and IRD800 labeled secondary anti-mouse antibody (Li-cor Biosciences GmbH, Bad Homburg, Germany). CYP1A2 apoprotein was quantified using cDNA-expressed CYP1A2 (BD Biosciences Discovery Labware) 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) and Odyssey software version 2.1B. All of the samples were measured at least in duplicate, and mean values were calculated and expressed in picomole per milligrams of microsomal protein.

**Results**

In the presence of NADPH, 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 protein, with a mean value of 561 pmol/min/mg of protein (Fig. 2). 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 protein, respectively (Fig. 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 ($r = 0.85$, $p < 0.0001$) and with immunologically determined CYP1A2 protein content ($r = 0.74$, $p < 0.0001$) (Fig. 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 Fig. 2, regular smokers had significantly increased levels of

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**TABLE 1**

**Analytical parameters for CYP1A2, CYP2C8, CYP2C9, and CYP2E1 assays**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Analyte, Concentration, and SIM Mass</th>
<th>Internal Standard, Concentration, and SIM Mass</th>
<th>Ionization Mode</th>
<th>Capillary Voltage</th>
<th>Fragmentor Voltage</th>
<th>Mobile Phase</th>
<th>Gradient, %B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Acetaminophen (0.25–20 μM) m/z 152</td>
<td>Acetaminophen-D4 (1 μM) m/z 156</td>
<td>ESI+</td>
<td>2.5</td>
<td>60</td>
<td>0.1% (v/v) Formic acid (A) and 0.1% (v/v) formic acid in acetonitrile (B)</td>
<td>2(0) → 30(5) → 80(7)</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>N′-Deethylamodiaquine (0.1–20 μM) m/z 328</td>
<td>N′-Deethylamodiaquine-D5 (0.5 μM) m/z 333</td>
<td>ESI+</td>
<td>1.5</td>
<td>80</td>
<td>5 mM Ammonium acetate, pH 3.0 (A), and acetonitrile (B)</td>
<td>30(0) → 55(9)</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>4-Hydroxydiclofenac (0.05–10 μM) m/z 310</td>
<td>4-Hydroxydiclofenac-D4 (1 μM) m/z 314</td>
<td>ESI−</td>
<td>3.5</td>
<td>50</td>
<td>5 mM Ammonium acetate, pH 3.0 (A), and acetonitrile (B)</td>
<td>5(0) → 35(5)</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>6-Hydroxychlorzoxazone (0.1–10 μM) m/z 184</td>
<td>6-Hydroxychlorzoxazone-13C6 (2 μM) m/z 190</td>
<td>ESI−</td>
<td>3.5</td>
<td>80</td>
<td>Water (A) and acetonitrile (B)</td>
<td>70 Isocratic</td>
</tr>
</tbody>
</table>
CYP1A2 protein (120 versus 53 pmol/mg microsomal protein; \( p = 0.04 \)), phenacetin \( O \)-deethylation activity (3137 versus 1119 pmol/min/mg protein; \( p = 0.005 \)), and 6-MNA formation (744 versus 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 weak because 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, 10, and 100 \( \mu \)M concentration levels, respectively. The effect of other inhibitors was less than 15% to the 6-MNA activity (Fig. 4). In immunoinhibition experiments with diverse anti-P450 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 (Fig. 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 formation activity (Fig. 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 \( \mu \)M in HLMs and recombinant CYP1A2, respectively (Fig. 7).

A screen of 11 human recombinant P450s showed that all the other recombinant P450s tested except CYP2A6, CYP2C8, CYP2C9, CYP3A4, and CYP3A5 were able to catalyze the formation of 6-MNA (Fig. 8). Taken into account the average human hepatic P450 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 P450s contributed only approximately 10% of the total activity (Table 4). The kinetics of 6-MNA formation was further assessed in recombinant CYP1A2 and was found to follow a first-order Michaelis-Menten kinetics with apparent \( K_m \) and \( V_{max} \) values of 45.1 \( \mu \)M 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 characterized the enzymes responsible for 6-MNA formation in the presence of HLMs and recombinant P450 enzymes. Based on the present findings, nabumetone was found to be metabolized to 6-MNA predominantly by CYP1A2.

At the enzyme activity level, the strongest correlation with 6-MNA formation was seen with CYP1A2-associated phenacetin O-demethylation 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 because of 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 as a result of common regulation mechanisms and cofactors (e.g., P450 reductase). In addition, verapamil N-demethylation to norverapamil is catalyzed 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, 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 toward 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 and 4.1 $\mu$M in HLMs and recombinant CYP1A2, respectively. Usually higher IC$_{50}$ and/or $K_i$ values in HLMs compared with recombinant enzymes suggest the contribution of multiple P450s in the metabolic reaction. In addition, in a situation where multiple enzymes participate in the biotransformation process, diagnostic inhibitors tend to lose their power in identifying the particular P450s involved. Taking into account the slight discrepancy observed in fluvoxamine inhibition and the fact that also recombinant CYP1A1, CYP2B6, CYP2C19, CYP2D6, and CYP2E1 were able to catalyze 6-MNA formation, it also is likely that other P450s besides CYP1A2 may play a minor role in the metabolism of nabumetone to 6-MNA. Inhibition of CYP1A2 was found to result in an approximately 60% decrease in 6-MNA formation. It is also of note that CYP2A6 was found to inhibit 6-MNA formation. Usually higher IC$_{50}$ and/or $K_i$ values in HLMs compared with recombinant enzymes suggest the contribution of multiple P450s in the metabolic reaction. In addition, in a situation where multiple enzymes participate in the biotransformation process, diagnostic inhibitors tend to lose their power in identifying the particular P450s involved. Taking into account the slight discrepancy observed in fluvoxamine inhibition and the fact that also recombinant CYP1A1, CYP2B6, CYP2C19, CYP2D6, and CYP2E1 were able to catalyze 6-MNA formation, it also is likely that other P450s besides CYP1A2 may play a minor role in the metabolism of nabumetone to 6-MNA. Inhibition of CYP1A2 was found to result in an approximately 60% decrease in 6-MNA formation. Inhibition of P450 enzymes is the most common cause of drug-drug interactions and has led to the removal of several drugs from the market during the past years (Lasser et al., 2002; Issa et al., 2007). P450 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 drug-drug interaction 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 P450 interaction studies concerning nabumetone are basically nonexistent. 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/induc-

**FIG. 5.** Effects of P450 isoenzyme-specific monoclonal antibodies (MABs) on the formation of 6-MNA in a pool of HLMs (50 μM nabumetone).

**FIG. 6.** Effects of 10 μM furafylline (FUR) in combination with selected chemical P450 inhibitors (white bars) and anti-CYP1A2 antibody in combination with selected P450 antibodies (gray bars) into 6-MNA formation in HLMs (50 μM nabumetone, 5 μl of each antibody per 100 μg of HLM). TIC, ticlopidine; 3-BN, (+)-N-3-benzyl-nirvanol; PYR, pyridine.

**FIG. 7.** Dixon plots for the inhibition of 6-MNA formation by fluvoxamine in a pool of HLMs (a) and in cDNA-expressed human CYP1A2 (b). Nabumetone concentrations are selected to correspond to the mean $K_{m}/2$, $K_{m}$ and $2 \times K_{m}$ for 6-MNA formation. Each point represents the mean of duplicate measurements.

**FIG. 8.** Formation of 6-MNA in a panel of cDNA-expressed human P450s. Each bar represents a mean ± S.D. of triplicate samples (50 μM nabumetone).
tion 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 P450 involved in the 6-MNA formation, induction of minor P450s 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., 2008).

Besides the aspects of drug interactions, characterization 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; Gunes and Dahl, 2008). The difference between individuals has been traditionally considered to be mainly a result of 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). For instance, in the case of the 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 toward the same substrate may differ, and their tissue expression patterns are highly distinct (Tassaneeyakul et al., 1993; Ding and Kaminsky, 2003). Also with nabumetone, both recombinant CYP1A1 and CYP1A2 were active in 6-MNA formation, but the difference in catalytic activity was notable. Regarding other extrahepatic P450s, a 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 P450s 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 P450s in the liver, it is likely that the role of CYP2J2—and CYP1A1—to the overall metabolism of nabumetone remains relatively small. However, CYP1A1 may possess some role in the first pass of nabumetone because 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 P450 enzymes involved in the metabolism of nabumetone to the pharmacologically active compound 6-MNA in the present study. The data strongly suggest a central role of CYP1A2 in the nabumetone bioactivation. In addition, other P450s including CYP2B6, CYP2C19, CYP2D6, and CYP2E1 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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References.


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