CYP-Dependent Metabolism of PF9601N, A New Monoamine Oxidase-B Inhibitor, by C57BL/6 Mouse and Human Liver Microsomes

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ABSTRACT - Purpose. The selective monoamine oxidase-B (MAO-B) inhibitor, l-deprenyl, is still used for treating Parkinson's patients, however, a disadvantage of its use lies in the formation of l-amphetamine and l-methamphetamine. Subsequently, this has promoted the design of a novel, more potent, MAO-B inhibitor PF9601N, which also has neuroprotective and antioxidant properties. The aim of this work was to investigate the effect of treatment with PF9601N on its own phase I hepatic metabolism. Kinetic parameters of PF9601N CYP-dependent N-dealkylation reaction was also studied and compared with those of l-deprenyl. Methods. C57BL/6 mice were treated with PF9601N for 4 days. After CYP content and related monoxygenase activities were assayed in liver microsomes of control and treated animals. Results. CYP activities, cytochrome b₅ content, NADPH-cytochrome P450 reductase and various monooxygenase activities were unaffected by in vivo PF9601N treatment. With microsomes from both control and treated mice, the PF9601N-dealkylation product, FA72, was the only detected metabolite with its formation rate following an hyperbolic, Michaelis-Menten curve. Among various inhibitors, only ketoconazole inhibited the FA72 formation rate, indicating a major involvement for CYP3A. Apparent Km and Vmax values generated by human liver microsomes were similar to those found with mouse microsomes. Ketoconazole inhibition indicates that CYP3A is one of the major enzymes involved in PF9601N metabolism also by human liver microsomes. In mouse liver microsomes, the intrinsic clearance of PF9601N was significantly lower than that of l-deprenyl suggestive of an improved bioavailability for the former. Conclusion. The observed favourable metabolic profile may suggest suitability of PF9601N for clinical use.

INTRODUCTION

l-Deprenyl is a selective monoamine oxidase-B (MAO-B) inhibitor. Owing to its capacity to increase striatal dopamine levels, it has been used for treating Parkinson's patients (1). In patients with early, untreated Parkinson's disease, controlled clinical trials have demonstrated that l-deprenyl delays the disability and decrease the rate of progression of signs and symptoms of this condition (2).

l-Deprenyl undergoes 90% presystemic clearance, hence, its bioavailability is low (3). In addition the observation that pretreating animals with l-deprenyl protects them against the neurotoxic effects of compounds such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine and N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) has stimulated studies aimed at clarifying the pharmacological and biochemical properties of this drug (4). Although other MAO-B inhibitors also act as neuroprotectors in experimental models of parkinsonism at concentrations lower than those required for MAO-B inhibition (5), l-deprenyl is the only MAO-B inhibitor available for therapeutic use. Its metabolites include l-amphetamine and l-methamphetamine, which can have adverse side-effects, and for some years, there has been some controversy as to whether l-deprenyl should continue to be part of Deprenyl and Tocopherol Antioxidative Therapy for Parkinson’s (DATATOP) treatment (6).

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Therefore, the search for new MAO-B inhibitors without potential amphetamine-like properties is a matter of great therapeutic interest. N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine, or PF 9601N, has been synthesized in the Laboratorio de Radicales Libres (Instituto de Chimica Organica General, CSIC, Madrid, Spain) by Cruces et al. (7). It has been shown to behave in vitro as a suicide MAO-B inhibitor (8), so that its effect could only be overcome by de novo MAO-B synthesis. It has also been shown that this novel MAO-B inhibitor easily crosses the blood–brain-barrier and results in in vivo, properties similar to those described in vitro (8).

Furthermore, PF9601N blocks dopamine uptake in human caudate and rat synaptosomal fractions with similar potency to that of l-deprenyl (8), whereas rasagiline does not (9). PF 9601N also enhanced the duration of L-DOPA-induced contralateral turning in 6-hydroxydopamine (6-OHDA) lesioned rats (10).

In a recent study (11) it has been shown that PF9601N and its putative cytochrome P450-dependent metabolite, 2-(5-benzyloxy-indolyl) methylamine (FA72) (figure 1) exerted antioxidant and neuroprotective activities in human neuroblastoma SHSY5Y cell line injured by dopamine oxidative stress. The concomitant structure-activity relationship study allowed us to conclude that the presence of a benzyloxy-, or a hydroxy- or methoxy- group in 5-position of the indol ring enhanced its antioxidant features, showing an increasing order of antioxidant activity for the primary > secondary > tertiary amine (11).

Due to the possible relevant pharmacological properties of the cytochrome P450 (CYP)-dependent metabolites of PF9601N, a study was performed in order to clarify the metabolism of this MAO-B inhibitor and envisage any interactions with other drugs at CYP system level. The effects on liver CYP isozymes of pre-treating animals with PF9601N were investigated on C57BL/6 mice, a widely accepted rodent model for Parkinsonism induced with MPTP. The present results indicate that PF9601N does not impair monooxygenase activities and has a lower intrinsic clearance as compared to l-deprenyl, thus suggesting that its use in Parkinson’s patients is devoid of some of l-deprenyl disadvantages.

METHODS

Materials

Human pooled liver microsomes were purchased from SIGMA Chemical Co (Milan, Italy), N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine (PF 9601N), 2-(5-benzyloxy-indolyl) methylamine (FA72) and N-(2-butynyl)-2-(5-benzyloxy-indolyl) methylamine (FA 74) were synthesized in Laboratorio de Radicales Libres (Instituto de Chimica Organica General, CSIC, Madrid) (7), L-(-)-N,α-dimethyl-N-2-propynylphenethyl-amine hydrochloride (l-deprenyl-HCl) and L-(-)-N,α-methyl-N-2-propynylphenethyl-amine (l-nordeprenyl) were gifts from Chinnoin Chemical Works (Budapest, Hungary), ketoconazole from Jannsen (Roma, Italy); NADPH, NADP, and glucose-6-phosphate dehydrogenase were provided by Boehringer (Mannheim, Germany). All other chemicals and solvents were of the highest grade available and obtained from common commercial sources.

Drug treatments

The animal protocols used were reviewed and approved by the Animal Care and Ethics Committee of the Università degli Studi di Siena and Ministry of Health, Rome, Italy. Male C57BL/6 mice (20-25 g body weight), obtained from Charles River (Milan, Italy), were housed in standard cages in a temperature- and light-controlled facility and allowed free access to food (M.I.L. from Morini, S.Polo d’Enza, Italy) and water. After an acclimatisation period of 10 days, one group of 10 mice was treated daily i.p. with PF9601N 10 mg x Kg\(^{-1}\) in normal saline and DMSO (9:1) for 4 days, as reported for l-deprenyl study in Valoti et al. (12). The control group consisted of 10 mice treated i.p with vehicle (saline: DMSO, 9:1).

Preparation of liver microsomes

All mice were fasted overnight prior to the sacrifice by CO\(_2\) asphyxia. The abdominal cavity was opened and the liver perfused in situ, through the vena cava, with ice-cold physiological saline solution. Livers were excised and weighed. They were then combined in pairs, chopped, suspended in 4 volumes of 0.25 M sucrose and homogenized in a Potter-Elvehjem homogeniser fitted with a teflon pestle. Homogenates were centrifuged at
10,000 g for 20 min and the resulting supernatants were centrifuged at 105,000 g for 1 h. The resulting microsomal pellets were suspended in 0.01 M Tris-HCl buffer, pH 7.6, containing 151 mM KCl, 1 mM EDTA and 20% glycerol, and stored in liquid nitrogen until use.

**Assay procedures**

CYP and cytochrome b₅ contents were measured from the CO-difference spectra of the microsomal preparation by the method of Omura and Sato (13) at A: 450-490 nm (ε: 91 mM) and A: 424-490 nm (ε: 112 mM), respectively. The minimum amount of CYP and cytochrome b₅ detectable was 0.05 nmoles·mg⁻¹ protein (CV 7.4%) and 0.07 nmoles·mg⁻¹ protein (CV 6.3%), respectively. NADPH-cytochrome P450 reductase activity was measured by following the cytochrome c reduction at 550 nm according to Masters et al. (14). The minimal amount detectable was 1 nmol·min⁻¹·mg⁻¹ protein (CV 5.6%). Testosterone hydroxylase activity was determined by an HPLC method (15) after 30 min incubation in presence of 0.2 mM testosterone, minimal amount detectable was 2 pmol·min⁻¹·mg⁻¹ protein (CV 8.3%).

Erythromycin demethylase (ErD) was assayed by following the formation of formaldehyde, trapped with the semicarbazide and measured with the Nash reagent at 412 nm, minimal amount detectable was 50 pmol·min⁻¹·mg⁻¹ protein (CV 9.7%). Methoxyresorufin O-demethylase (MROD), ethoxyresorufin O-deethylation (EROD), pentoxyresorufin O-depentylase (PROD) and benzyloxyresorufin O-debenzylase (BROD) activities were determined by measuring the formation of the fluorescence product resorufin at excitation and emission wavelength of 522 and 586 nm, respectively (17). The minimum amount detectable was 0.2 pmol·min⁻¹·mg⁻¹ protein (CV 3.6%). Cumarin hydroxylase activity (CoH) was determined by measuring the fluorescence of 7-OH-cumarin at excitation and emission wavelength of 370 and 450 nm, respectively as reported by Ko et al. (18). The minimum amount of compound detectable was 1.2 pmol·min⁻¹·mg⁻¹ protein (CV 6.8%). p-Nitrophenol (pNPH) hydroxylase activity was assayed by recording after 30 min period the formation of p-nitrocatechol according to the procedure describe by Reinke and Moyer (19). The minimum amount detectable was 0.1 nmol·min⁻¹·mg⁻¹ protein (CV 8.1%).

![](image1.png)

**Figure 1.** CYP-dependent N-dealkylation of PF9601N (panel A) and L-deprenyl (panel B).
Ex vivo and in vitro PF9601N metabolism

To investigate PF9601N N-dealkylation reaction, incubation mixture consisted of 0.5 mg x ml⁻¹ of liver microsomal protein, a range concentration of PF9601N (3-300 μM) and 100 mM phosphate buffer, pH 7.4, in a final volume of 0.5 ml. The reaction was started by adding 100 μl of a solution (NADPH-generating system) containing 1mM NADP⁺, 4mM glucose-6-phosphate and 1U x ml⁻¹ glucose-6-phosphate-dehydrogenase in 48 mM MgCl₂. After 30 min incubation in a shaking water bath thermostatted at 37°C, the reaction was terminated by adding 0.1 ml of 5M NaOH and cooled in ice-bath. Blanks containing boiled microsomes were incubated under the same conditions as the drug.

The PF9601N N-dealkylation reaction by human liver microsomes was carried out as above, by extending the reaction up to 60 min. Reaction mixtures were spiked with 100 nmol FA74 as internal standard and extracted once with 6 ml ethylacetate. The recovery of both parent drug and metabolites was subsequently determined by HPLC.

HPLC analysis

HPLC analysis was performed on a Shimadzu LC-10AD liquid chromatograph equipped with a EC150/4,6 Nucleosil 100 C18 (150 x 4.6 mm, 5 μm) capillary column at a flow rate of 1 ml x min⁻¹. The mobile phase consisted of 10 mM KH₂PO₄/CH₃CN/CH₃OH (60:35:5), pH 2. Ultraviolet detector (Shimadzu LC-10AV6) was set at 270 nm. The sample was injected by means of a reodyne valve with a 20 μl loop. Calibration curves were obtained by adding different amounts of the analytes to heat-inactivated microsomes.

Under the conditions described above retention times for PF9601N and its N-depropynylated metabolite, FA72, were 7.8 and 5.5 min, respectively. The internal standard FA74 presented a peak at 10.1 min. The recovery of analytes after incubation was 85% for PF9601N, 78% for FA72 and 80% for FA74. The sensitivity limit of the method, taken as the ratio between peak height of compounds and the background noise height ≥10 was 1.26 nmol injected. The relationship between analytes concentration and peak areas ratio versus area of the internal standard was linear up to 100 μM of the metabolite and 300 μM of the parent compound solution. Analysis of 6 standard curves performed over a week gave a correlation coefficient (r²) 0.9625. Day by day, the coefficient of variation of the slope of the calibration curves was 7.6%.

In vitro l-deprenyl metabolism

When l-deprenyl was used as a substrate the incubation mixture consisted of 0.5 mg x ml⁻¹ of mouse liver microsomal protein, a range concentration of l-deprenyl (3-300μM) and 100 mM phosphate buffer, pH 7.4, in a final volume of 1 ml. The reaction was started by adding 100 μl of NADPH-GS solution. After 15 min incubation in a shaking water bath thermostatted at 37°C, the reaction was stopped by adding 0.2 ml of 5M NaOH and cooling in ice-bath. Blanks containing boiled microsomes were incubated under the same conditions as the drug incubations. The metabolites and parent compounds were determined by GLC analysis, as reported elsewhere (20). The sensitivity limit of the method, taken as the ratio between peak height of compounds and the background noise height ≥10 for the parent compound and the metabolites was 5 pmol injected, day by day, the CV% of the slope of the calibration curves (range 0.05-100 μM) was 3% for l-deprenyl, 12% methamphetamine and 8.5% for nordeprenyl.

In vitro inhibition of PF9601N metabolism by mouse and human liver microsomes

Inhibition experiments were carried out by incubating mouse liver microsomal preparations (n=5) with 50μM inhibitors (α-naphthoflavone, 8-methoxypsoralen, sulfaphenazole, quinidine, 4-methylpyrazole, ketoconazole and secobarbital) (21-26), considered to be relatively specific for some CYP enzymes (CYPs 1A, 2A6, 2C9, 2D6, 2E1, 3A4 in human and 2B1 in rat, respectively). Inhibition rates were assessed at 80 μM PF9601N. The reaction mixture contained 0.5 or 1mg x ml⁻¹ of mouse or human microsomal protein, respectively, 50 μM inhibitor and 100 mM phosphate buffer, pH 7.4. The inhibitor was added first to microsomes and preincubated for 5 min, the reaction was started with PF9601N and 100 μl NADPH-GS in a shaking water bath thermostatted at 37°C. The reaction was stopped after 30 min incubation, for mouse, and 1h for human microsomes by adding 0.2 ml of 5 M NaOH.
Statistical analysis

Kinetic parameters were calculated according to the Michaelis-Menten equation for one or two enzymes by nonlinear regression analysis (Prism 3.02 Graphpad Software Inc, San Diego, CA, USA). All values are presented as means ± standard error of the mean (S.E.M.). The significance of differences between treatment and control groups was established by the Student's t-test.

RESULTS

Mixed function oxidase activities in liver microsomes from C57BL/6 control and treated mice

The 4-days treatment with PF9601N did not significantly alter either body or liver weight gain (results not shown). CYPs and cytochrome b₅, NADPH-cytochrome P450 reductase and several monoxygenase activities in liver microsomes from control and treated mice are given in Table 1. Either CYP and cytochrome b₅ content or NADPH-cytochrome P-450 reductase activity turned out to be unaffected by treatment. Furthermore, CYP related activities namely pNPH (CYP2E1), CoH (CYP2A5), EROD (CYP1A1), MROD (CYP1A2), PROD (CYP2B), ErD (CYP3A) were not significantly changed by treatment.

Kinetics analysis of PF9601N CYP-dependent N-dealkylation by C57BL/6 mice and human liver microsomes

A typical chromatogram of PF9601N after 30 min incubation with mice liver microsomes is reported in Figure 2. FA72 appeared to be the only detectable metabolite of PF9601N under our experimental conditions. Furthermore, two additional unknown peaks, with retention time of 2.9 and 3.7 min, respectively, were evident. However, they were not related to PF9601N CYP-dependent metabolism, since they were present also in the blank samples. CYP-dependent N-dealkylation of PF9601N under the conditions described above was linear with time, up to 60 min, both in mice and in human microsomal preparations; initial rates being proportional to the amount of protein added up to 2 mg x ml⁻¹.

When PF9601N was incubated with liver microsomal fractions prepared either from control and treated animals, at all substrate concentrations tested, FA72 turned out to be the only detected metabolite, the reaction following a hyperbolic, Michaelis-Menten equation thus suggesting that only one CYP isozyme is involved in PF9601N metabolism. In conjunction with these observations (Figure 3 inset), linear Eadie-Hofstee plots (27) were obtained. Similar results were observed when kinetic analyses were performed with microsomes from in vivo PF9601N-treated mice (Figure 3). These microsomes, showed lower though not significant Vmax and Km values as compared to those observed in control preparations. Relative Vmax/Km ratios, taken as an index of intrinsic clearance, were of the same order of magnitude in either preparations (Table 2).

When PF9601N was incubated with human microsomes FA72 formation rate followed a single-substrate kinetic, as shown in Figure 4. The apparent Km and Vmax values were of the same order of magnitude as those observed with mouse microsomes (Table 2).

Table 1. Mixed function oxidase activities in liver microsomes from C57BL/6 mice treated with vehicle (Control) or PF9601N 10 mg x Kg⁻¹ i.p (Treated) for 4 days.

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome-P450</td>
<td>0.794±0.069</td>
<td>0.707±0.059</td>
</tr>
<tr>
<td>Cytochrome b₅</td>
<td>0.654±0.080</td>
<td>0.653±0.040</td>
</tr>
<tr>
<td>NADPH-P450 reductase</td>
<td>124.085±20.632</td>
<td>151.621±34.260</td>
</tr>
<tr>
<td>pNPH</td>
<td>2.129±0.558</td>
<td>2.095±0.366</td>
</tr>
<tr>
<td>CoH</td>
<td>0.045±0.005</td>
<td>0.042±0.007</td>
</tr>
<tr>
<td>EROD</td>
<td>0.025±0.004</td>
<td>0.024±0.005</td>
</tr>
<tr>
<td>MROD</td>
<td>0.094±0.017</td>
<td>0.075±0.013</td>
</tr>
<tr>
<td>PROD</td>
<td>0.0105±0.001</td>
<td>0.017±0.006</td>
</tr>
<tr>
<td>BROD</td>
<td>0.075±0.002</td>
<td>0.084±0.003</td>
</tr>
<tr>
<td>ErD</td>
<td>0.544±0.102</td>
<td>0.591±0.154</td>
</tr>
</tbody>
</table>

*pNPH, p-nitrophenol hydroxylase; CoH, coumarin hydroxylase; EROD, ethoxyresorufin O-deethylase activity; MROD, methoxyresorufin O-demethylase activity; PROD, pentoxyresorufin O-depentylase activity; BROD, benzylxoyresorufin O-debenzylase activity; ErD, erythromycin demethylase activity.
Table 2. Kinetic constants for PF9601N metabolism by mouse and human liver microsomal preparations.

<table>
<thead>
<tr>
<th>Metabolite formed</th>
<th>Liver microsomes preparations</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>$CL_{I}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA72 mouse</td>
<td>37.0±4.8</td>
<td>26.0±1.1</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>FA72 pre-treated mouse*</td>
<td>34.2±5.0</td>
<td>20.1±0.9</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>FA72 human</td>
<td>11.0±2.2</td>
<td>10.0±0.4</td>
<td>0.9</td>
<td></td>
</tr>
</tbody>
</table>

* Pre-treated for 4 days with PF9601N (10 mg x Kg$^{-1}$ i.p., daily); data are means from experiments performed from 5 different preparations. The intrinsic clearance ($CL_{I}$) was calculated as $V_{max}/K_m$ ratio for each group of animals.

**Kinetic analysis of l-deprenyl CYP-dependent metabolism by C57BL/6 mice liver microsomes**

A preliminary assay of l-deprenyl N-dealkylation by mice liver microsomes, gave rise to the primary metabolites l-methamphetamine and l-nordeprenyl, the amount of products formed increased linearly with time up to 45 min and the initial rates were proportional to the amount of microsomal protein added up to 1 mg x ml$^{-1}$. The formation of low amounts of the secondary metabolite, l-amphetamine, was also evident.

Kinetic analysis of l-deprenyl N-dealkylation rates indicated that at all the concentrations of l-deprenyl tested, l-methamphetamine turned out to be the major metabolite formed, its concentration in the assay mixture being about 1.5-2.0 fold greater than that of l-nordeprenyl. The kinetic analysis of l-deprenyl metabolism data suggests that for both N-dealkylation reactions at least two components -one characterized by low and the other by high affinity- were describing substrate concentration vs. metabolite formation rate relationship. Accordingly, as shown in the inset of Figure 5, biphasic Eadie-Hofstee plots were obtained (27).

As depicted in Table 3, the high affinity component ($K_{m1}$) for both l-nordeprenyl and l-methamphetamine formation had similar $K_m$ values. Also the low affinity ($K_{m2}$) components had similar apparent $K_m$ values, but of about one order of magnitude higher than those of the high affinity component.

![Figure 2](image-url)
Figure 3. Kinetic analysis of FA72 formation rate by liver microsomes prepared from control and in vivo PF9601N treated mice. Data fitting was performed according to the Michaelis-Menten equation, while the inset shows the Eadie-Hofstee plots of the same data. Data refers to the mean ± SEM, derived from five different microsomal preparations.

Figure 4. Kinetic analysis of FA72 formation in human pooled liver microsomes. Fitting of data from three different microsomal preparations was performed according to the Michaelis-Menten equation, while the inset shows the Eadie-Hofstee plot of the same data. Data are reported as mean ± SEM.
Table 3. Kinetic constants for \( l \)-deprenyl metabolism by mouse liver microsomal preparations.

<table>
<thead>
<tr>
<th>Metabolite formed</th>
<th>( K_m^1 ) (( \mu \text{M} ))</th>
<th>( K_m^2 ) (( \mu \text{M} ))</th>
<th>( V_{\text{max}^1} ) (nmol x h(^{-1}) x mg(^{-1}) protein)</th>
<th>( V_{\text{max}^2} ) (nmol x h(^{-1}) x mg(^{-1}) protein)</th>
<th>( CL_{I1} ) (h(^{-1}))</th>
<th>( CL_{I2} ) (h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methamphetamine</td>
<td>23.9±7.3</td>
<td>443.7±1.7</td>
<td>254.8±6.2</td>
<td>995.4±1.1</td>
<td>10.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Nordeprenyl</td>
<td>14.6±4.2</td>
<td>518.7±1.1</td>
<td>123.6±2.0</td>
<td>912.0±8.6</td>
<td>8.5</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Data are means from experiments performed from 5 different preparations. The intrinsic clearance of the high affinity component (\( CL_{I1} \)) and of the low affinity component (\( CL_{I2} \)) was calculated as \( V_{\text{max}}/K_m \) ratio for each group of animals.

Figure 5. Kinetic analysis of methamphetamine and nordeprenyl formation rate by C57BL/6 mice liver microsomes incubated with \( l \)-deprenyl. Data fitting were performed according to the Michaelis-Menten equation, while the inset shows the Eadie-Hofstee plots of the same data. Data refer to the mean ± SEM, derived from five different microsomal preparations.

As to the low affinity component, \( V_{\text{max}} \) value for \( l \)-methamphetamine formation of the high affinity component (\( V_{\text{max}^1} \)) was about two fold higher than that for the formation of \( l \)-nordeprenyl. The ratio \( V_{\text{max}}/K_m \) as an index of intrinsic clearance (\( CL_i \)), for both metabolites was one order of magnitude higher for the high affinity component (\( K_m^1 \)) as compared to that for the low affinity component (\( K_m^2 \)). This suggests that the high affinity component is the major determinant of \( l \)-deprenyl N-dealkylation oxidation by mice liver microsomes.

Inhibition of PF9601N metabolism by mice and human liver microsomes

In order to identify CYP isozymes involved in PF9601N N-dealkylation, an inhibition study was performed by incubating mice or human liver microsomes with 80 \( \mu \text{M} \) PF9601N (2 times the \( K_m \) concentration) in presence of specific inhibitors of some CYP isoenzymes. 8-Methoxypsoralen, was used to inhibit mouse CYP2A5, closely related to human CYP2A6 (21), whilst 4-methylpyrazole and ketoconazole were used to inhibit mouse CYP2E1 and 3A isoforms, respectively (22-23). \( \alpha \)-Naphthoflavone,
sulfaphenazole, quinidine, and secobarbital were used to inhibit specifically CYPs 1A, 2C, 2D, and 2B, respectively (24-25-26).

As shown in Figure 6, only ketoconazole promoted a significant inhibition of about 35% of PF9601N N-dealkylation. CYP-dependent testosterone hydroxylase activities were also tested in presence of 80 µM PF9601N. As shown in Figure 7, PF9601N significantly inhibited the formation of 6β-hydroxytestosterone, marker of CYP3A activity, by about 78% as compared to control values. Furthermore the 30% inhibition of 15β-hydroxytestosterone formation, catalysed by CYP3A confirmed the previous data (28). The inhibition study performed on human liver microsomes showed that, as observed in mouse liver microsomal preparations, only ketoconazole significantly inhibited by about 30% PF9601N N-dealkylation rate (Figure 8).

DISCUSSION

Acetylenic compounds, including some MAO inhibitors, have been proven to affect CYP activities and it has been suggested that they interact with other drugs by inhibiting CYP isoenzymes (29). This can have important clinical consequences, since CYPs inactivation often leads to severe and long-lasting impairment of metabolic drug clearance and clinically meaningful drug-drug interactions (30).

In the present study, the 4-days treatment of C57BL/6 mice with a novel acetylenic MAO-B inhibitor, PF9601N, did not modify cytochrome P450 and b5 content, and did not change NADPH-CYP-reductase and CYP2E1, 2A5, 1A1, 2B, 3A activities. Furthermore, CYP-dependent metabolism of PF9601N by liver microsomes from either control or treated mice gave rise only to the formation of the N-dealkylated metabolite, FA72, identified either by HPLC retention time and MS analysis. Microsomal preparations, when tested for PF9601N N-dealkylation, at various substrate concentrations, exhibited a simple hyperbolic kinetic behavior which was confirmed by monophasic Eadie-Hofstee plots from treated and control mice.

These results indicate that PF9601N does not affect its own metabolism. This is contrary to what is known for other MAOIs, such as tranylcypromine, phenelezine and moclobemide (31). Moreover, we have previously shown that a 3-days treatment of C57BL/6 mice with l-deprenyl caused a significant decrease in liver microsomal CYP content as well as pNPH activity (12).

**Figure 6.** Effects of α-naphthoflavone (ANF), 8-methoxypsoralen (8-MP), sulfaphenazole (SPZ), quinidine (QND), 4-methylpyrazole (4-MPY), ketoconazole (KTZ) and secobarbital (SECO) on FA72 formation rate. PF9601N (80 µM) was incubated with C57BL/6 mouse liver microsomes at 37°C for 30 min in presence 50 µM inhibitor. Data are the means ± SEM (bars) from five different microsomal preparations. The comparison between metabolite formation in absence (CT) and in presence of inhibitor was performed by Student t test; *p<0.05.
Figure 7. Testosterone hydroxylase activity by C57BL/6 microsomes. Data are means ±SEM (bars) of five liver microsomal preparations. Incubations were carried out at 37°C for 30 min with 1 mg x ml⁻¹ microsomal protein. 6α/β, 6α/β-hydroxytestosterone; 6β, 15β, 15β-hydroxytestosterone; 7α, 7α-hydroxytestosterone, 16α/β, 16α/β-hydroxytestosterone; 11α, 11α-hydroxytestosterone; 2α/β, 2α/β-hydroxytestosterone and 17-OT, androst-4-ene-3,17-dione were detected. The comparison between metabolite formation rate in absence and in presence of PF9601N was performed by using Student t test; **p < 0.01.

Figure 8. Effects of α-naphthoflavone (ANF), 8-methoxypsoralen (8-MP), sulfaphenazole (SPZ), quinidine (QND), 4-methylpyrazole (4-MPY), ketoconazole (KTZ) and secobarbital (SECO) on FA72 formation rate. PF9601N (80 µM) was incubated with human pooled liver microsomes at 37°C for 1h in presence of 50 µM of inhibitor. The experiments were performed using three different microsomal preparations. The comparison between metabolite formation rate in absence (CT) and in presence of inhibitor was performed by Student t test; *p < 0.05.

In the present study, the kinetic analysis of l-deprenyl N-dealkylation by mouse liver microsomes gave rise to a biphasic Eadie-Hofstee plot thus indicating that at least 2 or more enzymes catalyse the formation of methamphetamine and nordeprenyl. The kinetic constants, reported in Table 3, underline the presence of both a high and a low affinity component in the formation of l-deprenyl derivatives. Moreover, even if the apparent Km...
for the high affinity component \((K_{m1})\) of both methamphetamine and nordepryl formation was close to the values for PF9601N metabolism, a great difference was observed in the apparent \(V_{\text{max}}\) values that were one order of magnitude higher for both metabolites of \(l\)-depryl as compared to that of FA72. This gave rise to a much higher (by one order of magnitude) intrinsic clearance of \(l\)-depryl (for the high affinity component, \(CL_{I1}\)) as compared to that of PF9601N.

The high intrinsic clearance observed for \(l\)-depryl in mice is in agreement with what has been observed in humans (32), wherein \(l\)-depryl has a very low oral bioavailability (10%) as the result of extensive hepatic first-pass metabolism (3). The high intrinsic clearance of \(l\)-depryl in humans, indeed, makes it difficult to achieve clinically effective plasma concentrations following oral doses. For these reasons, transdermal \(l\)-depryl formulations have been developed recently, which, give rise to persistent (minimal peak-trough fluctuations), high plasma levels of the parent compound, with high amounts of the drug being delivered to the brain and a low metabolite production (33-34).

As compared with \(l\)-depryl, PF9601N, has a lower intrinsic clearance. Neither the new drug influences CYP contents and related monoxygenase activities. This suggests a higher metabolic stability and a lower chance to give rise to drug-drug interactions for PF9601N as compared with \(l\)-depryl. PF9601N, therefore, may be a suitable candidate for development as an alternative to \(l\)-depryl. Moreover, similarly to what has been observed in mouse, CYP-dependent metabolism of PF9601N by human liver microsomes gave rise to a linear Eadie-Hofstee plot suggesting the involvement of only one isozyme, with \(K_m\) and \(V_{\text{max}}\) values very close to the values observed in the mouse (Table 2). In contrast, the apparent \(K_m\) values detected with \(l\)-depryl in mouse were quite different from the values given by human liver microsomes (35-36). This suggests that the use of mouse liver microsomes to predict \(l\)-depryl metabolism in humans is inconsistent. The inhibition study performed in mouse liver microsomes showed a significant inhibition by ketoconazole only of FA72 formation, suggesting the major involvement of CYP3A in PF9601N metabolism. The role of CYP3A was confirmed by the marked inhibition of the formation of \(6\)-\(\beta\) and \(15\)-\(\beta\)-hydroxytestosterone derivatives. The weak inhibition of \(16\)-\(\beta\)-hydroxytestosterone formation rate, however, is not indicative of the involvement of CYP2B as confirmed by the finding that secobarbital, a selective rat 2B1 inhibitor, was not able to inhibit PF9601N metabolism by mouse liver microsomes (37-38). The inhibition study performed on human liver microsomes suggests the major involvement of CYP 3A family, as previously observed in mouse liver microsomes.

In conclusion these results suggest the use of PF9601N, a non amphetamine-like MAO-B inhibitor, be promoted in Parkinson’s patients. Furthermore, this study indicates that the mouse is a good animal model to predict the metabolism of PF9601N in humans.

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