Regular Article

Metabolism of N,N-Dipropyl-2-[4-Methoxy-3-(2-Phenyl-Ethoxy)-Phenyl]-Ethyl-Amine-Monohydrochloride (NE-100), A Novel Sigma Ligand: Contribution of Cytochrome P450 Forms Involved in the Formation of Individual Metabolites in Human Liver and Small Intestine

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Summary: In the present study, human cytochrome P450 (CYP) forms involved in producing the primary metabolites of NE-100 were identified. Major metabolites of NE-100 in human liver microsomes (HLM) were N-depropylation of NE-100 (NE-098), p-hydroxylation of phenyl group of NE-100 (NE-152), m-hydroxylation of phenyl group of NE-100 (NE-163) and O-demethylation of NE-100 (NE-125). Judging from the correlation and inhibition studies, NE-125 and NE-152 + 163mix formations were predominantly mediated by CYP2D6 and NE-098 formation was mediated by multiple CYP forms at a low NE-100 concentration (0.1 μM) in the HLM. According to relative activity factor (RAF) approaches, all these reactions were predominantly catalyzed by CYP2D6 at a substrate concentration assuming a plasma level of NE-100 (Km ≈ S) in case of the human liver. Depending on the increase in NE-100 concentrations, the rate of contribution for NE-098 and NE-125 + 163mix formations increased in CYP3A4, although the predominant contribution of CYP2D6 for NE-125 formation did not change. In human intestinal microsomes (HIM), NE-100 was mainly metabolized to NE-098 and NE-152 + 163mix by CYP3A4. The intrinsic clearance for their formations in HIM was 3.2 and 14.9 times less than those in HLM, respectively, and no formation of NE-125 was observed in HIM. These results strongly suggest that CYP2D6 is the predominant form for NE-100 metabolism in the human liver in vitro conditions (Km ≈ S) and the liver plays a more important role than does the small intestine in the first pass metabolism.

Key words: NE-100, cytochrome P450, CYP2D6, relative activity factor, contribution rates

Introduction

NE-100 (N,N-dipropyl-2-[4-methoxy-3-(2-phenylethoxy)phenyl]-ethyl-amine-monohydrochloride) is a novel sigma ligand that has been developed as a therapeutic drug for treating subjects with schizophrenia.1,2 We have reported that the clinical effective level (concentration) of NE-100 required for therapy of schizophrenic subjects might be low due to high affinity for sigma receptor.3 In pre-clinical pharmacokinetic in vivo conditions (Km ≈ S) and the liver plays a more important role than does the small intestine in the first pass metabolism.
experiments, NE-100 rapidly disappeared from the in vitro rat intestinal loop and bioavailability of NE-100 after oral administration to rats was low (unpublished data). Metabolites of NE-100 were detected only as high polarity compounds in rat plasma and urine, and dog plasma. Primary metabolites of NE-100 and their metabolizing enzymes remained to be identified in rats and humans. In our previous study using various recombinant CYP forms, NE-100 metabolism was shown to be mainly mediated by multiple CYP forms, CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Based on the kinetic study, NE-100 metabolism by human liver microsomes (HLM) was catalyzed by CYP2D6 (high affinity) and other CYPs (low-affinity), but the metabolism by human intestinal microsomes (HIM) was mainly catalyzed by CYP3A4 (low affinity). These data suggested that CYP2D6 as a high-affinity enzyme and CYP3A4 as a low-affinity enzyme play an important role in NE-100 disappearance in HLM and in HIM, respectively. The contribution of these CYP forms for primary metabolites of NE-100 remained to be quantitatively predicted. CYP2D6 accounts for only 2% of the total CYP content in the liver, but it is involved in 30% metabolism of clinically relevant drugs. In addition, CYP2D6 as well as CYP2C19 has genetic polymorphism, which may bring a serious effect on pharmacokinetic, pharmacodynamic and toxicologic behavior. CYP3A4 is the most abundant form among all known CYP forms and accounts for about 20–30% of the total P450 in the human liver and it participates in oxidative biotransformation of over 50% of commercially relevant drugs.4–6 In recent studies, intestinal metabolism has been attracting attention regarding the first pass metabolism of orally administered drugs.23 Various drug-metabolizing enzymes have been detected in the small intestinal mucosa. In particular, of the CYP forms involved in drug metabolism, CYP3A4 is the most abundant form and the expression is about 70% of the total CYP content in intestinal microsomes. Therefore a number of drugs metabolized by CYP3A4 have low bioavailability after oral administration, for example felodipine, verapamil, saquinavir, cyclosporin, monoclonal anti-CYP2D6 and CYP3A4 antibodies have been synthesized at Taisho Pharmaceutical Co. Ltd. (Saitama, Japan). Furafylline, sulfaphenazole, s-mephenytoin, quinidine and ketocana- zole, and 6-hydroxyclozapoxide were purchased from Ultrafine Chemicals Co. (Manchester, UK). Acetaminophen, 7-hydroxycoumarin, 7-ethoxy-4-trifluoromethylcoumarin, dextromethorphan, dextrophan, chlorozoxazone, testosterone, lauric acid, 12-hydroxydocanoic acid, troleandomycine, omeprazole and lansoprazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-Hydroxy-omeprazole and proadifen hydrochloride (SKF-525A) were from Fujisawa Astra Co. (Osaka, Japan) and from Funakoshi Co. Ltd. (Tokyo, Japan), respectively. 6β-Hydroxytestosterone was from Sumika Chemical Analysis Service (Osaka, Japan). All other chemicals and solvents used were of the highest quality commercially available. 7-Hydroxy-4-trifluoro-methylcoumarin, monoclonal anti-CYP2D6 and CYP3A4 antibodies were obtained from BD Gentest Corporation (Woburn, MA, USA). Antiserum against rat CYP2C13 and human CYP2D6 were from Daiichi Pure Chemicals (Tokyo, Japan).

Materials and Methods

Chemicals and reagents: NE-100 hydrochloride and its metabolites, NE-125 (N,N-dipropyl-2-[4-hydroxy-3-(2-phenyl-ethoxy)phenyl]-ethyl-amine), NE-098 (N,N-dipropyl-2-[4-methoxy-3-(2-phenyl-ethoxy)phenyl]-ethyl-amine), NE-152 (N,N-dipropyl-2-[4-methoxy-3-(2-[4-hydroxy-phenyl)]ethoxy)phenyl]-ethyl-amine) and NE-163 (N,N-dipropyl-2-[4-methoxy-3-(2-[3-hydroxy-phenyl)]ethoxy)phenyl]-ethyl-amine), were synthesized at Taisho Pharmaceutical Co. Ltd. (Saitama, Japan). [14C]NE-100, labeled at the photoxy ring (97.5%, radiochemical purity, 8.53 MBq/mg), was synthesized at Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Arachidonic acid, phenacetin, coumarin, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and NADP+ were from Wako Pure Chemical Industries (Osaka, Japan). Furafylline, sulfaphenazole, s-mephenytoin, quinidine and ketocana- zole, and 6-hydroxyclozapoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-Hydroxy-omeprazole and proadifen hydrochloride (SKF-525A) were from Fujisawa Astra Co. (Osaka, Japan) and from Funakoshi Co. Ltd. (Tokyo, Japan), respectively. 6β-Hydroxytestosterone was from Sumika Chemical Analysis Service (Osaka, Japan). All other chemicals and solvents used were of the highest quality commercially available. 7-Hydroxy-4-trifluoro-methylcoumarin, monoclonal anti-CYP2D6 and CYP3A4 antibodies were obtained from BD Gentest Corporation (Woburn, MA, USA). Antiserum against rat CYP2C13 and human CYP2D6 were from Daiichi Pure Chemicals (Tokyo, Japan).

Enzyme: Pooled human liver microsomes (HLM; H161-lot.3) were obtained from BD Gentest Corporation (Woburn, MA, USA). Individual human livers were from SRI International Toxicology Laboratory (Menlo Park, CA, USA) and the Department of Anatomic Pathology (School of Medicine, Tohoku University, Sendai). Experiments on human livers were approved by the Tohoku University Ethics Committee. Microsomes of a human B lymphoblast cells expressing recombinant human CYP enzymes (i.e. CYP1A2, 2C9-Arg, 2C19, 2D6*-1-Val and 3A4) were purchased from BD Gentest Corporation (Woburn, MA, USA). Human intestinal microsomes were from Tissue Transformation
Kinetic study of NE-100 metabolism by recombinant human CYP forms: Kinetic studies for the formation of each metabolite of NE-100 were done using recombinant CYPs microsomes (CYP1A2, 2C9-Arg, 2C19, 2D6-Val and 3A4). The reaction contained recombinant CYP2D6, CYP2C9 or CYP3A4 microsomal proteins of 0.20, 0.46 or 0.43 mg/mL, respectively, was initiated by adding NE-100 at concentrations ranging from 0.05 to 20 μM. Incubation times are for 0.167–30 min in CYP2D6 and for 0.167–30 min in CYP2C9 and CYP3A4. Other reaction conditions were the same as described for HLM.

Correlation study: NE-100 metabolism was assessed in incubation mixtures containing 0.1 mg/mL microsomes, the NADPH-generating system and 1 μM NE-100 in 50 mM potassium phosphate buffer (pH 7.4) for 5 min. Formation activities of each metabolite in HLM were compared with phenacetin O-deethylation (CYP1A1/2), coumarin 7-hydroxylation (CYP2A6), 7-ethoxy 4-trifluoromethyl coumarin O-deethylation (CYP2B6), omeprazole 5-hydroxylation (CYP2C19), dextromethorphan 6-hydroxylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), testosterone 6β-hydroxylation (CYP3A4) and lauric acid ω-hydroxylation activity (CYP4A11), using microsomes obtained from 28 individual human livers as described previously.

Immunoinhibition study: The inhibitory effects of monoclonal anti-CYP2D6 and anti-CYP3A4 antibodies on the formation of the metabolite were assessed using HLM. After preincubation with HLM (0.2 mg/mL) and an antibody on ice for 15 min, the reaction was carried out by adding NE-100 (0.1, 1 and 10 μM) at 37°C for 5, 10 and 15 min, respectively. For HIM, a monoclonal antibody CYP3A4 antibody or anti-CYP2C13 serum were preincubated with HIM (0.25 mg/mL) on ice for 15 min, or at room temperature for 30 min, respectively. The reactions were carried out by addition of NE-100 (10 and 200 μM) at 37°C for 20 and 30 min, respectively. All reactions were stopped using 1 mL of methanol.

Chemical inhibition study: Inhibitory effects of selective inhibitors for CYP forms on each formation of metabolites of NE-100 in HLM and HIM were undertaken with 0.1 μM, 10 μM (for HLM) and 0.2 μM, 10 μM, 200 μM NE-100 (for HIM). Concentration of inhibitors used and reaction procedures were based on
our descriptions given elsewhere.\(^3\)

**Data analysis:** Assuming one- or two-enzyme models as described by Nakajima \textit{et al.},\(^3\) kinetic data of enzyme, \(K_m\) and \(V_{max}\) values \(K_m\) and \(V_{max}\) for high affinity component, \(K_m\) and \(V_{max}\) for low affinity component) for formation of each metabolite were estimated using residual analysis on Eadie-Hofstee plots based on visual inspection. Intrinsic clearance \((CL_{int})\) was calculated as ratio of the \(V_{max}\) to the \(K_m\). Correlation between the formation activity of each metabolite and the metabolite formation activities of the respective CYP forms-specific substrates were examined by least-squares linear regression analysis. The statistical significance of differences between the control and inhibitor treatments was determined by Dunnett’s test (SAS system for windows, ver 6.1, SAS Institute Inc., Cary, NC, USA). A p-value <0.05 was considered to be statistically significant.

**Relative contribution rate of each CYP form to formation activity of each metabolite in HLM:**

1) Rate of contribution under linear conditions for the formation of metabolites

The contribution rates of each CYP form to the formation of each metabolite were estimated based on kinetic data obtained from recombinant CYP forms, using the RAF proposed by Crespí.\(^3\) The intrinsic clearances \((CL_{int, CYP-X})\) for the formation of each metabolite in HLM were calculated as follows:

\[
CL_{int, CYP-X} = CL_{int, cDNA-CYP-X} \times RAFCYP-X
\]

where \(CL_{int, cDNA-CYP-X}\) and RAFCYP-X are intrinsic clearance for the formation of each metabolite obtained from recombinant CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, and RAF values for each CYP form, respectively. The total intrinsic clearance for the formation \((CL_{int, HLM})\) of each metabolite in HLM was expressed as the sum of the \(CL_{int, HLM-CYP-X}\) for each CYP form. The contribution rates of each CYP form to the \(CL_{int, HLM-CYP-X}\) for the formation of each metabolite were estimated as the percent of \(CL_{int, HLM-CYP-X}\) to \(CL_{int, HLM}\):

2) Relative rate of contribution of each CYP form depending on substrate concentration

The relative rates of contribution of each CYP form to the formation activity of each metabolite in HLM, depending on substrate concentration, were predicted using a kinetic parameter \((K_m, V_{max})\) from recombinant CYP forms and RAF values. The formation velocity of the metabolite \((V_{HLM-CYP-X})\) for each CYP form in HLM at various substrate concentrations was calculated as follows:

\[
V_{HLM-CYP-X} = (V_{HLM-cDNA-CYP-X} \times RAFCYP-X \times S) / (K_m + S)
\]

where \(S, K_m, V_{HLM-cDNA-CYP-X}, \) \(V_{HLM-cDNA-CYP-X}\) and RAFCYP-X are substrate concentration, affinity constants, the maximum formation velocities of each metabolite for each recombinant CYP microsome and RAF values for each CYP, respectively. The total formation velocities \((V_{HLM, tot})\) of each metabolite at various substrate concentrations in HLM are expressed as the sum of the \(V_{HLM-CYP-X}\) for each recombinant CYP form. The contribution rates of each CYP form to the formation velocity of each metabolite in HLM at various substrate concentrations were estimated as the percent of \(V_{HLM-CYP-X}\) to \(V_{HLM, tot}\):

**Results**

**Kinetic study of NE-100 metabolism by human liver and intestinal microsomes:** In the co-chromatography using 2D-TLC and HPLC analysis, the metabolites detected in a reaction sample with HLM corresponded to the authentic synthesized standards in \(R_f\) values on 2D-TLC and retention time on HPLC chromatograms. Possible metabolic pathways of NE-100 in HLM were \(N\)-depropylation (NE-098), hydroxylation of the phenyl ring portion (NE-152 and NE-163) and \(O\)-demethylation (NE-125) (Fig. 1). As NE-152 and NE-163 could not be quantitatively separated by the TLC method in this experiment, these metabolites were measured as a mixture of NE-152 and NE-163 and expressed as NE-152 + 163mix.

Eadie-Hofstee plots for NE-098, NE-152 + 163mix and NE-125 formations in HLM, and HIM are shown in Fig. 2. The plots for NE-098 and NE-152 + 163mix formations in HLM showed biphasic curves (high- and low-affinity components), while plots for NE-125 formation showed a monophasic curve. \(K_m\) (high affinity component) values of NE-098 and NE-152 + 163mix formations were 110.1 and 45.6 times smaller than those in HLM, while \(V_{max}\) values of NE-098 and NE-152 + 163mix formations were 3.2 and 14.9 times smaller than those in HLM, respectively.

In HIM, NE-100 was mainly metabolized to NE-098 and NE152 + 163mix. Eadie Hofstee plot for NE-098 was monophasic while that for NE152 + 163mix was biphasic. Intrinsic clearance \((CL_{int, HIM})\) of NE-098 formation was 3.9 times larger than that in case of the NE-152 + 163mix. \(CL_{int, HIM}\) of these metabolites in HIM were 3.2 and 14.9 times smaller than those in HLM, respectively.

**Correlation study:**
Correlation between activities of NE-098, NE-152 + 163mix or NE-125 formation and specific CYP substrate in 28 liver samples is shown in Table 2. Among the specific substrate activities

\(\text{Table 2}\)
Fig. 1. Possible primary metabolic pathways of NE-100 in HLM. NE-152 + 163mix expresses the mixtures of the two hydroxylated metabolites (NE-152 and NE-163).

**Human liver microsomes**

(A) NE-098 formation

(B) NE-152 + 163mix formation

(C) NE-125 formation

**Human intestinal microsomes**

(A) NE-098 formation

(B) NE-152 + 163mix formation

Fig. 2. Eadie-Hofstee plots for NE-100 metabolites in pooled HLM and HIM. Ranges of substrate concentrations used were from 0.05 to 20 μM for HLM or from 0.2 to 200 μM for HIM. V; Formation activity of each metabolite, S; NE-100 concentration. Data are the mean of duplicate determinations.

Contribution of CYPs to NE-100 Metabolism
examined, NE-098 and NE-125 formation activities significantly correlated with activities of testosterone 6β-hydroxylase (r² = 0.680) and dextromethorphan O-demethylation (r² = 0.826), respectively, at 1 μM NE-100. NE-152 + 163mix formation activity significantly correlated with both activities of dextromethorphan O-demethylation and testosterone 6β-hydroxylase (r² = 0.811, and 0.474, respectively).

Immunoinhibition study by HLM and HIM: The inhibitory effect of anti-CYP2D6 and anti-CYP3A4 antibodies on the formation of each metabolite are shown in Fig. 3(A) – (C) at three substrate concentrations (0.1, 1 and 10 μM) in HLM. At 0.1 μM NE-100 (which is corresponding to K_m, value of a high-affinity component), the anti-CYP2D6 antibody inhibited the NE-098, NE-152 + 163mix and NE-125 formations by 17.5%, 90.8% and 93.2%, respectively. The inhibitory effect of anti-CYP2D6 antibody on NE-125 formation did not differ among these three concentrations of NE-100. However, the inhibitory effect on NE-152 + 163mix formation changed depending on concentration of the substrate. The inhibitory effect on NE-098 formation also changed depending on the concentration but was less than that seen with NE-152 + 163mix formation. On the other hand, the anti-CYP3A4 antibody inhibited the NE-098 formation at 0.1 μM NE-100 by 42.5%, but weakly inhibited NE-152 + 163mix and NE-125 formations. Depending on increase in the substrate concentration, the anti-CYP3A4 antibody inhibited the formation activity of NE-152 + 163mix as well as NE-098.

The inhibitory effects of anti-CYP3A4 antibody and anti-CYP2C13 serum on NE-098 and NE-152 + 163mix formations in HIM are shown in Fig. 4. The antiserum raised against CYP2C13 cross-reacts with CYP2C8, 2C9 and 2C19 (the data sheet provided by the manufacturers). Anti-CYP3A4 antibody inhibited the NE-098 formation at 10 and 200 μM NE-100 by 71.6% and 45.0%, respectively. Anti-CYP2C13 serum inhibited it at both concentrations by 16.2% and 14.3%, respectively.

Table 1. Kinetic parameters for the formation of NE-100 metabolites in pooled HLM and HIM

<table>
<thead>
<tr>
<th>CYP form</th>
<th>Specific activity</th>
<th>NE-098</th>
<th>NE-125</th>
<th>NE-152 + 163mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1/2</td>
<td>Phenacetin O-deethylase</td>
<td>0.103</td>
<td>0.123</td>
<td>0.095</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin 7-hydroxylase</td>
<td>0.020</td>
<td>0.025</td>
<td>0.031</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>7-Ethoxy-4-trifluoromethyl coumarin O-deethylase</td>
<td>0.460</td>
<td>0.500</td>
<td>0.530</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>Omeprazole 5-hydroxylase</td>
<td>0.205</td>
<td>0.171</td>
<td>0.240</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan O-demethylase</td>
<td>0.200</td>
<td>0.193</td>
<td>0.209</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorozoxazone 6-hydroxylase</td>
<td>0.018</td>
<td>0.019</td>
<td>0.020</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone 6β-hydroxylase</td>
<td>0.680</td>
<td>0.420</td>
<td>0.474</td>
</tr>
<tr>
<td>CYP4A11</td>
<td>Lauryl acid co-oxysulfase</td>
<td>0.217</td>
<td>0.316</td>
<td>0.282</td>
</tr>
</tbody>
</table>

Table 2. Correlation coefficients between enzyme activity and formation activities of three metabolites of NE-100

<table>
<thead>
<tr>
<th>CYP forms</th>
<th>Specific activity</th>
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<th>NE-152 + 163mix</th>
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<td>0.500</td>
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<tr>
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<td>0.205</td>
<td>0.171</td>
<td>0.240</td>
</tr>
<tr>
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<td>0.316</td>
<td>0.282</td>
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</table>

Correlation coefficient was shown as r² value. * p<0.0001.

NE-100 (1 μM) was incubated with human liver microsomal samples (n = 28).
Fig. 3. Effect of antibodies raised against CYP2D6 and CYP3A4 on formation activities of NE-100 metabolites in HLM. Initial substrate concentrations used were 0.1 (A), 1 (B) and 10 μM (C). For inhibition by antibodies, closed circles represent the inhibitory effect of antibody for CYP2D6 and open circles represents the inhibitory effect of antibody for CYP3A4. Each point represents the mean ± S.E. of triplicate determinations.

Fig. 4. Effect of antibodies raised against CYP3A4 (A, B) and CYP2C13 (C, D) on the NE-098 and NE-152 + 163mix formation activities in HIM. Initial substrate concentrations used were 10 μM (A, C) and 200 μM (B, D). The antiserum raised against CYP2C13 cross-reacts with CYP2C8, 2C9 and 2C19 (the data sheet provided by the manufacturers). Each point represents the mean of duplicate determinations.
NE-100 by 71.7% and 69.6%, respectively. Anti-CYP2C13 serum inhibited it at both concentrations by 6.7% and 18.8%, respectively.

Chemical inhibition study in HLM and HIM: The effects of selective CYP inhibitors on the formation activities of NE-098, NE-152 + 163mix and NE-125 in HLM were examined at two substrate concentrations (0.1 mM and 10 mM). As shown in Fig. 5, 100 μM SKF-525A (non-selective CYP inhibitor) completely inhibited all metabolite formations at a 0.1 mM substrate concentration. At the low substrate concentration (0.1 μM corresponding to $K_{m1}$ value of a high-affinity component), the formation activity of NE-098 was inhibited by 48.2% and 35.2% by ketoconazole and troleandomycin (selective CYP3A4 inhibitors), respectively, and was inhibited by 22.5% by quinidine (a selective CYP2D6 inhibitor). On the other hand, the formation activity of NE-152 + 163mix was strongly inhibited by quinidine and was weakly inhibited by ketoconazole and troleandomycin. The formation activity of NE-125 was clearly inhibited by quinidine.

At a high substrate concentration (10 mM corresponding to $K_{m2}$ value of a low-affinity component), the formation activity of NE-098 was strongly inhibited by 78.8% and 49.7% by ketoconazole and troleandomycin, respectively, and the inhibitory percentage was much greater than those that at the low substrate concentration. Furthermore, the formation activity of NE-098 was slightly inhibited by sulfaphenazole (a selective CYP2C9 inhibitor) and S-mephenytoin (a substrate of CYP2C19). The formation activity of NE-152 + 163mix at the high substrate concentration was mainly reduced by ketoconazole and troleandomycin, but not by quinidine. The formation activity of NE-125 at a high substrate concentration was not significantly inhibited by typical CYP inhibitors used in this study.

Effects of selective CYP inhibitors on NE-098 and NE-152 + 163mix formations in HIM were also evaluated at 0.2, 10 and 200 μM NE-100. Ketoconazole strongly inhibited the formation activity of NE-098 by 88.3%, 86.3% and 81.2% at 0.2, 10 and 200 μM NE-100, respectively. For other inhibitors, sulfaphenazole and lansoprazole (for CYP2C19, 2 μM) inhibited the formation activity of NE-098 at 10 μM NE-100 by 6.6% and 9.5%, respectively. The formation activity of NE-152 + 163mix at 0.2 μM, 10 μM and 200 μM NE-100 was also inhibited by ketoconazole and the inhibitory rates were 46.6%, 84.1% and 71.0%, respectively. Although arachidonic acid (for CYP2J2, 100 μM) inhibited the NE-152 + 163mix formation at 0.2, 10 and 200 μM NE-100 by 38.4%, 43.2% and 24%, respectively.
Kinetic study of NE-100 metabolism by recombinant human cytochrome P450 4a40: Kinetic parameters (Km, Vmax and CLHLM) for NE-098, NE-152 and NE-125 were determined using the RAF approach. The RAF values were calculated based on the ratio of the specific activity for a relatively specific reaction in HLM (H161) divided by the activity for that same reaction in recombinant CYP. The specific activity for each CYP form used for calculating the RAF values has been described in the materials supplied by the manufacturer.

Table 3. Kinetic parameters of formation activity of NE-100 metabolites in recombinant CYPs microsomes and the predicted CLHLM for each CYP form

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>CYP forms</th>
<th>Recombinant microsomes</th>
<th>Human liver microsomes (H161-lot.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>K&lt;sub&gt;m&lt;/sub&gt; (μM)</td>
<td>V&lt;sub&gt;max&lt;/sub&gt; (pmol/min/mg protein)</td>
</tr>
<tr>
<td>NE-098</td>
<td>2D6</td>
<td>0.10</td>
<td>5.89</td>
</tr>
<tr>
<td></td>
<td>3A4</td>
<td>15.33</td>
<td>156.51</td>
</tr>
<tr>
<td></td>
<td>2C19</td>
<td>29.20</td>
<td>36.34</td>
</tr>
<tr>
<td></td>
<td>1A2</td>
<td>15.11</td>
<td>12.47</td>
</tr>
<tr>
<td>NE-098</td>
<td>2D6</td>
<td>0.07</td>
<td>77.17</td>
</tr>
<tr>
<td></td>
<td>3A4</td>
<td>9.78</td>
<td>19.06</td>
</tr>
<tr>
<td>NE-098</td>
<td>2C19</td>
<td>23.62</td>
<td>10.85</td>
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<tr>
<td></td>
<td>1A2</td>
<td>93.94</td>
<td>942.34</td>
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</tbody>
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Kinetic studies on the formation of NE-098, NE-152 and NE-125 were done using substrate concentration from 0.05 to 20.0 μM. For CYP1A2 and CYP2C19, the kinetic parameters were estimated using the results obtained from a preliminary study on recombinant P450s. For CYP2D6, CYP2C9 and CYP2C19, the kinetic parameters were calculated from kinetic parameters on recombinant CYPs using RAF approaches, as described in the materials and methods.

For NE-098 formation, the predicted CLHLM = CLHLM-2D6 + CLHLM-3A4 + CLHLM-2C19 + CLHLM-1A2 + CLHLM-2C9 = 0.126 mL/min/mg protein. For NE-125 formation, the predicted CLHLM = CLHLM-2D6 + CLHLM-3A4 + CLHLM-2C19 + CLHLM-1A2 + CLHLM-2C9 = 0.342 mL/min/mg protein. —: Not detected.

Relative contribution rate of CYP forms to the formation activity of metabolites in human liver microsomes: As shown in Table 3 and Fig. 6, predicted CLHLM values for each metabolite were 0.126 (for NE-098 formation), 0.201 (for NE-152 formation) and 0.342 mL/min/mg protein (for NE-125 formation) and agreed well with data on HLM. As described in the materials and methods, the contribution rates of CYP2D6, CYP3A4 and CYP2C9 were very low compared with activities for CYP2D6 and CYP2C9. The contribution rate of CYP2D6 was estimated to be 93.6% and 4.4%, respectively. Moreover, the NE-125 forma-
Hepatic intrinsic clearance and relative contribution for each metabolite formation in pooled HLM. The clearance was calculated by extrapolating in vitro kinetic data with recombinant CYP forms to human liver activity using a RAF approach. Predicted $CL_{intrinsic, HLM-CYP-X}$ was calculated from kinetic parameters on recombinant CYPs using RAF approach as described under Materials and Methods. The RAF values for each CYP form were calculated as the ratio of the specific activities described in the data sheet supplied by the manufacturer in HLM and recombinant CYPs microsomes. Figure 6(C) represents the expanded ranges from concentrations near 0 mM (5 $K_m$) to 1 mM. The contribution rate of the formation of each metabolite for the entire metabolism of NE-100 is estimated based on the ratio of formation activity for each metabolite to sum of activities of NE-125, NE-152 + 163mix and NE-098 at each substrate concentration.

Fig. 6. Hepatic intrinsic clearance and relative contribution for each metabolite formation in pooled HLM. The clearance was calculated by extrapolating in vitro kinetic data with recombinant CYP forms to human liver activity using a RAF approach. Predicted $CL_{intrinsic, HLM-CYP-X}$ was calculated from kinetic parameters on recombinant CYPs using RAF approach as described under Materials and Methods. The RAF values for each CYP form were calculated as the ratio of the specific activities described in the data sheet supplied by the manufacturer in HLM and recombinant CYPs microsomes. Figure 6(C) represents the expanded ranges from concentrations near 0 mM ($S < K_m$) to 1 mM. The contribution rate of the formation of each metabolite for the entire metabolism of NE-100 is estimated based on the ratio of formation activity for each metabolite to sum of activities of NE-125, NE-152 + 163mix and NE-098 at each substrate concentration.

Discussion

To clarify NE-100 metabolism in humans, CYP forms were identified. In our previous data, NE-100 metabolism was shown to be mediated by CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. However, CYP2D6 and CYP3A4 were predominant forms involved in NE-100 metabolism in the liver and in the small intestine, respectively. These data support the possibility that NE-100 receives the considerable first pass effect in the small intestine. In the previous study, however, we were unable to quantify individual metabolites and metabolism of NE-100 was monitored by substrate disappearance. For further elucidation, we identified metabolites and predicted the contribution of CYP forms for the first pass effect in liver and small intestine. Four primary metabolites (NE-098, NE-125, NE-152 and NE-163) were detected in reaction mixtures, including liver microsomes (Fig. 1). Judging from inhibition studies with antibodies against CYP forms (Fig. 3) and typical CYP inhibitor (Fig. 5), at a low NE-100 concentration (0.1 mM), all metabolites were predominantly catalyzed by CYP2D6. This finding supports our previous data for which the disappearance of NE-100 was measured. On the other hand, at a high NE-100 concentration, kinetic data on individual metabolites differed.
Contribution of CYPs to NE-100 Metabolism

The contribution rates of CYP forms involved in the formation of NE-100 metabolites (Table 3) were estimated using RAF approaches. The predicted contribution rates of CYP forms for individual metabolites change in humans depending on NE-100 concentrations. In this study, although we assumed the formation of NE-152 and NE-163 to be the mixture of two metabolites, different CYP forms might be involved in their formations. The contributions of the CYP form involved in the formation of NE-100 metabolites in HLM have been estimated using RAF approaches. The contribution rates of CYP forms involved in the formation of each metabolite were predicted using kinetic parameters ($K_m$, $V_{max}$) with recombinant CYP forms and RAF values. The predicted change in contribution rates of CYP forms for individual metabolites is shown in Fig. 7. At the NE-100 concentration corresponding to in vivo conditions, the NE-125 and NE-152 + 163mix formations were predominantly catalyzed by CYP2D6 (Fig. 7(E) and 7(F)). In contrast, multiple CYP forms appear to contribute to the NE-098 formation (Fig. 7(C)). At the high substrate concentration, the contribution rates of CYP2D6, CYP2C9 and CYP3A4 for NE-125 formation were predicted to be 81.2%, 15.2%, and 3.6%, respectively. The rates for the NE-152 + 163mix formation decreased in CYP2D6 and it increased in CYP3A4, depending on the increase in NE-100 concentrations. Similar results were also observed regarding NE-098 formation. These findings support results obtained in chemical inhibition studies and suggest that major CYP forms involved in NE-100 metabolism differ between the low and high substrate concentrations. As shown in Fig. 7(A) and (B), the contribution rate of CYP2D6 to the entire metabolism of NE-100 in HLM was large in case of a low substrate concentration, but the rate of CYP3A4 was large at a high substrate concentration. Thus, the multiple metabolites are formed at a low concentration, however at a high concentration, NE-098 is a major metabolite (Fig. 6(B) and (C)). We reported that the CYP form catalyzing N-demethylation of diazepam predominantly depended on the drug concentration and we suggested that the concentration used is most critical for in vitro...
metabolic studies. Thus, estimation of the contributions of CYP forms and determination of major metabolites at low and high substrate concentrations are important for considerations between pharmacokinetics and toxicokinetics, drug-drug interactions or the polymorphism of certain enzymes.

On the other hand, in our previous kinetic study, we suggested that NE-100 disappearance in HIM was predominantly mediated by CYP3A4, although CYP2C19 and CYP2J2 seem to be less than that for the liver. Considering the metabolism in the liver significantly contributed to the biotransformation of NE-100, compared to the intestinal metabolism, the metabolism in the liver was not formed in the HIM. Thus, the contributions of intestinal metabolism for the first pass effect are likely to be less than those for hepatic metabolism.

Due to the high contribution rate of CYP2D6 for metabolism in the liver, the metabolism of NE-100 might be inhibited by drugs that are metabolized by CYP2D6 such as beta-blockers, tricyclic antidepressants antiarrhythmic and antipsychotic drugs in livers. In addition, large inter-individual differences in plasma concentrations of NE-100 occur between an extensive metabolizer and a poor metabolizer of CYP2D6. Therefore estimation of contribution rates of CYP forms involved in drug metabolism is very important to consider the relationship between plasma concentration and efficacy or side effects.

In conclusion, four primary metabolites (NE-098, NE-125, NE-152 and NE-125mix) and NE-100 were mainly metabolized into NE-098 in a patient (Km = 3.2 times and 14.9 times smaller than for CYP2D6) in the NE-098 and NE-152 + 163mix, but not into NE-125 mix in HIM. Therefore, estimation of contribution rates of CYP forms involved in drug metabolism is very important to consider the relationship between plasma concentration and efficacy or side effects.

In conclusion, four primary metabolites (NE-098, NE-152 and NE-163) and CYP forms involved in NE-100 metabolism were identified. All these metabolites were mainly catalyzed by CYP2D6, at a substrate concentration assuming plasma level of substrate concentration. The intestinal metabolism for the first pass effect are likely to be less than those for hepatic metabolism.

References


13) Fitzsimmons, M. E. and Collins, J. M.: Selective biotransformation of the human immunodeficiency virus protease inhibitor saquinavir by human small-intestinal...