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Metabolism of Selegiline Hydrochloride, a Selective Monoamine B-type Inhibitor, in Human Liver Microsomes

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Summary: The participation of cytochrome P-450 (CYP) isoforms in the metabolism of selegiline was investigated. Experiments using recombinant CYP isoforms expressed in human lymphoblastoid cells showed CYP2B6 to be the major CYP isoform involved with the metabolism of selegiline. CYP1A2 and CYP3A4 also contributed to the metabolism of selegiline but their catalytic activities were much less than that of CYP2B6. CYP2B6 had a higher affinity for both N-depropagation \( K_m = 21.4 \mu M \) and N-demethylation \( K_m = 25.2 \mu M \) of selegiline than CYP3A4 and CYP1A2. In immunoinhibition studies using mixed human hepatic microsomes, selegiline N-depropagation activity was most strongly inhibited by anti-CYP2B and anti-CYP3A antibodies, while selegiline N-demethylation activity was most inhibited by anti-CYP2B antibody. In CYP2B6-rich human hepatic microsomes, anti-CYP2B antibody had the strongest inhibitory effects on both activities. Selegiline inhibited CYP2B6-mediated (S)-mephenytoin N-demethylation activity and CYP2C19-mediated (S)-mephenytoin 4'-hydroxylation activity. These findings suggest that attention should be paid to the drug-drug interaction associated with CYP2B6 and CYP2C19.

In conclusion, CYP2B6 participates in the metabolism of selegiline but the degree of its contribution varies with the level of its expression in human liver.

Key words: selegiline; human; cytochrome P450; drug-drug interactions

Introduction

Selegiline hydrochloride \([(-)-(R)-N,\alpha\text{-dimethyl-}N\text{-2-propynylphenethylamine hydrochloride}]\) is a selective and irreversible inhibitor of monoamine oxidase type B (MAO-B).1-4 Selegiline is used both alone and together with levodopa for the treatment of Parkinson’s disease.2-3 Selegiline is metabolized to N-desmethylselegiline and l-methamphetamine followed by l-amphetamine (Fig. 1). CYPs (cytochrome P450 isoforms) participate in this metabolism of selegiline in rats.4-5 In humans, CYP2D6 and CYP3A4 have been reported to be involved with the metabolism of selegiline.6-7 However, Scheinin showed that CYP2D6 did not contribute to this metabolism: in that study, pharmacokinetic parameters of selegiline such as \( C_{\text{max}} \), \( t_{\text{max}} \), \( t_{1/2} \) and AUC did not differ significantly between poor and extensive metabolizers of debrisoquine.8 The contribution of CYP1A2 and CYP3A4 to the formation of N-desmethylselegiline and participation of CYP3A4 to the formation of l-methamphetamine have been reported.9,10 The most recently, CYP2B6 and CYP2C19 have

\[ \text{selegiline} \rightarrow \text{N-desmethylselegiline} \]
\[ \text{N-demethylation} \]
\[ \text{N-depropagation} \]
\[ \text{\rightarrow l-methamphetamine} \]
\[ \text{\rightarrow l-amphetamine} \]

Fig. 1. Major metabolic pathway of selegiline.
been reported to be the major enzymes responsible for the metabolism of selegiline.\textsuperscript{11} It is not clear which CYP isoforms actually contribute, to what extent, to the metabolism of selegiline in humans.

In this study, we investigated the participation of CYP isoforms in the metabolism of selegiline using human hepatic microsomes and recombinant CYP isoforms expressed in human lymphoblastoid cells. The possibility of drug-drug interactions attributed to the inhibition of CYP-mediated metabolism by selegiline was also examined.

Materials and Methods

Materials: Selegiline hydrochloride [(−)-(R)-Nα-dimethyl-N-2-propynylphenethylamine hydrochloride] was obtained from the Chinoin Pharmaceutical and Chemical Works Company (Budapest, Hungary). N-Desmethyselgiline hydrochloride and l-amphetamine hydrochloride were synthesized by Fujimoto Pharmaceutical Co. Ltd (Osaka, Japan). l-Methamphetamine hydrochloride was purchased from Dainippon Pharmaceutical Co., Ltd (Osaka, Japan). The human hepatic microsomes designated HHM-195, HHM-223 and HHM-224 were obtained from the International Institute for the Advancement of Medicine (NJ, USA) while those designated as Ms-28 and Ms-17 were prepared from human livers obtained from the Karolinska Institute (Sweden). The preparation of these hepatic microsomes was performed as described elsewhere.\textsuperscript{12} Pooled human hepatic microsomes and microsomes of human lymphoblastoid cells expressing CYP isoforms (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 (Val), CYP2E1, CYP3A4 and CYP4A11) were purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Antibodies against rat CYP isoforms (CYP1A1, CYP2A, CYP2B2, CYP2C, CYP2D, CYP2E and CYP3A) were raised in female Japanese White rabbits obtained from Biotech (Saga, Japan) as described previously.\textsuperscript{13} The following compounds were used as either substrates, metabolites or inhibitors: (S)-mephenytoin, (S)-nirvanol, 4-hydroxytolbutamide, 4'-hydroxymephenytoin, 1'-hydroxybufuralol, 6-hydroxychlorzoxazone, testosterone, 6β-hydroxytestosterone and ketoconazole from Daiichi Pure Chemicals Co., Ltd (Tokyo, Japan), methoxyphenamine hydrochloride, methoxsalen, 7-ethoxyresorufin, diethylthiocarbamate, α-naphtoflavone, 7-hydroxycoumarin, orphenadrine, coumarin, tolbutamide, sulfaphenazole, quinidine, chlorzoxazone and tranlycypromine from SIGMA Chemical Co. (St. Louis, MO), and resorufin from Aldrich Chemical Co. (Milwaukee, WI). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP (nicotinamide adenine dinucleotide phosphate) and NADPH were purchased from Oriental Yeast Co. (Tokyo, Japan). All other chemicals and organic solvents were obtained from Nacalai Tesque Co. (Kyoto, Japan).

Metabolism of selegiline by recombinant CYP isoforms: An incubation mixture containing microsomes (5.4 to 42 μL) of human lymphoblastoid cells (10 pmol of each CYP isoform) and 5 μL of 5 mM selegiline (final concentration, 100 μM) was adjusted to a final volume of 230 μL by addition of 100 mM potassium phosphate buffer (pH 7.4). The reaction was started by adding 20 μL of 10 mM NADPH. The mixture was incubated with gentle shaking for 15 min at 37°C. The reaction was terminated by adding 5 μL of 60% perchloric acid and then 5 μL of 100 μM methoxyphenazine solution was added to the mixture as an internal standard. The mixture was centrifuged at 12,000 × g for 4 min and the supernatant was analyzed by HPLC-UV as follows to determine the amount of selegline and its metabolites. A 50-μL aliquot of the supernatant was applied to a reversed phase column (TSKgel ODS-80Ts, 4.6 × 250 mm, TOSOH, Tokyo, Japan) using an autosampler (AS-8000, TOSOH, Tokyo, Japan). The column temperature was maintained at 45°C. The mobile phase consisting of 4 mM perchloric acid and acetonitrile (85:15) was delivered at a flow rate of 1.0 ml/min. The detection was carried out using a UV detector (SPD-6A, Shimadzu, Kyoto, Japan) at a wavelength of 215 nm. In the kinetic experiments, several concentrations of selegline ranging from 25 to 700 μM were used to determine the kinetic parameters.

Inhibition study with anti-CYP antibodies: Inhibition studies using anti-CYP antibodies and human hepatic microsomes were carried out as follows. An incubation mixture containing 100 μg of microsomal protein and each anti-CYP antibody (50, 100 and 200 μg of IgG) was adjusted to a final volume of 225 μL by addition of 100 mM potassium phosphate buffer (pH 7.4). After the preincubation for 20 min at room temperature, 20 μL of 10 mM NADPH was added and the reaction was started by adding 5 μL of 5 mM selegline (final concentration, 100 μM). The conditions for both the reaction and HPLC were as described above.

CYP isoform-specific enzyme assays: Pooled human hepatic microsomes prepared from ten human livers were used in the assays. The following CYP isoform-specific enzyme assays were employed; 7-ethoxyresorufin O-deethylation (CYP1A1/2), coumarin 7-hydroxylation (CYP2A6),\textsuperscript{14} (S)-mephenytoin N-deethylation (CYP2B6),\textsuperscript{15} tolbutamide 4-hydroxylation (CYP2C9),\textsuperscript{16} (S)-mephenytoin 4'-hydroxylation (CYP2C19),\textsuperscript{15} bufuralol 1'-hydroxylation (CYP2D6),\textsuperscript{17} chlorzoxazone 6-hydroxylation (CYP2E1)\textsuperscript{18} and testosterone 6β-hydroxylation (CYP3A4).\textsuperscript{13} The reference inhibitors used in each assay were α-naphtoflavone (CYP1A2),\textsuperscript{19} methoxsalen (CYP2A6),\textsuperscript{20} orphenadrine (CYP2B6),\textsuperscript{21,22} sulfaphenazole
Metabolism of Selegiline by Human CYP Isoforms

Other methods: The enzyme kinetic parameters (\(K_m\) and \(V_{max}\)) were determined according to a nonlinear least squares regression analysis. The amount of CYP protein in human hepatic microsomes (HHM-195, HHM-223, HHM-224, Ms-17 and Ms-28) was measured by Western blot analysis.26)

Results

Metabolism of selegiline by recombinant CYP isoforms: The metabolites of selegiline, methamphetamine and desmethylselegiline, were identified by comparing their retention times on HPLC with those of the authentic compounds. \(\beta\)-Methamphetamine, \(N\)-desmethylselegiline and methoxyphenamine (internal standard) were eluted at 14.0, 21.3 and 23.2 min, respectively (Fig. 2). Selegiline at a final concentration of 100 \(\mu\)M was incubated in the microsomes of human lymphoblastoid cells expressing CYP isoforms and the catalytic activities for \(N\)-depolyglation and \(N\)-demethylation were measured (Fig. 3). Among 11 recombinant CYP isoforms examined, CYP2B6 showed the highest level of activity for both \(N\)-depolyglation (5.70 pmol/min/\(\mu\)mol P450) and \(N\)-demethylation (5.36 pmol/min/\(\mu\)mol P450). The catalytic activities of other recombinant CYP isoforms were less than 40% of those of CYP2B6. A contribution by CYP1A2 and CYP3A4 to the metabolism of selegiline was also observed, though to a lesser extent. Although CYP1A1 showed similar catalytic activity to CYP1A2 and CYP3A4, its contribution to the metabolism was considered to be minor because of its markedly low expression level in human liver.27) The kinetic analysis was further carried out for CYP2B6, CYP1A2 and CYP3A4, and their kinetic parameters for the \(N\)-depolyglation and \(N\)-demethylation of selegiline are listed in Table 1. The \(K_m\) values for the \(N\)-depolyglation and \(N\)-demethylation by CYP2B6 were 21.4 and 25.2 \(\mu\)M, respectively, much smaller than those of CYP1A2 and CYP3A4. The \(V_{max}\) values for both the reactions differed little among the three CYP isoforms. Consequently, \(V_{max}/K_m\) values for both the \(N\)-depolyglation and \(N\)-demethylation of selegiline by CYP2B6 were much larger than those by CYP1A2 and CYP3A4.

Inhibition study with anti-CYP antibody: The inhibitory effect of anti-CYP antibody on the \(N\)-
Fig. 3. Metabolism of selegiline by recombinant CYP isoforms expressed in human lymphoblastoid cells. Open column: \(N\)-depropagation, closed column: \(N\)-demethylation.

Selegiline concentration: 100 \(\mu\)M. Amount of CYP isoform in each reaction mixture: 10 pmol. Each column represents the mean \(\pm\) S.D. of triplicate assays.

Table 1. Kinetic parameters for \(N\)-propagation and \(N\)-demethylation of selegiline by recombinant human CYP isoforms

<table>
<thead>
<tr>
<th>CYP</th>
<th>(K_m) ((\mu)M)</th>
<th>(V_{max}) (pmol/min/pmol P450)</th>
<th>(V_{max}/K_m) (ml/min/(\mu)mol P450)</th>
<th>(K_m) ((\mu)M)</th>
<th>(V_{max}) (pmol/min/pmol P450)</th>
<th>(V_{max}/K_m) (ml/min/(\mu)mol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>225 (\pm) 81.9</td>
<td>2.10 (\pm) 0.27</td>
<td>10.09 (\pm) 3.04</td>
<td>190 (\pm) 148</td>
<td>1.06 (\pm) 0.20</td>
<td>7.39 (\pm) 3.72</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>21.4 (\pm) 2.78</td>
<td>5.70 (\pm) 0.27</td>
<td>269.1 (\pm) 23.6</td>
<td>25.2 (\pm) 4.41</td>
<td>5.36 (\pm) 0.33</td>
<td>215.6 (\pm) 24.5</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>2025 (\pm) 36.4</td>
<td>13.64 (\pm) 0.46</td>
<td>6.74 (\pm) 0.15</td>
<td>1095 (\pm) 198</td>
<td>1.87 (\pm) 0.20</td>
<td>1.73 (\pm) 0.14</td>
</tr>
</tbody>
</table>

Each value represents the mean \(\pm\) S.D. of triplicate experiments.

depropagation and \(N\)-demethylation of selegiline in human hepatic microsomes (a mixture of HHM-195, HHM-223 and HHM-224) was investigated. Anti-CYP2B and anti-CYP3A antibodies inhibited the \(N\)-depropagation more potently than the other antibodies (Fig. 4), while anti-CYP2B antibodies had the strongest effect on \(N\)-demethylation.

To examine the inhibitory effect of anti-CYP antibody on the two catalytic activities in human hepatic microsomes rich in CYP2B6 or in CYP1A2, Ms-28 and Ms-17 were used (Fig. 5). The amount of CYP protein in these microsomes is shown in Table 2. In Ms-28 rich in CYP2B6, anti-CYP2B antibody had the strongest inhibitory effect on both the \(N\)-depropagation and \(N\)-demethylation of selegiline of all the anti-CYP antibodies examined (Figs. 5A and 5B), with 56.9% and 67.0% inhibition, respectively, at 20 \(\mu\)L of antibody. In Ms-17 rich in CYP1A and poor in CYP2B6, anti-CYP1A antibody inhibited the two catalytic activities most potently than the other antibodies examined (Figs. 5C and 5D). Anti-CYP2B antibody showed little inhibition of either activity in Ms-17.

Inhibitory effect of selegiline on the catalytic activity of CYP isoforms: The influences of selegiline (5, 20 and 100 \(\mu\)M) on 7-ethoxyresorufin \(O\)-deethylation (CYP1A1/2), coumarin 7-hydroxylation (CYP2A6), (S)-mephenytoin \(N\)-demethylation (CYP2B6), tolbutamide 4-hydroxylation (CYP2C9), (S)-mephenytoin 4’-hydroxylation (CYP2C19), bufuralol 1’-hydroxylation (CYP2D6), chlorozoxazone 6-hydroxylation (CYP2E1) and testosterone 6\(\beta\)-hydroxylation (CYP3A4) in pooled human hepatic microsomes were examined (Fig. 6). An inhibitory effect was observed on (S)-mephenytoin \(N\)-demethylation dependent on the
Fig. 4. Inhibitory effect of anti-CYP antibody on N-depropagation and N-demethylation of selegiline in human hepatic microsomes. Antibody: (○) CYP1A2, △(CYP2A), (○)(CYP2B), (○)(CYP2C), ▲(CYP2D), ■(CYP2E), ●(CYP3A), ●(control).

Selegiline concentration: 100 μM. Microsomes: mixed human hepatic microsomes (HHM-195, HHM-223, HHM-224). Control activities of selegiline N-depropagation and selegiline N-demethylation in HHM-195, HHM-223, HHM-224 without antibody were 1.636 ± 0.137 nmol/min/mg protein and 1.157 ± 0.103 nmol/min/mg protein, 0.689 ± 0.041 nmol/min/mg protein and 0.481 ± 0.031 nmol/min/mg protein, and 1.241 ± 0.067 nmol/min/mg protein and 0.756 ± 0.032 nmol/min/mg protein, respectively.

Each point represents the mean ± S.D. of triplicate assays.

Table 2. Amount of CYP protein in human hepatic microsomes

<table>
<thead>
<tr>
<th>CYP isoforms (pmol/mg protein)</th>
<th>1A2</th>
<th>2A6</th>
<th>2B6</th>
<th>2C9</th>
<th>2D6</th>
<th>2E1</th>
<th>3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHM-195</td>
<td>4.9</td>
<td>42.8</td>
<td>15.7</td>
<td>45.9</td>
<td>18.2</td>
<td>15.4</td>
<td>70.1</td>
</tr>
<tr>
<td>HHM-223</td>
<td>1.3</td>
<td>43.3</td>
<td>6.0</td>
<td>26.8</td>
<td>6.4</td>
<td>18.4</td>
<td>37.1</td>
</tr>
<tr>
<td>HHM-224</td>
<td>0.8</td>
<td>49.1</td>
<td>10.1</td>
<td>14.9</td>
<td>6.2</td>
<td>20.7</td>
<td>99.0</td>
</tr>
<tr>
<td>Ms-17</td>
<td>11.8</td>
<td>19.3</td>
<td>4.4</td>
<td>56.7</td>
<td>1.5</td>
<td>21.9</td>
<td>68.3</td>
</tr>
<tr>
<td>Ms-28</td>
<td>7.2</td>
<td>29.6</td>
<td>25.6</td>
<td>78.7</td>
<td>3.2</td>
<td>18.4</td>
<td>66.2</td>
</tr>
</tbody>
</table>

Amount of each CYP isoform was measured by Western blot analysis.

Concentration of selegiline, with 25.0%, 51.5% and 72.8% inhibition at 5, 20 and 100 μM of selegiline, respectively. The activity of (S)-mephénytoin 4′-hydroxylation was also inhibited 67.3% only at the highest concentration of selegiline (100 μM). The other CYP-mediated reactions were inhibited less than 32.0%. The reference inhibitors used as positive controls inhibited the activities of the respective CYP-mediated reactions in a concentration-dependent manner; from 50% to 80% at 20 and 100 μM (data not shown).

Discussion

Several CYP isoforms such as CYP1A2, CYP2B6, CYP2C19, CYP2D6 and CYP3A4 have been known to contribute to the metabolism of selegiline, but it is not clear which isoforms are most involved in the metabolism in humans. In this study, we investigated the contribution of CYP isoforms to the N-depropagation and N-demethylation of selegiline using recombinant CYP isoforms and human hepatic microsomes.

Among the recombinant CYP isoforms expressed in human lymphoblastoid cells, CYP2B6 contributed markedly to both the N-depropagation and N-demethylation compared with other CYP isoforms including CYP1A2, CYP2C19, CYP2D6 and CYP3A4. In the kinetic analysis, CYP2B6 displayed a much smaller K_m value and much larger V_max/K_m value for both N-depropagation and N-demethylation than either CYP1A1 or CYP3A4. These results suggest that CYP2B6 is a high-affinity CYP isoform for the metabolism of selegiline in humans.

The participation of CYP2B6 in the metabolism of selegiline was confirmed by the following; anti-CYP2B
Fig. 5. Inhibitory effect of anti-CYP antibody on N-depropagation and N-demethylation of selegiline in CYP2B-rich or CYP1A-rich human hepatic microsomes.

(A), (B): CYP2B6-rich human hepatic microsomes (Ms-28). Control activities of selegiline N-depropagation and selegiline N-demethylation in Hs-28 were 0.986 ± 0.078 nmol/min/mg protein and 0.711 ± 0.062 nmol/min/mg protein, respectively.

(C), (D): CYP1A2-rich human hepatic microsomes (Ms-17). Control activities of selegiline N-depropagation and selegiline N-demethylation in Hs-17 were 0.438 ± 0.008 nmol/min/mg protein and 0.278 ± 0.007 nmol/min/mg protein, respectively.

Antibody: (○) CYP1A, △ (CYP2A), ◻ (CYP2B), ◇ (CYP2C), ▲ (CYP2D), ■ (CYP2E), ● (CYP3A), ● (control).

Selegiline concentration: 100 μM. Each point represents the mean ± S.D. of triplicate assays.
antibody (100 µg IgG) inhibited both the N-depropagation activity and N-demethylation activity of selegiline more than any other anti-CYP antibody in the mixed human hepatic microsomes. In addition, its inhibitory effect on both catalytic activities was strengthened in the CYP2B6-rich microsomes. These results indicate a major role for CYP2B6 in the metabolism of selegiline. However, anti-CYP1A antibody strongly inhibited both N-depropagation and N-demethylation activities in the CYP1A2-rich and CYP2B6-poor human hepatic microsomes. Consequently, CYP2B6 is not the main isoform involved in the metabolism of selegiline in individuals with very low levels of CYP2B6 in liver. These immunoinhibition studies indicate that CYP2B6 is most involved in the metabolism of selegiline, but the degree of its contribution varies with the level of its expression in the liver.

The fact that selegiline inhibited CYP2B6-mediated (S)-mephenytoin-N-demethylation and CYP2C19-mediated (S)-mephenytoin 4′-hydroxylation activities suggests that attention should be paid to the drug-drug interactions associated with CYP2B6 and CYP2C19. The most recently, Hidestrand et al. reported that CYP2B6 and CYP2C19 are the major enzymes responsible for the metabolism of selegiline.11 There is the difference in the contribution of CYP2C19 to the metabolism of selegiline between this recent report and our results, but the reason for this difference is presently unknown.

In conclusion, CYP2B6 is closely involved in both the N-depropagation and N-demethylation of selegiline, while CYP3A4 and CYP1A2 contribute to a lesser extent. However, the degree to which CYP2B6 contributes to the metabolism of selegiline in humans varies with the level of its expression in the liver. Consequently, contribution by CYP3A4 and CYP1A2 to the metabolism of selegiline is possible in case that the expression level of CYP2B6 is low.

References

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