A Study of the Biological Pharmacology of IFO, a New Selective and Reversible Monoamine Oxidase-B Inhibitor

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ABSTRACT—3-[4-[3-(1H-Imidazol-1-yl)propoxy]phenyl]-5-trifluoromethyl-1,2,4-oxadiazole (IFO), designed to be a novel selective inhibitor of monoamine oxidase (MAO), showed highly selective inhibition for type-B (MAO-B); its IC50 was approximately > 200 \( \mu \)M and 30 nM for type-A (MAO-A) and MAO-B, respectively, in the standard assay using mitochondrial preparations from rat brain or liver. The in vitro MAO-B inhibition by IFO was time-independent, non-competitive and tight-binding; and furthermore, in the presence of sodium cholate its inhibition was not tight-binding and was reversible. Oral administration of IFO (0.5 – 100 mg/kg) produced a dose-dependent MAO-B inhibition in mouse brain; its ED50 (p.o., 1 hr) was 1.6 mg/kg, while l-deprenyl inhibited the enzyme with the ED50 of approximately 8.0 mg/kg. The ED50 for MAO-A was > 100 mg/kg for either IFO and l-deprenyl. The MAO inhibitive effect of IFO in mouse liver was the same as that in the brain, but that of l-deprenyl in mouse liver was different from that in the brain as shown by the ED50 values of 35 mg/kg and 0.6 mg/kg for MAO-A and MAO-B, respectively. In mice, IFO increased the striatal concentrations of 2-phenylethylamine (2-PEA) and showed almost the same protective efficacy as l-deprenyl against the lethality and striatal dopamine (DA) depletion induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). These results indicate that IFO appears to be a potent inhibitor of MAO-B in mouse brain.

Keywords: IFO, MAO-B, l-Deprenyl, Parkinson's disease, MPTP

Biogenic endogenous and exogenous monoamines are inactivated by monoamine oxidase (MAO: EC 1.4.3.4). MAO exists in two functional isoenzymic forms termed MAO-A and MAO-B, which are differentiated by their selectivity towards various substrates and inhibitors. The MAO-A isoenzyme preferentially oxidizes 5-hydroxytryptamine (5-HT) and norepinephrine, whereas the MAO-B isoenzyme prefers trace amines such as 2-phenylethylamine (2-PEA) and octopamine. Dopamine (DA) and tryptamine seem to be oxidized by both MAO-A and MAO-B. Selective inhibitors of the two different forms of MAO have been discovered (e.g., clorgyline for MAO-A, l-deprenyl for MAO-B), and the enzymes show different pharmacological profiles (1). When a non-selective inhibitor is used, one must consider the hazards of its combination with a variety of foods and drugs, and the use of a MAO-A inhibitor has also been frequency accompanied by a number of other serious side effects based on the activation of the 5-HTergic system (2). The selective inhibitors of MAO-B, however, being free from these side effects (2, 3), are considered to be safe drugs for human use. Treatment with MAO-B inhibitor is thought to be suitable for the treatment of Parkinson's disease (PD) because nigrostriatal DA is thought to be inactivated predominantly by MAO-B in humans (4) and it is remarkably deficient in parkinsonian patients. Additionally, the turnover of DA by MAO increases in the surviving nigrostriatal neurons of parkinsonian patients, which consequently results in an increased formation of hydrogen peroxide that may lead to neuronal degeneration (5, 6). Recently, it has been found that platelet MAO-B activity is significantly raised in untreated parkinsonians with age-matched controls, and that the level of the endogenous MAO-B inhibitor isatin in their cerebrospinal fluid was decreased compared to that in the controls (7). Moreover, it has been found that endogenous 2-PEA may modulate the DAergic transmission in nigrostriatal neurons and that this modulation is enhanced by the increased striatal concentration of 2-PEA induced by l-deprenyl (8). Therefore, DA-turnover repression and optimum stimulation of DA neurons in parkinsonian nigrostriata are required, and the regulation of MAO-B activity with some selective
inhibitor may prove to be a suitable medical treatment to satisfy this requirement. The usefulness of l-deprenyl for suppressing the progression of this disease has already been demonstrated. It has been reported that the l-dopa (levodopa) treatment combined with l-deprenyl has reduced the side effects and prolonged parkinsonian lifespan as compared to treatment with levodopa alone (9). A potent and reversible MAO-B inhibitor would provide a safe adjuvant that is devoid of the hazards of unwanted side effects. However, most of the currently available potent MAO-B inhibitors, like l-deprenyl, are irreversible and long-lasting.

The present report is about the pharmacological evaluation of 3-[4-[3-(1H-imidazol-1-yl)propoxy]phenyl]-5-trifluoromethyl-1,2,4-oxadiazole (IFO) (Fig. 1), a new potent, selective and reversible MAO-B inhibitor. We describe the characteristics of the inhibitory effect of IFO on MAO-B activity in vitro and in vivo, and the protective efficacy of this compound against the DAergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the mouse (10).

**Fig. 1. Chemical structure of IFO.**

In the ex vivo MAO determination, mice were sacrificed at the indicated time, and then the isolated tissues were homogenized in 50 mM potassium phosphate buffer, pH 7.4. MAO-A activity was determined with 5-HT as the substrate by the fluorometric assay method of Hidaka et al. (13). MAO-B activity was determined with kynuramine as the substrate according to the fluorometric method of Kraml (14) under the inhibition of MAO-A activity by 1 μM clorgyline.

**Monoamine and its metabolites determinations**

After decapitation of the mice, the brain striata were quickly removed, frozen on dry-ice and stored at −80°C until used. Striatal DA, its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT) were determined by high-performance liquid chromatography with electrochemical detection as follows: Striata were homogenized in 0.2 ml of 0.1 M perchloric acid containing 3,4-dihydroxybenzylamine as an internal standard, 1 mM disodium EDTA, and 1 mg/ml sodium bisulfite. After standing for 20 min in ice cold water, the homogenate was centrifuged. The supernatant was eluted in a 5 μm Capcell ODS® (Shiseido, Tokyo) column of 4 mm x 250 mm with a mobile phase (pH 3.2) consisting of 4% acetonitrile, 75 mM sodium nitrate, 75 mM citric acid, 50 mM sodium acetate, 4 mM di-n-butylamine, 1 mM sodium octylsulfate and 0.15 mM disodium EDTA at a flow rate of 0.7 ml/min. 2-PEA only was determined by gas chromatography of the pentafluorobenzoyl derivative with electron capture detection according to the method of Reynolds et al. (15) after extraction with 0.2 ml of 0.1 M perchloric acid containing 3-phenylpropylamine as an internal standard.

**Drug treatments**

Male C57 black mice, 5- to 6-week-old, obtained from Clea Japan, Inc. (Tokyo) were used for in vivo and ex vivo experiments. Details for each experiment are described in the legend for each figure and table. MPTP hydrochloride was dissolved in saline solution and administered by s.c. injection. IFO suspended in 0.5% methylcellulose solution was administered orally at various doses. At the indicated time, mice were decapitated and their whole brains (or striata) and livers were removed quickly and frozen on dry ice, and they were stored at −80°C until used.

**Drugs**

IFO was synthesized at our company. L-Deprenyl hydrochloride and MPTP hydrochloride were obtained from Research Biochemicals, Inc. (Natick, MA, USA). Clorgyline was obtained from Sigma Chemical Co. (St.
Louis, MO, USA). All other chemicals used were reagent grade and commercially available.

Data analysis
Analysis of data was performed with Student’s t-test. Values were expressed as the mean±S.E.M. IC50 values were determined graphically from log-probit plots of five to eight different concentrations of test compound. ED50 (i.e., 50% decrease of the enzyme activity) values were determined graphically from semilogarithmic dose-inhibition plots. MAO activity in the ex vivo experiment was expressed as the percent relative to that found in the vehicle-treated control group.

RESULTS

Inhibition of MAO by IFO in vitro
In rat brain and liver mitochondrial preparations, the concentration of IFO required to inhibit the oxidation of BZ was lower than when it acted in the oxidation of 5-HT (Table 1). L-Deprenyl also potently inhibited MAO-B activity in these mitochondrial preparations, but did not inhibit more selectively than IFO. Under the different periods of preincubation, the IC50 value of IFO was not altered when BZ was used as a substrate, whereas that of L-

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Table 1. In vitro inhibition by IFO and L-deprenyl of MAO activity in mitochondrial fraction from rat brain and liver

<table>
<thead>
<tr>
<th>Compound</th>
<th>Preincubation time (min)</th>
<th>IC50 (μM)</th>
<th>Brain</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MAO-A</td>
<td>MAO-B</td>
<td>MAO-A</td>
</tr>
<tr>
<td>IFO</td>
<td>4</td>
<td>&gt;200</td>
<td>0.027</td>
<td>&gt;200</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>&gt;200</td>
<td>0.025</td>
<td>&gt;200</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>&gt;200</td>
<td>0.035</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Deprenyl</td>
<td>4</td>
<td>8.5</td>
<td>0.08</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.4</td>
<td>0.007</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.5</td>
<td>0.0035</td>
<td>0.4</td>
</tr>
</tbody>
</table>

IC50 values were determined graphically from log-probit plots of five to eight different concentrations of the compounds. Using the standard assay, the mitochondrial fractions of rat brain and liver were preincubated with various concentrations of the compounds for different periods of time, and the remaining MAO activity was determined for each type of the enzyme. 5-HT and BZ were used as selective substrates for MAO-A and -B, respectively.

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Fig. 2. Lineweaver-Burk plots on the MAO inhibitory activity of IFO in rat liver mitochondrial fraction in the absence of (A) and in the presence of (B) sodium cholate. The mitochondrial protein (0.54 mg/ml) was preincubated with (●) or without (○) IFO for 5 min at 37°C, and the initial velocity of MAO-B activity was determined by measuring oxygen consumption at varying concentrations of BZ. B: sodium cholate was present at a concentration of 1% in the preincubation and the activity determination.
deprenyl altered time-dependently under the same conditions. MAO activity in the mixture of IFO and a liver mitochondrial fraction did not recover after dialysis against (or washing with) potassium phosphate buffer, but recovered fully after dialysis in the presence of sodium cholate (not shown). The MAO-B inhibition activity of IFO in the mitochondrial preparation was non-competitive toward BZ in either the presence (Fig. 2B) or absence (Fig. 2A) of sodium cholate. In the absence of sodium cholate, the Henderson plot gave parallel lines supporting tight-binding behavior (16) (Fig. 3), and the IC\textsubscript{50} values obtained from the relationship between \( \text{Vi} \) and [IFO] were 30 and 86 nM at the concentrations of 0.54 and 1.08 mg/ml of protein, respectively. These results indicate that IFO acts as an irreversible inhibitor of MAO. The \( K_c \) value calculated from the slope obtained from plots of [IFO]/(1 – \( \text{Vi} \)/\( \text{Vo} \)) against [Vo/\( \text{Vi} \)] (Fig. 3) was 33 nM, and Fig. 2A shows the same value in the secondary plot of the slopes of the line against IFO concentrations. On the other hand, the line-separation depending on the protein concentration, although there was no precise line-crossing at the origin of the axes, was not obtained in the presence of sodium cholate (Fig. 3). The presence of sodium cholate created an IC\textsubscript{50} value of 49 nM at either protein concentration. The \( K_c \) value in the presence of sodium cholate was 90 – 180 nM in the Lineweaver-Burk and Henderson plots, so in the presence of sodium cholate, a higher concentration of IFO was required to inhibit MAO-B.

**Inhibition of MAO after a single administration of IFO in mice**

In mouse brain and liver ex vivo, IFO and l-deprenyl selectively and dose-dependently inhibited MAO-B. MAO-B was inhibited selectively in contrast with MAO-A by IFO as shown in Table 2. l-Deprenyl inhibited selectively MAO-B in the brain and liver; however, its inhibitory activity for MAO-A and -B in the liver was larger than that in the brain. When mice were administered a single dose of IFO (5 mg/kg, p.o., a dose 3 times higher than the ED\textsubscript{50} for MAO-B activity), the MAO-B inhibitory effect continued for about 8 hr with little influence on the MAO-A activity in the brain and liver (Fig. 4). The oral administration (0.5 – 30 mg/kg) of IFO to mice produced a dose-dependent increase in the striatal concentration of 2-PEA (Fig. 5).

**Protective effect of IFO against the dopaminergic neurotoxicity of MPTP**

The protective effect against MPTP toxicity was tested in mice pretreated with some doses of IFO and l-deprenyl. Table 3 shows that IFO produced protection against an acute lethal toxicity of MPTP (100 mg/kg, s.c., a dose sufficient to kill all mice used in the present experiment). Its effective dose was about the same as the ED\textsubscript{50} value for brain MAO-B activity in Table 2. The dose of IFO required to increase the survival of the mice tended to be smaller than that of l-deprenyl in relation to their ED\textsubscript{50} for MAO-B activity. At 5 mg/kg, IFO as well as l-deprenyl completely protected the mice against MPTP lethal toxicity and protected against the depletion of DA and its metabolites, except for 3-MT, in mice brain striata induced by repeated subcutaneous injection of MPTP, as shown in Table 4. The metabolite ratio (DOPAC+HVA)/DA showed little alteration with either inhibitor in MPTP-treated mice, and 3-MT/DA was recovered to the normal value by either inhibitor in spite of its increase by MPTP.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Brain ED\textsubscript{50} (mg/kg)</th>
<th>Liver ED\textsubscript{50} (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFO</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Deprenyl</td>
<td>&gt;100</td>
<td>&gt;35</td>
</tr>
</tbody>
</table>

ED\textsubscript{50} values were determined from the dose-response curves (0.5 – 100 mg/kg, p.o.) for inhibition of MAO measured ex vivo. Mice were given a single oral dose of either of the compounds and MAO determinations made 1 hr later for the brain and liver homogenate. 5-HT and BZ were used as selective substrates for MAO-A and MAO-B, respectively.
treatment. In normal mice, l-deprenyl diminished the levels of all metabolites to about 70% each of control group as both metabolite/DA ratios diminished significantly, but IFO affected neither of them in this experiment.

**DISCUSSION**

The present study demonstrated that IFO inhibits selectively MAO-B in rodent brain and liver in vitro and in vivo. IFO produced time-independent inhibition, but its binding to the mitochondrial fraction was quite tight with no change after dialysis (or washing) with buffer. If
MAO-B catalyzed the conversion of IFO in the same manner as its conversion of other irreversible inhibitors (17), which have been divided into four chemical types: substituted hydrazine, cyclopropylamine, propargylamine and allylamine derivatives, IFO and its product would covalently bind to the active site of this enzyme (17). However, covalent binding is not supported by the fact that the tight binding behavior of IFO, as shown in the Henderson plot (16), disappears in the presence of sodium cholate, which causes the inhibitor to dissociate from the enzyme. Therefore, it is considered that the hydrophobic property of IFO is important for its MAO-B inhibitory activity. Yagi and Naoi (18) reported that soluble MAO-containing apoenzyme, partially purified and obtained after removal of lipids by a surfactant, was increased by dissociable flavin adenine dinucleotide (FAD), and the dissociation constant of FAD was also influenced by phospholipids. IFO may influence the adherence of free FAD and lipids to the enzyme without formation of a covalent adduct.

The reversibility of IFO was supported by its short-lasting activity: its duration of activity was 6-8 hr in the ex vivo experiment for mouse brain MAO-B inhibition and in our preliminary in vivo experiment for striatal 2-PEA concentration, at a single dose of 5 mg/kg, p.o. However, the characteristics of IFO do not seem to reflect its in vitro tight binding behavior. Considering this disparity, one must determine if IFO may be carried away from the tissue or if it is possibly metabolized in vivo. The reversibility of IFO may be a unique feature as compared to the characteristics of known reversible and irreversible MAO inhibitors (1, 17).

MAO inhibition by IFO was virtually equipotent in the brain and liver after oral administration in mice; on the other hand, l-deprenyl inhibited liver MAO-B much more potently than the brain enzyme in mice. IFO was not much more potent than l-deprenyl with sufficient preincubation in the in vitro experiment, but was virtually equipotent to l-deprenyl in the inhibition of mouse brain MAO-B in vivo. In this point, between l-deprenyl and IFO, the in vitro inhibition data (in Table 1) for the brain and liver MAO fractions disagreed with the ex vivo inhibition data (in Table 2) in mouse brain and liver after oral administration. Accordingly, a possible reason for this is that there may be differences in the manners by which these compounds are translocated into/from the tissues.

The protection by l-deprenyl against MPTP lethality in mice has been reported (19), and IFO tended to prevent the neurotoxic effects of MPTP as shown in Table 3. This result shows that IFO protects against the acute lethality of MPTP in mice and against the marked depletion in striatal DA and its metabolites at a dose similar to that of l-deprenyl in mice. Moreover, even though the metabolite ratio should be increased by the metabolite releasing by MPTP (20), MAO-B inhibition by IFO and l-deprenyl in the metabolism of DA to DOPAC and HVA seemed to be so effective that the recovery from the abnormal (DOPAC + HVA)/DA ratio to the normal one was repressed in MPTP-treated mice. In normal mice, we recognized the ineffectiveness of IFO contrary to the effectiveness of l-deprenyl in metabolite ratio, which might be due to differences between these compounds with respect to their duration of action and selectivity towards both MAO forms that oxidize DA well. The effectiveness of l-deprenyl is thought to be due to the MAO-A inhibition effect since MAO-B is not involved in the majority of DA metabolic processes in DA neurons in rodents (21-23). In spite of its ineffectiveness on the metabolite ratio, IFO showed protective efficacy against MPTP toxicity, suggesting that the time of its effectiveness is thought to sufficiently cover the time required for the disappearance of MPTP from the mouse brain (20, 24) and that MPTP is converted selectively to a toxic metabolite by MAO-B in the brain (25). Accordingly, the effectiveness of IFO is considered

### Table 4. Effect of IFO and l-deprenyl on DA, its metabolites and metabolite/DA ratios in MPTP-treated mice striata

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DA</th>
<th>DOPAC</th>
<th>HVA</th>
<th>3-MT</th>
<th>(DOPAC+HVA)/DA</th>
<th>3-MT/DA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>73.8 ± 3.9</td>
<td>32.1 ± 2.2</td>
<td>14.2 ± 1.0</td>
<td>15.2 ± 0.6</td>
<td>0.69 ± 0.02</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>IFO</td>
<td>69.5 ± 1.2</td>
<td>29.9 ± 0.8</td>
<td>12.9 ± 0.2</td>
<td>13.7 ± 1.1</td>
<td>0.62 ± 0.02</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>Deprenyl</td>
<td>73.8 ± 4.2</td>
<td>22.1 ± 1.1*</td>
<td>9.9 ± 0.4*</td>
<td>10.5 ± 1.4</td>
<td>0.45 ± 0.02**</td>
<td>0.15 ± 0.01*</td>
</tr>
<tr>
<td>MPTP</td>
<td>23.1 ± 0.5***</td>
<td>3.6 ± 0.3***</td>
<td>5.7 ± 0.3***</td>
<td>17.6 ± 3.0</td>
<td>0.40 ± 0.02***</td>
<td>0.75 ± 0.13**</td>
</tr>
<tr>
<td>MPTP + IFO</td>
<td>86.8 ± 3.8***</td>
<td>19.6 ± 1.5*</td>
<td>11.0 ± 0.4***</td>
<td>17.7 ± 2.5</td>
<td>0.36 ± 0.02***</td>
<td>0.22 ± 0.05***</td>
</tr>
<tr>
<td>MPTP + Deprenyl</td>
<td>85.2 ± 3.9***</td>
<td>19.2 ± 1.2*</td>
<td>11.6 ± 0.2***</td>
<td>17.1 ± 1.1</td>
<td>0.37 ± 0.02***</td>
<td>0.20 ± 0.04***</td>
</tr>
</tbody>
</table>

IFO and l-deprenyl, respectively (5 mg/kg), were administered p.o. to mice 1 hr before treatment with MPTP (2 injections of 30 mg/kg/injection, s.c., at 24-hr intervals). Mice were sacrificed 10 hr after the second administration of inhibitor, and concentrations of striatal DA and its metabolites were determined. Results are the mean ± S.E.M. of five mice. Significance: *P < 0.05, **P < 0.01 and ***P < 0.001 vs. control; #P < 0.01 and ##P < 0.001 vs. vehicle group, in MPTP-treated mice.
to be due to its blockage of the conversion of MPTP to the toxic metabolite through the inhibition of MAO-B in the mouse brain.

Most of the early symptoms of PD patients derive considerable benefit from levodopa treatment, but their DA-neuronal output pathways from the striatum, although these are spared (26), are considered to be gradually degenerated by oxidative stress associated with the increased DA turnover by its repeated treatment (6). The available period of PD-therapy will be extended if the onset of levodopa-treatment in patients with early symptoms is deferred by another treatment of stimulating DAergic neurotransmission and if the levodopa-resistance is prevented by a habitual lower dose of levodopa supported by a new adjuvant. It is possible that these stimulation and supporting strategies will be achieved by DA-turnover inhibition. MAO-B inhibition by IFO increased the striatal 2-PEA concentration in mice. In the human brain, striatal DA is oxidized preferentially by MAO-B (4), so that the 2-PEA-increasing efficacy of IFO may affect the DAergic transmission. Accordingly, if we make another attempt to study the effect of IFO in an animal model, we should use the PD model as animal response to levodopa.

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