RS-2232, a Compound with a Reversible and Specific Type-A Monoamine Oxidase Inhibiting Property in Mouse Brain

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Accepted April 24, 1987

Abstract—Effects of RS-2232 on monoamine oxidase (MAO) activities in mouse brain and liver were investigated with 5-hydroxytryptamine (5-HT), β-phenylethylamine (PEA), and in some cases, kynuramine as substrate. IC50s of RS-2232 for 5-HT (100 μM) and PEA (20 μM) deaminations in brain mitochondrial preparations were 0.14 μM and 52 μM, respectively. RS-2232 was found to be a competitive inhibitor of 5-HT deamination in the preparation, and its Kᵢ was 0.054 μM. The inhibitions of MAO in both brain and liver homogenate by RS-2232 in vitro measured with kynuramine (100 μM) were independent of the prolonged preincubation. 5-HT deaminations in the brain homogenates of mice treated with RS-2232 were decreased significantly by 15% and 59% at 10 and 30 mg/kg (p.o.) of the compound, respectively. On the other hand, PEA deaminations were not changed at the same doses. Pressor responses induced by intravenous tyramine (0.1–1.0 mg/kg) in anesthetized rats was little affected by oral administration of RS-2232 (3–30 mg/kg) once daily for two weeks. These results reveal that RS-2232 has a reversible and specific type-A MAO inhibiting property in mouse brain, and they suggest that RS-2232 is relatively safe in tyramine-potentiation.

In the preceding paper (1), we have shown that RS-2232 (4-(4-cyanophenyl)amino-6,7-dihydro-5H-cyclopentapyrimidine hydrochloride), a new candidate for an antidepressant drug, caused not only increases in levels of monoamines such as norepinephrine (NE), dopamine (DA) and 5-hydroxytryptamine (5-HT), but also caused decreases in levels of monoamine metabolites such as 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) in the mouse whole brain, 60 min after oral administrations of the drug (3, 10 and 30 mg/kg). In addition, reserpine (2 mg/kg, s.c.)-induced changes in the levels of monoamines and their related metabolites in the mouse whole brain were antagonized, in a dose-dependent manner, when reserpine was administered to mice along with RS-2232 (3, 10 and 30 mg/kg, p.o.). Furthermore, increase in monoamines produced by 10 mg/kg of RS-2232 was restored to the normal levels within a few hours. Thus, these results suggest that RS-2232 has a reversible monoamine oxidase (MAO) inhibiting property in vivo in mouse brain.

It is generally accepted that MAO exists in at least two different forms: type-A MAO has preference for NE and 5-HT as substrates and is inhibited by a low concentration of the drug clorgyline, while type-B MAO has a preference for benzylamine and β-phenylethylamine (PEA) as substrates and is inhibited by a low concentration of the drug deprenyl. Tyramine, however, is a substrate for both types of enzyme (2–5). These findings raised the possibility that reversible and specific type-A MAO inhibitors could be effective for the treatment of depression without causing a severe hypertensive crisis called the cheese effect (6).

We describe in this paper the effects of RS-2232 on mouse brain MAO and tyramine-
induced pressor response examined as a model of the “cheese effect”, in anesthetized rats.

Materials and Methods

Radiochemical assay of MAO activity: Male ddY mice were sacrificed, and their whole brains were removed and homogenized in cold 0.32 M sucrose and 10 mM sodium phosphate buffer, pH 7.4. The mitochondrial fraction was obtained by the differential centrifugation technique to yield a protein concentration of 1 mg/ml and stored at −20°C until used. The protein content was determined according to the method of Lowry et al. (7), using bovine serum albumin as a standard.

MAO activity was determined by the radiochemical technique essentially as described by Wurtmann and Axelrod (8). Incubation tubes containing 50 mM sodium phosphate buffer (pH 7.4), various amounts of RS-2232 and the mitochondrial preparation were preincubated at 38°C for 20 min in air. Then radiolabelled substrate, 14C-5-HT or 14C-PEA, was added to the tubes, and incubation was continued for another 20 min. After the reaction was stopped with 2 M HCl, the labelled metabolites were extracted into ethyl acetate — toluene (1:1 V/V) and counted for radioactivity.

In ex vivo MAO determination, mice were orally administered RS-2232 (3, 10 and 30 mg/kg), and 1 hr later, they were sacrificed. Preparation of the homogenates and determination of MAO activity were carried out essentially by the methods described above, except for the reaction time of 5 and 3 min for 5-HT and PEA, respectively.

Fluorimetric assay of MAO activity: Mice were sacrificed, and their whole brain and liver were removed and homogenized in cold 62.5 mM sodium phosphate buffer (pH 7.4) to yield a tissue concentration of 10 mg wet weight/ml and stored at −20°C until used.

MAO activity in both homogenates was determined according to the method of Kraml (9), using kynuramine dihydrobromide as substrate. The assay mixture contained 62.5 mM sodium phosphate buffer (pH 7.4), either of the homogenates and various amounts of RS-2232 was preincubated for 0, 10, 20, 40 or 80 min at 37°C in air. Then the reaction was started by the addition of kynuramine dihydrobromide, and incubation was continued for another 30 min. After the reaction was stopped with 10% (V/V) trichloroacetic acid, the fluorescence of 4-hydroxyquinoline formed was determined at 370 nm with an excitation wavelength of 315 nm.

Under all experimental conditions, product formation was linear with incubation time and amount of enzyme preparation used.

Effects of repeated administration of RS-2232 on the pressor response to tyramine in anesthetized rats: Male Wistar rats weighing about 300 g were used. RS-2232 was suspended in saline containing 0.3% (W/V) carboxymethylcellulose (CMC) and administered orally once daily for 2 weeks to rats in a volume 0.1 ml per 100 g body weight. The mean arterial blood pressure was directly monitored by inserting a cannula into the common carotid artery of the anesthetized rats. Tyramine hydrochloride dissolved in saline was injected into the femoral vein in a volume of 0.1 ml per 100 g body weight, 2 hr after final administration of RS-2232.

Compounds: Clorgyline hydrochloride was a gift from May & Baker, Ltd. (Dagenham, U.K.). 1-Deprenyl was a gift from Prof. J. Knoll, Semmelweis University of Medicine (Budapest, Hungary). Isocarboxazid was purchased from Takeda Pharm. Ind., Ltd. (Osaka, Japan). 14C-5-HT (5-hydroxy (side chain 2-14C) tryptamine creatinine sulfate) and 14C-PEA (2-phenyl (1-14C) ethylamine hydrochloride) were obtained from the Radiochemical Centre (Amersham, U.K.). Kynuramine dihydrobromide was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A). Other chemicals used were of analytical grade.

Results

Concentration-dependent inhibition of MAO activity by RS-2232 in mouse brain mitochondrial fraction in vitro: Clorgyline, a specific type-A MAO inhibitor, inhibited MAO activity with 100 μM of 14C-5-HT as substrate in mouse brain mitochondrial fraction at considerably lower concentrations than those required to inhibit the activity with
20 \mu M of \textsuperscript{14}C-PEA as substrate, and vice versa for deprenyl, a specific type-B MAO inhibitor (Fig. 1). All the inhibitions by clorgyline and deprenyl were concentration-dependent, and single sigmoidal curves were obtained. Thus, it is certain that deamination of 5-HT and PEA observed under these experimental conditions represents MAO activity of type-A and type-B MAO, respectively, since 5-HT and PEA are also metabolized by type-B and type-A MAO, respectively, if appropriate concentrations of the substrates are not used in the assay (10).

Effects of increasing concentrations of RS-2232 are shown in Fig. 2. RS-2232 inhibited 5-HT deamination by 50% at about 0.1 \mu M and complete inhibition was observed at 10 \mu M of the compound, whereas PEA was inhibited by 30% and 60% at concentrations of 10 and 100 \mu M, respectively. The IC50s (concentrations of the inhibitors to inhibit MAO activity by 50%) of clorgyline, deprenyl and RS-2232 were calculated from the inhibition-curves in Figs. 1 and 2 and are presented in Table 1. The IC50s of clorgyline for 5-HT and PEA were 0.0012 \mu M and 0.55 \mu M, respectively. The ratio of IC50 for PEA to that for 5-HT was 458, showing its preferential inhibition toward type-A MAO. On the other hand, the IC50 of deprenyl for 5-HT and that for PEA were 1.8 \mu M and 0.005 \mu M, respectively, the ratio 0.0028 showing its preferential inhibition toward type-B MAO.

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{fig1}
\caption{Concentration-dependent inhibitions of MAO activity by clorgyline and deprenyl in mouse brain mitochondrial fraction in vitro. Reaction mixture containing 30 \mu g of mitochondrial protein and varying amounts of the inhibitor in a total volume of 270 \mu l of sodium phosphate buffer (pH 7.4) was preincubated at 38\textdegree C for 20 min. Then, 30 \mu l of either \textsuperscript{14}C-5-HT or \textsuperscript{14}C-PEA was added to the mixture, and reaction was performed for 20 min. Each point is the mean value for triplicate determinations. Substrates used were: \(\bullet\) (100 \mu M of 5-HT) and \(\circ\) (20 \mu M of PEA).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{fig2}
\caption{Effects of RS-2232 on MAO activity in mouse brain mitochondrial fraction in vitro. MAO activity with 100 \mu M of \textsuperscript{14}C-5-HT (\(\bigcirc\)) and 20 \mu M of \textsuperscript{14}C-PEA (\(\bullet\)) were examined as for Fig. 1.}
\end{figure}

\begin{table}
\centering
\caption{IC50 values of clorgyline, deprenyl and RS-2232 toward 5-HT and PEA demainations in the mouse brain mitochondrial fraction in vitro}
\begin{tabular}{lccc}
\hline
Compounds & \multicolumn{2}{c}{IC50 (\mu M)} & \\
 & 100 \mu M of 5-HT (A) & 20 \mu M of PEA (B) & IC50 (B)/IC50 (A) \\
\hline
Clorgyline & 0.0012 & 0.55 & 458 \\
Deprenyl & 1.8 & 0.005 & 0.0028 \\
RS-2232 & 0.14 & 52 & 371 \\
\hline
\end{tabular}
\footnote{IC50 values were calculated from the concentration-inhibition curves shown in Figs. 1 and 2.}
\end{table}
Table 2. Effects of RS-2232 administration on ex vivo MAO activities in mouse brain homogenates

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg, p.o.)</th>
<th>Ex vivo MAO activities (% of control)</th>
<th>100 μM of 5-HT</th>
<th>20 μM of PEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC</td>
<td>0</td>
<td>100.3±2.1</td>
<td>100.0±2.9</td>
<td></td>
</tr>
<tr>
<td>RS-2232</td>
<td>3</td>
<td>95.4±1.7</td>
<td>96.5±3.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>84.5±4.3*</td>
<td>96.0±3.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>41.3±3.6****</td>
<td>93.5±4.4</td>
<td></td>
</tr>
</tbody>
</table>

Mice were killed by decapitation 1 hr after oral administration of RS-2232. The brain homogenates were incubated at 38°C with 100 μM of 5-HT and 20 μM of PEA as substrates for 5 and 3 min, respectively. All the values expressed as percents of the control (CMC alone-treated) are means±S.E. of four animals. *P<0.05, ****P<0.001, as compared to the control.

As for RS-2232, the IC50s for 5-HT and PEA were 0.14 μM and 52 μM, respectively, the ratio 371 suggesting that its specificity for type-A MAO is comparable to that of clorgyline.

Effect of preincubation time on the extent of MAO inhibition by RS-2232 in the brain and in the liver homogenate in vitro: It was examined whether MAO inhibition by RS-2232 is dependent on prolonged incubation time; brain or liver homogenate was preincubated with RS-2232 or isocarboxazid for 0, 10, 20, 40 or 80 min. Then 100 μM of kynuramine was added to the mixture, and the reaction was performed for 30 min. The results presented in Fig. 3 clearly show that 1 and 5 μM of RS-2232 inhibited MAO by 45% and 55%, respectively, in brain homogenate without preincubation, and they indicate that the extents of MAO inhibition by RS-2232 were independent of prolonged preincubation time up to 80 min. In contrast, an irreversible MAO inhibitor, isocarboxazid (0.1 and 0.5 μM), produced an increasing extent of MAO inhibition in brain homogenate as the preincubation was prolonged. Similar results were also observed in liver homogenate with RS-2232 (5 and 10 μM) and isocarboxazid (0.1 and 0.5 μM). It should be noted that 5 μM of RS-2232 inhibited MAO more strongly in the brain homogenate than in the liver homogenate.

Kinetics of the type-A MAO inhibitory effect of RS-2232 in brain mitochondrial fraction in vitro: Since RS-2232 was found to be a specific inhibitor of type-A MAO as shown in Fig. 2 and its MAO inhibition was suggested to be reversible in the brain as illustrated in Fig. 3, the reversible property toward type-A MAO was directly examined by the use of Lineweaver-Burk double-reciprocal plots of 5-HT deamination and presented in Fig. 4. The inhibitions of 5-HT
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Fig. 4. Lineweaver-Burk double-reciprocal plots for inhibition of 5-HT deamination by RS-2232 in mouse brain mitochondrial fraction in vitro. Varying amounts of 14C-5-HT and 30 μg of brain mitochondrial protein were incubated in the absence of (□) or presence of 0.03 (●), 0.06 (∆) and 0.09 (▲) μM of RS-2232 in a total volume of 0.3 ml of sodium phosphate buffer (pH 7.4) at 38°C for 20 min. Ordinate, 1/activity, expressed as arbitrary units; abscissa, 1 /mM 5-HT. Each point is the mean value for triplicate determinations.

deamination by various concentrations of RS-2232 were competitive toward 5-HT in the brain mitochondrial fraction. The secondary plot of the slopes of the graph against RS-2232 concentrations were linear and produced a Ks value of 0.0054 μM.

Dose-dependent MAO inhibitory effect of RS-2232 in brain homogenate ex vivo: To investigate whether RS-2232 has specificity toward type-A MAO in vivo as well as in vitro, ex vivo MAO activity was examined in the brain homogenates from RS-2232-administered mice, with 100 μM of 14C-5-HT and 20 μM of 14C-PEA as substrates. The results presented in Table 2 show that in the brain homogenates from mice given 10 and 30 mg/kg of RS-2232, 5-HT deaminations were significantly reduced by 15% and 59%, respectively. On the other hand, PEA deaminations were not changed. Thus, these results are indicative that as in vitro, RS-2232 has a specific inhibitory effect on type-A MAO in vivo.

Effects of repeated administration of RS-2232 on the pressor response to tyramine in anesthetized rats: In the control rats (CMC alone-treated), the mean arterial blood pressure was 95.7±2.0 mmHg under anesthetized condition, while they were 98.8±9.4, 99.7±8.7 and 99.3±5.6 mmHg in the rats treated with 3, 10 and 30 mg/kg of RS-2232, respectively. There is no significant differences between the control rats and the RS-2232-treated ones (data not shown).

As shown in Fig. 5, injections of 0.1, 0.3 and 1.0 mg/kg of tyramine hydrochloride dose-dependently increased the mean arterial blood pressure by 13.2±1.1, 23.3±2.5 and 61.5±5.6 mmHg, respectively, in the control rats. These pressor responses to tyramine were not potentiated in the rats treated with 3, 10 and 30 mg/kg of RS-2232 once daily for 2 weeks, although minor potentiation by 30 mg/kg of the compound was observed only with the 1.0 mg/kg of tyramine injection.

Discussion

The results from this study clearly revealed that RS-2232 had a specific type-A MAO inhibiting property both in vitro and in vivo in mouse brain. Furthermore, its specificity toward type-A MAO was found to be comparable to that of clorgyline. These
results are indicative of the clinical efficacy of RS-2232 in the treatment of depression, since clorgyline, a specific type-A MAO inhibitor, was reported to be an effective antidepressant (11), whereas deprenyl, a specific type-B MAO inhibitor was not (12).

This study also demonstrated that inhibitions of type-A MAO by varying concentrations of RS-2232 were competitive toward 5-HT in the brain mitochondrial fraction in vitro and that with kynuramine as substrate, inhibitions of MAO by RS-2232 were not influenced at all by prolonged preincubation time both in the brain and in the liver homogenates in vitro. These results, together with the preceding findings that the effect of RS-2232 (10 mg/kg, p.o.) on endogenous monoamine content disappeared within a few hours (1), indicate that RS-2232 possesses a reversible MAO inhibiting property in nature, in vitro and in vivo, and in the brain and liver.

It was reported that at least 80% reduction of MAO activity in the brain was necessary to cause changes in the levels of endogenous monoamine (13). Ex vivo determination of MAO activity in Table 2 shows that type-A MAO activity was reduced only 59% in the brain homogenate from mice given 30 mg/kg or higher dose of RS-2232 than that required to increase the monoamine content in the brain (1). The reason for this discrepancy seems to be that the substantial in vivo concentration of RS-2232 in the brain is attenuated by the process of both homogenization of brain tissue and assay procedure for determining MAO activity in the homogenate because of the reversible interaction between type-A MAO and RS-2232; thus, the apparent inhibition by RS-2232 in the homogenate is underestimated.

Classical MAO inhibitors were shown to be effective antidepressants, but their clinical usefulness was decreased mainly because of the hypertensive crisis induced by tyramine contained in food ingested by patients treated with the inhibitors (6). However, the discovery of the two types of MAO by Johnston in 1968 suggested that the hypertensive crisis of tyramine could be overcome by the use of reversible and specific type-A MAO inhibitors instead of classical MAO inhibitors which act on both types of the enzyme in an irreversible manner (6). Transmitter NE and 5-HT are believed to be related to the pathophysiology of depression (14–16) and are predominantly metabolized by type-A MAO, while tyramine is a good substrate for both types of the enzyme; thus injected tyramine can still be metabolized by type-B MAO even when there is inhibition of type-A MAO by its specific inhibitors. Moreover, the inhibition can easily be replaced by a high concentration of tyramine due to the reversible interaction between the inhibitor and type-A MAO.

Figure 5 shows that repeated administration of RS-2232 for 2 weeks caused rather weak tyramine-potentiation in anesthetized rats. In general, the clinical therapeutic action of antidepressants requires treatment for several weeks; therefore, an experimental schedule in which MAO inhibitors are administered to animals for at least several weeks is essential for determining if a particular inhibitor induces the “cheese effect” in patients. Since the ED50 values of RS-2232 for the antagonism against reserpine-induced ptosis in rats was 3.0 mg/kg, p.o. (I. Nakayama et al., unpublished observation), RS-2232 is suggested to be relatively safe in the chronic treatment of depressed patients.

Acknowledgment: We wish to gratefully acknowledge the technical assistance of Mr. T. Karube. Gratitude should also be expressed to Dr. T. Tonohiro for his great help in part of the present experiments and for critical discussions.

References
4 Knoll, J. and Magyar, K.: Some puzzling pharmacological effects of monoamine oxidase
Specific Inhibition of MAO-A by RS-2232


12 Mendis, N., Pare, C.M.B., Sandler, M., Glover, V. and Stern, G.M.: Is the failure of (−) deprenyl, a selective monoamine oxidase B inhibitor, to alleviate depression related to freedom from the cheese effect? Psychopharmacology (Berlin) 73, 87–90 (1981)


