INFLUENCE OF N-SUBSTITUTION OF 7-METHOXY-4-(AMINOMETHYL)-COUMARIN ON CYTOCHROME P450 METABOLISM AND SELECTIVITY

JENNIFER VENHORST, ROB C. A. ONDERWATER, JOHN H. N. MEERMAN, JAN N. M. COMMANDEUR, AND NICO P. E. VERMEULEN

Leiden/Amsterdam Center for Drug Research (LACDR), Division of Molecular Toxicology, Department of Pharmacochemistry, Vrije Universiteit, Amsterdam

(Received April 25, 2000; accepted August 15, 2000)

This paper is available online at http://www.dmd.org

ABSTRACT:

A series of six structural analogs of 7-methoxy-4-(aminomethyl)-coumarin (MAMC), a recently developed high-throughput substrate of P450 2D6 (CYP2D6), was synthesized to investigate the influence of N-substitution on the metabolism by cytochrome P450s, as well as on P450 selectivity. The analogs were obtained by introducing alkyl substituents at the amino group of MAMC and by replacing this moiety with a pyridine group. Competition experiments using heterologously expressed CYP2D6 demonstrated that the introduction and elongation of alkyl substituents strongly decreased the IC50 values toward dextromethorphan O-demethylation. Metabolism studies showed that the regioselectivity of metabolism was unaffected by the varying N substituents, as only O-dealkylation of the analogs and no N-dealkylation was observed. In excellent agreement with the competition experiments, metabolism studies also showed that elongation of the alkyl chain dramatically increased the affinity of the compounds toward CYP2D6, as indicated by an up to 100-fold decrease in Km values. The Vmax values displayed a much less pronounced decrease with an increasing N-alkyl chain, resulting in as much as a 30-fold increase in the Vmax/Km value. Interestingly, due to the higher fluorescent yield of the N-alkyl metabolites compared with the metabolite of MAMC, O-dealkylation of N-methyl MAMC by CYP2D6 can be measured with a more than 3-fold higher sensitivity. Studies on P450 selectivity showed that only CYP1A2 and CYP2D6 contribute to the O-dealkylation of the N-alkyl analogs in both heterologously expressed P450s and human liver microsomes. In sharp contrast to CYP2D6, N-dealkylation of MAMC did not significantly affect the Km values of O-dealkylation by CYP1A1, but it did result in higher Vmax values. Finally, CYP1A2 also N-dealkylated the analogs.

An important role in the development of new drugs concerns the preclinical study of drug metabolism (Rodrigues, 1997). Drug metabolism involves the superfamily of hepatic cytochrome P450s (P450s)1 (Spatzegger and Jaeger, 1995). Although the P450s generally function as a detoxification mechanism by converting their substrates into more easily excretable products (Lewis and Pratt, 1998), toxic metabolites can also be formed (Goeptar et al., 1995; Vermeulen, 1996). Other important features of P450s are drug-drug interactions, occurring when two simultaneously administered drugs compete for the same enzyme, and the highly variable activity of P450s among individuals (Tucker, 1994; Smith and Jones, 1999; Wormhoudt et al., 1999). Both will have an impact on the efficacy of administered drugs because of altered pharmacokinetics (Lin and Lu, 1997). Therefore, high-affinity binding to drug-metabolizing enzymes and metabolism by genetically polymorphic enzymes are often considered major criteria for aborting the development of a new chemical entity into a drug. Understanding the structure-activity relationship of ligands of the individual P450s is consequently of great value.

The human P450 isoforms mainly involved in the metabolism of exogenous compounds are CYP1A2, CYP3A4, CYP2C9, CYP2C19, and CYP2D6 (Smith and Jones, 1992; Spatzegger and Jaeger, 1995). Over the years, ample in vitro inhibition assays have been described for these enzymes, which have provided a vast source of information regarding the specificity and selectivity of ligand binding by individual P450s (Smith et al., 1997). Due to the recent availability of high-throughput inhibition assays, the number of identified ligands of P450s is now growing even more rapidly (Rodrigues et al., 1994; Crespi, 1999). Apart from studies on the binding characteristics of compounds, extensive efforts have also been made to elucidate the metabolic routes of the P450s. In the case of CYP2D6, it was originally thought that this enzyme only catalyzed C-hydroxylation and O-dealkylation reactions. More recently, however, it was shown that this enzyme is also capable of performing N-dealkylation reactions (Coutts et al., 1994; De Groot et al., 1995).

We recently described the development and application of a novel
NADPH were from Roche Molecular Biochemicals (Mannheim, Germany). 7-Hydroxy-4-(methylpyridinium)-coumarin was a generous gift from Yamamoto. 7-Methoxy-4-(bromomethyl)-coumarin was obtained from Acros (Geel, Belgium). MAMC were synthesized as described previously (Onderwater et al., 1999). CYP2D6 was investigated, and the corresponding enzyme kinetic parameters were determined. Finally, the effect of structural variation on the selectivity of the metabolism was tested with both heterologously expressed human P450s and human liver microsomes. The metabolism by significantly contributing P450s was further investigated.

### Materials and Methods

**Chemicals.** MAMC, HAMC, and 7-hydroxy-4-(chloromethyl)-coumarin were synthesized as described previously (Onderwater et al., 1999). 7-Methoxy-4-(bromomethyl)-coumarin was obtained from Acros (Geel, Belgium). 7-Hydroxy-4-(methylpyridinium)-coumarin was a generous gift from Yamamouchi (Leiderdorp, The Netherlands). Glucose-6-phosphate dehydrogenase and NADPH were from Roche Molecular Biochemicals (Mannheim, Germany) and Applichem (Darmstadt, Germany), respectively. Furafylline was obtained from RBI (Natick, MA). Quinidine was from Aldrich (Zwijndrecht, The Netherlands). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

### Synthesis of 4-Substituted 7-Methoxy-4-aminomethyl-coumarins.

A 5 times molar excess of the corresponding primary or secondary amine or pyridine was added to a suspension of 250 mg of 7-methoxy-4-(bromomethyl)-coumarin in 20 ml of acetonitrile. The reaction mixture was left stirring at room temperature for approximately 1 h, resulting in a colorless solution in the case of the N-alkyl amines. Progress of each reaction was followed by thin-layer chromatography using acetone as mobile phase. After completion of the reaction, the mixture was acidified with 6 drops of 47% hydrobromic acid, and the solvent evaporated. The resulting yellow solid was then recrystallized up to two times in 80% isopropanol, after which the beige product could be obtained. In the case of the 4-methylpyridinium analog, the product was obtained by filtration of the suspension, resulting in a white powder. The identity of each product was established by 1H NMR (dimethyl sulfoxide-d6) obtained with a Bruker AC 200 (200.1 MHz) using tetramethylsilane as internal standard. Purities of the products were additionally tested by means of HPLC with UV-absorption detection at 220 nm and fluorescence detection at excitation and emission wavelengths of 330 and 400 nm, respectively. Product yield varied between 25 and 60%, and purities were higher than 98%.

**N-Methyl 7-methoxy-4-(aminomethyl)-coumarin (MAMC):** \( \delta \) 7.75 (1H, d, \( J = 10 \) Hz), 7.10–6.90 (2H, m), 6.35 (1H, s), 4.45 (2H, s), 3.85 (3H, s), 2.65 (3H, s).

**N,N-Dimethyl 7-methoxy-4-(aminomethyl)-coumarin (dMMAMC):** \( \delta \) 7.90 (1H, d, \( J = 10 \) Hz), 7.15–7.00 (2H, m), 6.50 (1H, s), 4.55 (2H, s), 3.95 (3H, s), 2.90 (6H, s).

**N-Propyl 7-methoxy-4-(aminomethyl)-coumarin (PMAMC):** \( \delta \) 7.75 (1H, d, \( J = 10 \) Hz), 7.15–7.00 (2H, m), 6.50 (1H, s), 4.45 (2H, s), 3.85 (3H, s), 3.05 (2H, t, \( J = 10 \) Hz), 1.30–1.25 (2H, m), 0.95 (3H, t, \( J = 7 \) Hz).

**N,N-Dimethyl 7-methoxy-4-(aminomethyl)-coumarin (diPMAMC):** \( \delta \) 7.80 (1H, d, \( J = 10 \) Hz), 7.15–7.00 (2H, m), 6.50 (1H, s), 4.45 (2H, s), 3.86 (3H, s), 3.08 (2H, t, \( J = 7 \) Hz), 1.75–1.55 (2H, m), 1.50–1.25 (2H, m), 0.95 (3H, t, \( J = 8 \) Hz).

**7-Methoxy-4-(methylpyridinium)-coumarin (MMPyrC):** \( \delta \) 9.18 (2H, D, \( J = 8 \)), 8.82–8.65 (1H, m), 8.35–8.20 (2H, m), 7.75 (1H, d, \( J = 10 \) Hz), 7.15–7.00 (2H, m), 6.25 (2H, s), 5.75 (1H, s), 3.90 (3H, s).

### Synthesis of 4-Substituted 7-Hydroxy-4-aminomethyl-coumarins.

A 5 times molar excess of the corresponding N-alkyl amine was added under a nitrogen atmosphere to 1 g of 7-hydroxy-4-(chloromethyl)-coumarin dissolved in 60 ml of acetonitrile. The reaction mixture was left stirring for 48 h at room temperature, during which the progress of the reaction was followed by thin-layer chromatography using acetone as mobile phase. After completion of the reaction, the mixture was acidified with 6 N hydrochloric acid and the solvent evaporated. The resulting yellow solid was taken up in 50 ml of H2O and extracted three times with 50 ml of ethyl acetate to remove unreacted 7-hydroxy-4-(chloromethyl)-coumarin. The combined water layers were subsequently evaporated to dryness. The resulting yellow solid was then recrystallized twice in isopropanol, resulting in the beige product. The identity of the products was established by 1H NMR (dimethyl sulfoxide-d6) obtained with a Bruker AC 200 (200.1 MHz) using tetramethylsilane as internal standard. Purities of the products were additionally tested by means of HPLC with UV-absorption detection at 220 nm and fluorescence detection at excitation and emission wavelengths of 370 and 470 nm, respectively. Product yield varied between 20 and 40%, and purities were higher than 98%.

**N-Methyl 7-hydroxy-4-(aminomethyl)-coumarin (MAMC):** \( \delta \) 10.85 (1H, s), 7.70 (1H, d, \( J = 10 \) Hz), 7.00–6.80 (2H, m), 6.40 (1H, s), 4.45 (2H, s), 2.65 (3H, s).

**N,N-Dimethyl 7-hydroxy-4-(aminomethyl)-coumarin (diMAMC):** \( \delta \) 10.90 (1H, s), 7.88 (1H, d, \( J = 10 \) Hz), 7.05–6.75 (2H, m), 6.65 (1H, s), 4.55 (2H, s), 2.85 (6H, s).

**N-Propyl 7-hydroxy-4-(aminomethyl)-coumarin (PMAMC):** \( \delta \) 10.85 (1H, s), 7.70 (1H, d, \( J = 10 \) Hz), 6.95–6.70 (2H, m), 6.45 (1H, s), 4.45 (2H, s), 3.15 (2H, q, \( J = 12 \) Hz), 1.30 (3H, t, \( J = 10 \) Hz).
and the reaction was stopped by the addition of 10 μM glucose-6-phosphate dehydrogenase. After 0 and 45 min, samples were drawn, added to 15% perchloric acid, the linear gradient was started, increasing the methanol content to 15% at 25 min using a flow rate of 0.6 ml/min. Fluorescence was measured with 2% methanol/1% triethylamine/97% H2O, adjusted to pH 3 using 70% perchloric acid, for MMAMC, diMMAMC, and EMAMC delivered at a flow rate of 0.6 ml/min. For PMAMC and BMAMC, a gradient was used. After eluting for 10 min with 2% methanol/1% triethylamine/97% H2O, adjusted to pH 3 using 70% perchloric acid, the linear gradient was started, increasing the methanol content to 15% at 25 min using a flow rate of 0.6 ml/min. Fluorescence detection was performed at excitation and emission wavelengths of 370 and 470 nm (bandwidths 18 nm), respectively, for detecting products, as well as 330 and 400 nm (bandwidths 18 nm) for detecting P450s. The eluent applied for this compound consisted of 10% methanol/1% triethylamine/89% H2O, adjusted to pH 3 using 70% perchloric acid, with a flow rate of 0.5 ml/min.

Enzyme Kinetics of O-Dealkylation by CYP2D6 and CYP1A2. To investigate the metabolism of the 4-substituted 7-methoxycoumarins by CYP2D6 and CYP1A2, 100 μM of each compound was incubated in a 100 mM phosphate buffer (pH 7.4) with 20 μM dextrorphan and 1 mM NADPH, in the presence of human liver microsomes from three subjects (designated A9, A10, and A11) were a generous gift from Ph. Beaune (INSERM U75, Paris, France).

Calculation of Lipophilicities. The lipophilicity of the synthesized 7-methoxy analogs was calculated according to the hydrophobic fragmental constant approach used by Rekker and Mannhold (1992). The log P value of the cozymain structure was obtained from Hansch et al. (1995).

Microsomal Protein. Heterologously expressed human CYP1A2, -2B6, -2C9, -2C19, -2D6, -2E1, and -3A4 (catalog nos. P203, M110a, P258, P259, P171, M106k, and P202, respectively) were all obtained from Gentest Corp. (Woburn, MA). Human liver microsomes from three subjects (designated A9, A10, and A11) were a generous gift from Ph. Beaune (INSERM U75, Paris, France).

A10, and A11) were a generous gift from Ph. Beaune (INSERM U75, Paris, France). A10, and A11) were a generous gift from Ph. Beaune (INSERM U75, Paris, France). Human liver microsomes from three subjects (designated A9, A10, and A11) were a generous gift from Ph. Beaune (INSERM U75, Paris, France).

Calculation of Lipophilicities. The lipophilicity of the synthesized 7-methoxy analogs was calculated according to the hydrophobic fragmental constant approach used by Rekker and Mannhold (1992). The log P value of the cozymain structure was obtained from Hansch et al. (1995).

Microsomal Protein. Heterologously expressed human CYP1A2, -2B6, -2C9, -2C19, -2D6, -2E1, and -3A4 (catalog nos. P203, M110a, P258, P259, P171, M106k, and P202, respectively) were all obtained from Gentest Corp. (Woburn, MA). Human liver microsomes from three subjects (designated A9, A10, and A11) were a generous gift from Ph. Beaune (INSERM U75, Paris, France).

Calculation of Lipophilicities. The lipophilicity of the synthesized 7-methoxy analogs was calculated according to the hydrophobic fragmental constant approach used by Rekker and Mannhold (1992). The log P value of the cozymain structure was obtained from Hansch et al. (1995).
Excitation and emission maxima of the fluorescent 7-methoxy and 7-hydroxy 4-substituted coumarins

<table>
<thead>
<tr>
<th>Compound</th>
<th>Excitation Maximum</th>
<th>Emission Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAMC</td>
<td>326</td>
<td>396</td>
</tr>
<tr>
<td>MMAMC</td>
<td>342</td>
<td>402</td>
</tr>
<tr>
<td>EMAMC</td>
<td>342</td>
<td>403</td>
</tr>
<tr>
<td>PMAMC</td>
<td>342</td>
<td>403</td>
</tr>
<tr>
<td>BMAMC</td>
<td>342</td>
<td>403</td>
</tr>
<tr>
<td>DiMMAMC</td>
<td>342</td>
<td>405</td>
</tr>
<tr>
<td>MMPyrC</td>
<td>342</td>
<td>404</td>
</tr>
<tr>
<td>Metabolite</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAMC</td>
<td>370</td>
<td>470</td>
</tr>
<tr>
<td>BMHAC</td>
<td>373</td>
<td>472</td>
</tr>
<tr>
<td>EHAMC</td>
<td>378</td>
<td>473</td>
</tr>
<tr>
<td>PHAMC</td>
<td>378</td>
<td>473</td>
</tr>
<tr>
<td>BHAMC</td>
<td>378</td>
<td>473</td>
</tr>
<tr>
<td>DiMHMAC</td>
<td>382</td>
<td>475</td>
</tr>
<tr>
<td>MHPyrC</td>
<td>321</td>
<td>466</td>
</tr>
</tbody>
</table>

Fluorescent yield of 1 μM 7-hydroxycoumarins measured in a microplate reader at excitation and emission wavelengths of 405 (bandwidth 8 nm) and 460 nm (bandwidth 30 nm), respectively, in a 100 mM phosphate buffer (pH 7.4). The excitation wavelength of 405 nm was chosen to eliminate interference from background fluorescence of NADPH. HMPyrC could not be detected under these conditions.

Modeling. Structures of the analogs of MAMC and their low energy conformers were generated as described previously (Onderwater et al., 1999). An active site model of CYP2D6, based on a multi-alignment of CYP2D6 (to be published; available upon request) with the crystallographically resolved bacterial P450s terp (Hasemann et al., 1994), cam (Poulos et al., 1985), BM3 (Ravichandran et al., 1993), eryF (Cupp-Vickery and Poulos, 1995), and nor (Park et al., 1997), was built using the Homology Modeling Module of InsightII (Biosym/MSI, San Diego, CA). The protein model was relaxed by first minimizing the side chains, followed by a minimization with the backbone tethered using the default settings of the Discover program (Biosym/MSI).

The low energy conformers of each analog were manually docked into the active site model of CYP2D6 in such a way that the basic amino nitrogen atom of the substrate could form a hydrogen bond with either Asp90 or Glu116 and with the site of oxidation directed toward the heme iron atom. The complexes were subsequently energy-minimized and evaluated by means of the interaction energy between the substrate and active site residues and the distance between the site of oxidation and heme iron atom. The criterion for the latter was set at a maximal distance of 4.5 Å.

All minimizations were carried out with the Discover program (Biosym/MSI) using the conjugate gradient method and the consistent valence force field implemented with the heve parameters obtained from Paulsen and Ornstein (1991, 1992).

Results

Fluorescent Properties of the 4-Substituted Coumarins. The 4-substituted 7-methoxy coumarins and their putative 7-hydroxy metabolites, as shown in Fig. 1, were synthesized and their fluorescent characteristics determined. As shown in Table 1, the introduction of N-alkyl substituents consistently shifted the excitation maximum of the 7-methoxy analogs from 326 to 342 nm. The nature of the incorporated substituent did not affect the excitation maxima. Effects of N-substitution on emission maxima were less pronounced. In the case of the putative 7-hydroxy metabolites, alkylation of the nitrogen atom resulted in a smaller shift of the excitation maxima to longer wavelengths. In contrast, incorporation of a positively charged pyridine moiety at the 4-methyl position in the case of HMPyrC resulted in a shift of the excitation maximum to lower wavelengths. As a result, this product cannot be accurately quantified by fluorescence using a microplate reader due to interference with NADPH fluorescence. The relative fluorescence of the various 7-hydroxy compounds at a concentration of 1 μM, and excitation and emission wavelengths of 405 (bandwidth 8 nm) and 460 nm (bandwidth 30 nm), respectively, was also determined (Fig. 2). Interestingly, the intrinsic fluorescence of the 7-hydroxy N-alkyl analogs was 3.8 to 5.5 times higher than HAMC, the O-dealkylated metabolite of MAMC.

Competition Experiments. The relative binding affinities of MAMC and its synthesized analogs for CYP2D6 were determined by competition experiments in the presence of 10 μM of the CYP2D6 model substrate dextromethorphan. The observed IC50 values, as listed in Table 2, indicate that addition of a single N-alkyl substituent to MAMC, and subsequent enlargement of its chain length, markedly increases the affinity of the coumarins for CYP2D6. However, the introduction of a second N-methyl group in diMMAMC resulted in a reduction in affinity for CYP2D6 when compared with MAMC and the mono-N-methyl analog. Interestingly, MMPyrC had an affinity toward CYP2D6 similar to that of MAMC. When examining the calculated lipophilicities of the compounds (Table 2), an excellent correlation (R2 = 0.978) was obtained between log of and the log IC50 values (Fig. 3). On statistical grounds, diMMAMC was excluded from this correlation.

Metabolite Identification of CYP2D6-Mediated Metabolism. Because all of the MAMC analogs displayed affinity toward CYP2D6, our study was continued by investigating the metabolism of the structural analogs by this enzyme. Metabolite identification by HPLC analysis of the incubation mixtures, containing 100 μM of each analog and 20 nM CYP2D6, showed that in all cases O-dealkylation to the corresponding 7-hydroxy compounds occurred. Formation of MAMC and HAMC, which were anticipated as metabolites resulting from N-dealkylation (Fig. 4), in the case of the mono-N-alkyl analogs, was not detected. DiMMAMC was not O-dealkylated by CYP2D6, either.

Enzyme Kinetics of CYP2D6-Mediated O-Dealkylation. For determining the enzyme kinetics of O-dealkylation of the N-alkyl MAMC analogs by heterologously expressed CYP2D6, the incubations were carried out in real-time at 37°C, with the exception of MMPyrC. For this compound, enzyme kinetics was determined by means of HPLC analysis. Product formation was linear up to at least 45 min and displayed apparent Michaelis-Menten kinetics for all analogs. As shown in Table 3, all MAMC analogs are good substrates of CYP2D6, with no Km values above 16.31 ± 0.73 μM. It can also be seen that addition
**TABLE 2**

IC<sub>50</sub> values and lipophilicities of the 4-substituted 7-methoxycoumarins

The IC<sub>50</sub> measurements were performed with CYP2D6 expressed in human lymphoblasts, and dextromethorphan as reference compound. Values are the mean ± S.D. (n = 3). Lipophilicities (Σf) were calculated according to the hydrophobic fragmental constant approach by Rekker and Mannhold (1992). Because the fragmental constant of the pyridine structure was not available, the lipophilicity of MMPyrC could not be determined.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; Value</th>
<th>Σf</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAMC</td>
<td>132 ± 19</td>
<td>0.392</td>
</tr>
<tr>
<td>MMAMC</td>
<td>90 ± 9</td>
<td>0.642</td>
</tr>
<tr>
<td>EMAMC</td>
<td>26 ± 3</td>
<td>1.161</td>
</tr>
<tr>
<td>PMAMC</td>
<td>2.0 ± 0.1</td>
<td>1.680</td>
</tr>
<tr>
<td>BMAMC</td>
<td>0.51 ± 0.03</td>
<td>2.199</td>
</tr>
<tr>
<td>DiMMAMC</td>
<td>171 ± 16</td>
<td>1.106</td>
</tr>
<tr>
<td>MMPyrC</td>
<td>138 ± 23</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not determined.

**FIG. 3.** Correlation between log IC<sub>50</sub> and the calculated lipophilicities (Σf) of the mono-N-alkyl analogs of MAMC and MAMC itself (R<sup>2</sup> = 0.978).

DiMMAMC (□) was not included in this correlation.

and elongation of a single N-alkyl chain led to a smaller K<sub>m</sub> value, resulting in extremely low values of 0.20 ± 0.03 and 0.080 ± 0.020 μM for the N-propyl and N-butyl analog, respectively. Addition of a second N-methyl group in diMMAMC led to a somewhat higher K<sub>m</sub> value when compared with the mono-N-substituted analog MMAMC, i.e., 10.56 ± 0.36 versus 5.09 ± 0.94 μM. Another observation made was that N-dealkylation and subsequent elongation of the alkyl substituent resulted in a reduction in the maximal rate of metabolism by CYP2D6. The intrinsic clearance of the compounds, however, showed a more than 30-fold increase going from unsubstituted MAMC to the N-butyl-substituted analog. MMPyrC displayed the lowest intrinsic clearance.

**P450 Selectivity in the Metabolism of 4-Substituted 7-Methoxycoumarins.** The P450 selectivity of the novel substrates was first investigated with heterologously expressed P450s. Each of the MAMC analogs was incubated in a concentration of approximately 5 times its K<sub>m</sub> value for CYP2D6 in the presence of 10 nM of the most important drug-metabolizing enzymes, i.e., CYP1A2, -2B6, -2C9, -2C19, -2D6, -2E1, and -3A4. No metabolism could be detected in the case of CYP2B6, -2E1, and -3A4. The observed rates of metabolism by the other P450s is shown in Fig. 5. It can be seen that, at saturating conditions of CYP2D6, both MAMC and the analogs were also metabolized by recombinant CYP1A2 and CYP2C19, albeit to varying degrees. In the case of CYP1A2, only MAMC and MMAMC were metabolized at a lower rate than CYP2D6. The activity of CYP2C19 was, however, lower than that of CYP2D6 for all analogs except diMMAMC and BMAMC. Interestingly, CYP2C9 only metabolized the N-butyl analog of MAMC.

**Metabolism of 4-Substituted 7-Methoxycoumarins in Human Liver Microsomes.** In addition to heterologously expressed P450s, the metabolism of MMAMC and BMAMC was also investigated in human liver microsomes to determine relative contributions of the various P450s to O-dealkylation in this system. Liver microsomes of three subjects (designated A9, A10, and A11) were used. The incubations were performed with 25 μl of microsomal protein and 1 min of microsomal protein and 1 min of microsomal protein and 1 min of microsomal protein and 1 min of microsomal protein and 1 min of microsomal protein.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; Value</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; Value</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAMC</td>
<td>11.52 ± 1.32</td>
<td>2.81 ± 0.14</td>
<td>0.244 ± 0.039</td>
</tr>
<tr>
<td>MMAMC</td>
<td>5.09 ± 0.94</td>
<td>1.72 ± 0.12</td>
<td>0.338 ± 0.084</td>
</tr>
<tr>
<td>EMAMC</td>
<td>1.00 ± 0.08</td>
<td>1.41 ± 0.07</td>
<td>1.410 ± 0.183</td>
</tr>
<tr>
<td>PMAMC</td>
<td>0.20 ± 0.03</td>
<td>0.91 ± 0.03</td>
<td>4.550 ± 0.819</td>
</tr>
<tr>
<td>BMAMC</td>
<td>0.08 ± 0.02</td>
<td>0.70 ± 0.03</td>
<td>8.750 ± 2.537</td>
</tr>
<tr>
<td>DiMMAMC</td>
<td>10.56 ± 0.36</td>
<td>1.20 ± 0.02</td>
<td>0.114 ± 0.005</td>
</tr>
<tr>
<td>MMPyrC</td>
<td>16.31 ± 0.73</td>
<td>1.15 ± 0.02</td>
<td>0.071 ± 0.004</td>
</tr>
</tbody>
</table>

**FIG. 4.** Metabolic routes of the N-alkyl analogs of MAMC mediated by CYP2D6 and CYP1A2.

O-dealkylation of the N-alkyl 7-methoxycoumarins resulted in the corresponding 7-hydroxy compounds, whereas N-dealkylation resulted in MAMC formation. A combination of O- and N-dealkylation resulted in the formation of HAMC.
1.0 μM quinidine. HPLC analysis of incubation samples only showed the formation of the corresponding O-dealkylated product.

**Enzyme Kinetics of CYP1A2-Mediated O-Dealkylation.** Because CYP1A2, in addition to CYP2D6, was found to be the only physiologically relevant P450 enzyme contributing to the O-dealkylation of MMAMC and BMAMC, the metabolism of the N-alkyl analogs by CYP1A2 was investigated further. Product formation was linear for 30 min and 20 min, respectively, for MAMC and its analogs. Using these incubation times, apparent Michaelis-Menten kinetics was observed. As can be derived from the data in Table 4, addition and elongation of the N-alkyl chain had little influence on the $K_m$ values of the compounds, nor did the introduction of a second N-alkyl substituent. However, in contrast to CYP2D6, an increase in the corresponding $V_{max}$ values was observed with increasing chain length.

The effect of elongation of the mono-N-alkyl chain on the intrinsic clearance of the analogs was not as notable as the one observed for CYP2D6. Investigation of the time-dependent metabolism of MMPyrC, by HPLC analysis, showed that this compound is not metabolized by CYP1A2.

**Metabolite Identification of CYP1A2-Mediated Metabolism.** Metabolite identification of incubation mixtures containing 100 μM compound and 20 nM CYP1A2 demonstrated that all N-alkyl analogs were mainly metabolized to the corresponding O-dealkylated metabolites. After 45 min of incubation, metabolite peaks corresponding to MAMC, HAMC, and MMAMC (in the case of diMMAMC) could also be detected (Fig. 6). Thus, apart from O-dealkylation reactions, CYP1A2 also mediates the N-dealkylation of the analogs.

**Discussion**

Recently, we described the development of a novel CYP2D6 substrate, 7-methoxy-4-(aminomethyl)-coumarin (Onderwater et al., 1999). MAMC is selectively O-dealkylated by CYP2D6 to the corresponding 7-hydroxy compound, HAMC. Due to the significantly different fluorescent properties of HAMC and MAMC and the favor-
of the 4-(aminomethyl)-coumarin structure to investigate the effects of structural variations of MAMC on cytochrome P450 metabolism and selectivity.

The investigated set of structurally related compounds, shown in Fig. 1, was obtained by introducing alkyl substituents to the nitrogen moiety of the 4-aminomethyl group of MAMC. The increase in affinity toward CYP2D6, observed for all of the secondary amine analogs, upon addition and enlargement of a single N-alkyl group, is most likely due to additional hydrophobic interactions of the substituents with active site residues. This hypothesis is supported by the excellent correlation observed between the log IC$_{50}$ values and calculated lipophilicities of the compounds ($R^2 = 0.978$, Table 2 and Fig. 3), as well as by docking studies of the substrates in an active site model of CYP2D6. The latter indicated that the alkyl substituents of the mono-alkylated analogs mainly interact with hydrophobic residues located in the I helix (Ile$_{297}$ and Ala$_{300}$) of the enzyme (Fig. 7). The introduction of the second methyl group in diMMAMC resulted in a reduction in affinity. DiMMAMC was found to adopt a different orientation in the active site of CYP2D6 due to steric hindrance of the additional N-methyl group with I helix residues. Modeling studies suggested that, instead of forming a hydrogen bond with Asp$_{301}$, diMMAMC interacts with Glu$_{216}$ (Fig. 8). Amino acid Glu$_{216}$ has also been proposed to play a role in the binding of other ligands of CYP2D6 (Lewis et al., 1997; De Groot et al., 1999). The different
binding orientation of diMAMC may explain why this analog does not fit the above-mentioned correlation. MMPrC was found to have a binding orientation similar to those of the mono-N-alkyl-substituted analogs.

Both CYP2D6 and CYP1A2 were found to catalyze the O-dealkylation reaction of the studied mono- and di-N-alkyl analogs of MAMC. N-Dealkylation was not observed for CYP2D6, although this enzyme is intrinsically capable of performing this metabolic route (Coutts et al., 1994; De Groot et al., 1995). Thus, it can be concluded that the regioselectivity of the metabolism by CYP2D6 is not compromised by substitutions at the 4-aminomethyl group of MAMC. In contrast to CYP2D6, an additional metabolic route was observed for CYP1A2, as this enzyme also mediated the N-dealkylation of the compounds, resulting in several products (Fig. 4).

The present kinetic studies on the O-dealkylation of the analogs by CYP2D6 demonstrated that N-alkyl substitution of MAMC notably affected the $K_m$ and $V_{max}$ values (Table 3) of the compounds. The increase in intrinsic clearance values (i.e., $V_{max}/K_m$, Table 3) with elongation of the mono-N-alkyl chain was most marked. The trend in the $K_m$ values of the N-alkyl analogs corresponds well with the one observed for the IC$_{50}$ values (Table 2), indicating that the $K_m$ value reflects the affinity of the compounds for CYP2D6. An interesting feature with respect to the high-throughput screening prospective of the present analogs is that the fluorescent signal of the N-substituted 7-hydroxy compounds is higher than that of HAMC (Fig. 2). Thus, despite their somewhat lower $V_{max}$ values, MMAMC, diMAMC, and MAMC O-dealkylation can be measured in the microplate reader with a higher sensitivity than MAMC. In the case of MMAMC, the sensitivity was increased more than 3-fold (Fig. 9).

Apart from its high turnover, the most important advantage of MAMC over other fluorescent probes is its selectivity for CYP2D6 (Onderwater et al., 1999). Because the use of human liver microsomes as an in vitro system is imperative in the process of drug discovery and development, we also investigated whether the isozyme selectivity of MAMC was influenced by the introduction of N-alkyl substituents. In heterologously expressed systems and at saturating conditions of CYP2D6, MAMC and all of the N-alkyl analogs were also metabolized by CYP1A2 and CYP2C19 (Fig. 5). Only BMAMC was also metabolized by CYP2C9. Thus, the identity of participating P450s has remained unchanged upon addition of an N-alkyl group, with the exception of BMAMC. The ratio of metabolism by the various heterologously expressed P450s did differ between the different analogs (Fig. 5).

Incubations of MMAMC and BMAMC in human liver microsomes showed that these substrates were exclusively metabolized by CYP2D6 and CYP1A2. Regardless of its relative contribution, the latter could be totally inhibited by preincubating with furafylline, a selective CYP1A2 inhibitor. As MAMC was also shown to be exclusively metabolized by CYP1A2 and CYP2D6 in human liver microsomes (Onderwater et al., 1999), it can be concluded that introduction of an N-alkyl chain does not qualitatively affect the P450 selectivity of the investigated compounds in this system.

In conclusion, the addition of mono-N-alkyl substituents to MAMC leads to higher affinities toward CYP2D6. Although the structural variation introduced did not result in altered metabolic profiles, the CYP2D6-mediated O-dealkylation was strongly influenced by the substituents. The P450s contributing to O-dealkylation of the N-alkyl analogs of MAMC were found to be identical to those of MAMC itself (Onderwater et al., 1999) in both human liver microsomes and heterologously expressed P450s, with the exception of BMAMC. Thus, next to CYP2D6 only CYP1A2 contributed to the O-dealkylation of the N-alkyl compounds in human liver microsomes. Further investigation of the CYP1A2-mediated O-dealkylation showed that, in sharp contrast to CYP2D6, the $K_m$ value of the MAMC analogs was virtually unaffected by the structural variation introduced, whereas the turnover increased with elongation of the N-alkyl chain. An additional metabolic pathway was observed in the case of CYP1A2, as N-dealkylation was also detected.

Acknowledgment. We gratefully acknowledge Prof. Dr. R. F. Rekker for help regarding the lipophilicity calculations of the 7-methoxy 4-(aminomethyl)-coumarin analogs.

References


Ravichandran KG, Bodupalli SS, Hasemann CA, Peterson JA and Deisenhofer J (1993) Crystal
structure of hemoprotein domain of P450<sub>BM-3</sub> - a prototype for microsomal P450s. Science (Wash DC) 261:731–736.


