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IN VITRO EFFECTS ON MONOAMINE UPTAKE AND RELEASE BY THE REVERSIBLE MONOAMINE OXIDASE-B INHIBITORS LAZABEMIDE AND *N*-(2-AMINOETHYL)-*p*-CHLOROBENZAMIDE: A COMPARISON WITH L-DEPRENYL

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Abstract—To investigate whether the reversible monoamine oxidase-B (MAO-B) inhibitors lazabemide and Ro 16-6491 have any additional effect on monoamine uptake and release, *in vitro* experiments were performed on rat forebrain synaptosomes and blood platelets. The effects of the two drugs were compared with those of L-deprenyl, the well-known irreversible MAO-B inhibitor which is reported to affect amine uptake. Both lazabemide and Ro 16-6491 behaved as weak inhibitors of [^{3}H]monoamine uptake by synaptosomes, with a similar rank order of potency for amine uptake inhibition (noradrenaline (NA)≥ 5-hydroxytryptamine (5 HT) > dopamine (DA)). The IC_{50} values for lazabemide and Ro 16-6491, respectively, were: 86 μM and 90 μM for NA uptake; 123 μM and 90 μM for 5HT uptake; >500 μM and > 1000 μM for DA uptake. L-Deprenyl (rank order of inhibitory potency: NA > DA >5 HT) was four to 10 times more potent than either compound in inhibiting [^{3}H]catecholamine uptake (IC_{50} = NA 23 μM, DA 109 μM), and two to three times less potent in inhibiting 5 HT uptake (IC_{50} 233 μM). Lazabemide and Ro 16-6491 also differed from L-deprenyl in their ability to induce release of endogenous monoamines from synaptosomes. Thus, Ro 16-6491 (500 μM) induced a greater 5 HT release than did L-deprenyl, but was less effective than L-deprenyl in releasing DA. On the contrary, lazabemide was almost completely inactive on either 5 HT and DA release. The differential effect of the three MAO-B inhibitors on synaptosome 5 HT uptake and release was confirmed by [^{14}C]5HT uptake and liberation experiments with isolated rat platelets. The data indicate that the reversible MAO-B inhibitors lazabemide and Ro 16-6491 at relatively high concentrations possess amine uptake-inhibiting properties. With regard to the effects examined, lazabemide markedly differs from L-deprenyl since it does not interfere with DA uptake nor induce amine release from synaptosomes.

Key words: monoamine oxidase-B inhibitors; lazabemide; L-deprenyl, monoamine uptake; release; synaptosomes

MAO§ (flavin-containing, EC 1.4.3.4.) inhibitors of the new generation are characterized by their relative selectivity for type A and B MAO isoenzymes and in some instances by the reversibility of their action [1]. The 2-aminoethyl carboxamide derivatives lazabemide and Ro 16-6491 are short-acting reversible, mechanism-based MAO-B inhibitors [1, 2]. A series of studies both *in vitro* and *ex vivo* have demonstrated that lazabemide is the most potent and selective MAO-B inhibitor known to date [3–5].

In this work we have attempted to clarify if the two drugs, besides their MAO-inhibiting property, share any additional effect on monoamine systems in brain tissue with other MAO inhibitors [6, 7]. Rat brain crude synaptosome preparations, with added pargyline to block MAO activity, were used to study *in vitro* interference by lazabemide and Ro 16-6491 with monoamine uptake and release. The effect of the two drugs on 5HT uptake and release was also assessed on isolated blood platelets, a suitable model of amine re-uptake in brain 5HT neurons [8], which in the rat have the advantage of being practically devoid of MAO activity [9]. In the same experiments the effects of L-deprenyl, an acetylenic irreversible MAO-B inhibitor known to interfere with monoamine uptake [6], were also determined for comparison.

MATERIALS AND METHODS

Materials. Analytical reagents were from Merck

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§ Abbreviations: MAO, monoamine oxidase; 5HT, 5-hydroxytryptamine; NA, L-noradrenaline; DA, dopamine; DHBA, 3,4-dihydroxybenzylamine; tyramine, 4-hydroxyphenylethylamine; pargyline, *N*-methyl-*N*-2-propynylbenzylamine; Ro 16-6491, *N*-(2-aminoethyl)-*p*-chlorobenzamide hydrochloride; L-deprenyl, *R*(−)-*N*-dimethyl-*N*-2-propynylbenzene ethamine hydrochloride; lazabemide, *N*-(2-amino ethyl)-5-chloro-2-pyridine carboxamide hydrochloride; diclofensine, 7-methoxy1,2,3,4-tetrahydro - 2 - methyl - 4 - dichlorophenyl - isoquinoline; HPLC, high-performance liquid chromatography; PRP, platelet-rich plasma; MPTP, 1 - methyl - 4 - phenyl - 1,2,3,6-tetrahydropyridine.

(Darmstadt, Germany), except for sodium octyl sulphate (Fluka, Buchs, Switzerland). 5HT creatinine sulphate, NA, DA hydrochloride, DHBA hydrochloride, tyramine hydrochloride and pargyline hydrochloride were all from Sigma (St. Louis, MO, U.S.A.). D-Amphetamine sulphate was from Recordati (Milan, Italy). Ro 16-6491 and L-deprenyl were from Research Biochemicals Inc. (Natick, MA, U.S.A.). Lazabemide and diclofensine were a gift from Hoffmann-La Roche (Basel, Switzerland). [^{3}H]5HT creatinine sulphate (28.2 Ci/mmol), [^{14}C]-5HT creatinine sulphate (57 mCi/mmol), [^{3}H]DA (45 Ci/mmol) and [^{3}H]NA (30 Ci/mmol) were obtained from The Radiochemical Centre (Amersham, U.K.). Aqueous solutions were in reagent-grade water obtained by a Milli-Q System (Millipore, Bedford, MA, U.S.A.).

Synaptosome experiments. Male Sprague-Dawley rats (Charles Ribver, Calco, Italy) weighing 200 g were used. Animals were killed by cervical dislocation and the forebrains removed and homogenized in 10 volumes of ice-cold 0.32 M sucrose. The homogenates were centrifuged at 1000 *g* for 10 min at 4°. Supernatants were then centrifuged at 10,000 *g* for 5 min and the pellets washed once with ice-cold Krebs buffer and resuspended in a volume of Krebs equal to that of initial homogenate. Two hundred microlitres of this suspension were placed in Eppendorf tubes, to which were added 100 μL of a solution of pargyline (10 μM) and 700 μL of Krebs.

Radioactive amine uptake. Monoamine uptake was determined as previously described [10, 11] using [^{3}H]5HT, [^{3}H]DA and [^{3}H]NA as substrates (3 nM). Briefly, the samples were preincubated for 5 min at 37° in the presence or absence (controls) of the inhibitors under study. Then the radioactive amine was added and the incubation continued for an additional 4 min. After centrifugation and two washings at 4°, pellets were solubilized with Soluene-Instagel (Canberra Packard, Milan, Italy) before measurement of radioactivity by liquid scintillation spectrometry. The radioactivity in samples incubated at 4°, under otherwise identical conditions, was used as blank value. Proteins were determined by a spectrophotometric method [12].

Endogenous amine release. Since there is a high rate of amine leakage and re-uptake in synaptosomes kept at 37°, the releasing effect of drugs was studied in the presence of the amine uptake inhibitor diclofensine [13], so as to minimize the contribution of drug-induced amine re-uptake inhibition to the observed effects. In these synaptosomes used as control, diclofensine (1 μM) reduced 5HT, DA and NA content by a mean of 27, 23 and 10%, respectively. After preincubation at 37° for 5 min in the presence of diclofensine, the samples were incubated for an additional 15 min in the presence or absence (controls) of various concentrations of the drugs or D-amphetamine. Samples were then centrifuged at 4° and the pellets homogenized with 200 μL of 0.3 N perchloric acid containing DHBA as internal standard. After centrifugation, 20 μL aliquots of supernatant were assayed for NA, DA and 5HT in a single run by reversed-phase HPLC with coulometric detection (Coulochem 5100 A; ESA, Milford, MA, U.S.A.). The chromatographic conditions were as previously described [14], with slight modifications. The retention times for NA, DA and 5HT were 4.6, 10 and 27 min, respectively.

Blood platelets. The blood was taken from abdominal aorta of rats under pentobarbitone anaesthesia (45 mg/kg i.p.) and mixed with 5% disodium EDTA (1:10, v/v). PRP was obtained by centrifugation at 150 *g* for 15 min at room temperature. Since rat platelets, unlike synaptosomes, are devoid of MAO activity and efficiently retain stored 5HT *in vitro*, interference with [^{14}C]-5HT uptake and liberation processes by the MAO-B inhibitors tested was studied without the addition of further drug, as previously described [15]. For uptake experiments, 250 μL aliquots of PRP were preincubated at 37° for 5 min in the presence or absence (controls) of various concentrations of the drugs. Then [^{14}C]5HT (1 μM) was added and the incubation continued for an additional 5 min. At the end of incubation platelets were sedimented by centrifugation (1200 *g* for 10 min at 4°) and washed once with ice-cold modified Krebs buffer (devoid of $CaCl_2$ and containing disodium EDTA). The platelet pellets were then lysed in 1% sodium dodecyl sulphate and an aliquot of lysate used for measurement of radioactivity and proteins.

For liberation experiments, PRP was preincubated at 37° for 10 min with [^{14}C]5HT (1 μM), then centrifuged (1200 *g* for 10 min at 4°). The pellet was washed once with ice-cold modified Krebs buffer and resuspended in a volume of buffer equal to that of initial PRP. Aliquots of 250 μL of platelet suspension were reincubated at 37° for 60 min in the presence or absence (controls) of various concentrations of the drugs or tyramine. After sedimentation and washing, platelet pellets were lysed and radioactivity and protein determined as described above.

Values are expressed as means ± SEM. The differences between two means were evaluated by Student's *t*-test. The values of IC_{50} were calculated by two-parameter logistic non-linear regression.

RESULTS

Synaptosomes

Radioactive amine uptake. The effects of lazabemide and Ro 16-6491 on [^{3}H]5HT uptake by synaptosomes are shown in Fig. 1. Both MAO-B inhibitors blocked [^{3}H]5HT uptake in a dose-dependent fashion, Ro 16-6491 (IC_{50} 91 ± 17 μM) being slightly more effective than lazabemide (IC_{50} 123 ± 17 μM).

Figure 2 shows the effects of lazabemide and Ro 16-6491 on [^{3}H]DA uptake. Both drugs, particularly lazabemide, proved to be only weak inhibitors of DA uptake by synaptosomes ($IC_{50} > 500$ μM and >1000 μM for Ro 16-6491 and lazabemide, respectively).

The effects of lazabemide and Ro 16-6491 on [^{3}H]NA uptake are shown in Fig. 3. The dose–response curves for the two compounds were almost identical, indicating that the drugs were equally effective in inhibiting NA uptake (IC_{50} 86 ± 16 μM and 90 ± 12 μM for lazabemide and Ro 16-6491, respectively).

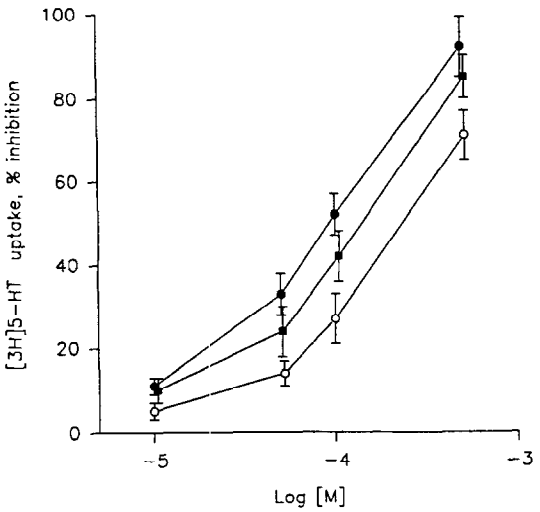

Fig. 1. Effect of different MAO-B inhibitors (■, lazabemide; ●, Ro 16-6491; ○, L-deprenyl) on the *in vitro* uptake of [^{3}H]5HT (3 nM)) by rat forebrain synaptosomes. Means ± SEM of three experiments. Synaptosomes were preincubated for 5 min at 37° in Krebs containing pargyline (10 μM) in the presence or absence (controls) of the drugs. Thereafter the tritiated amine was added and the incubation continued for an additional 10 min. Data are expressed as the percent inhibition of uptake with respect to controls. Absolute values of [^{3}H]5HT uptake by controls: 0.23 ± 0.02 pmol/mg protein.

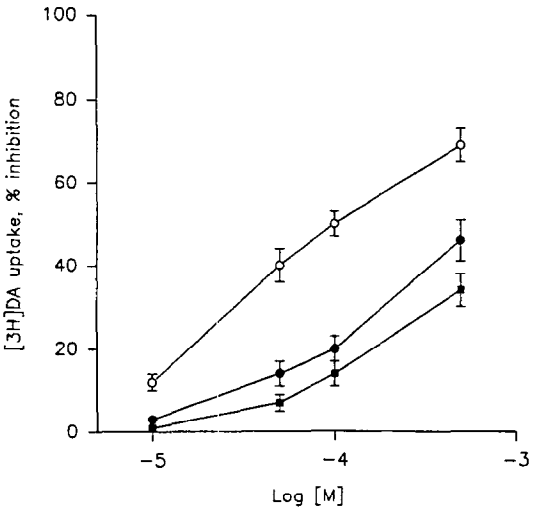

Fig. 2. Effect of different MAO-B inhibitors (■, lazabemide; ●, Ro 16-6491; ○, L-deprenyl) on the *in vitro* uptake of [^{3}H]DA (3 nM) by rat forebrain synaptosomes. Means ± SEM of three experiments. For experimental details see legend to Fig. 1. Absolute values of [^{3}H]DA uptake by controls: 0.32 ± 0.02 pmol/mg protein.

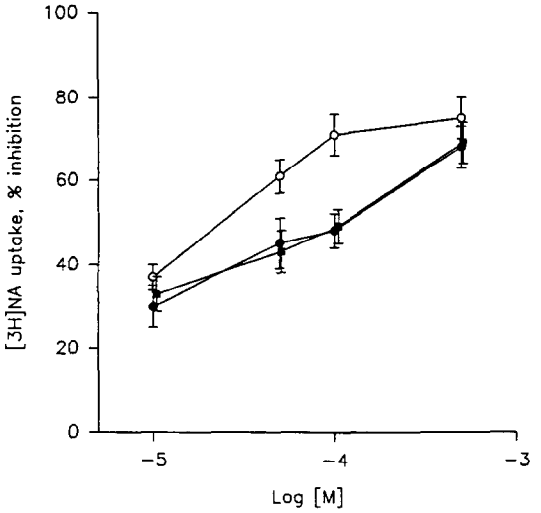

Fig. 3. Effect of different MAO-B inhibitors (■, lazabemide; ●, Ro 16-6491; ○, L-deprenyl) on the *in vitro* uptake of [^{3}H]NA (3 nM) by rat forebrain synaptosomes. Means ± SEM of three experiments. For experimental details see legend to Fig. 1. Absolute values of [^{3}H]NA uptake by controls: 0.16 ± 0.01 pmol/mg protein.

From the dose–response curves of L-deprenyl inhibition of [^{3}H]monoamine uptake (Figs 1–3), it is evident that this drug was more potent than Ro 16-6491 and lazabemide in inhibiting catecholamine uptake by synaptosomes (IC_{50} 109 ± 16 μM and 23 ± 8 μM for DA and NA uptake, respectively), whereas it was less effective than either compound in inhibiting 5HT uptake (IC_{50} 233 ± 56 μM).

Endogenous amine release. The results reported in Table 1 show that Ro 16-6491 was effective in decreasing both 5HT and DA content of synaptosomes, these effects being more marked at 500 μM than at 100 μM concentration. Under the same experimental conditions, lazabemide was almost completely inactive on 5HT and DA release.

L-Deprenyl was more effective than Ro 16-6491 in lowering DA, but less effective in lowering 5HT concentration. Indeed, a significant DA decrease was already present at 100 μM L-deprenyl, whereas a 5HT decrease was appreciable only at an L-deprenyl concentration of 500 μM.

In this synaptosome preparation only D-amphetamine, taken as a classical amine releaser, was able to reduce endogenous NA content, while all three MAO-B inhibitors tested left the concentration of this catecholamine unchanged (Table 1), even at the highest concentration assayed (1 mM, data not shown).

[^{14}C]*5HT uptake and liberation by platelets*

The results of experiments with platelets are shown in Table 2. Ro 16-6491 and lazabemide (IC_{50} < 500 μM for both) were more active than L-deprenyl (IC_{50} < 500 μM) in inhibiting [^{14}C]5HT uptake by isolated rat platelets.

At the highest concentration tested (500 μM),

Table 1. *In vitro* effects of different MAO-B inhibitors and D-amphetamine on the concentrations of endogenous 5 HT, DA and NA in rat forebrain synaptosomes

Drug	Concentration (μM)	Amine (% of controls) 5HT	DA	NA
Lazabemide	100	92 ± 4	96 ± 1	97 ± 4
	500	88 ± 5	94 ± 2	97 ± 4
Ro 16-6491	100	84 ± 4	87 ± 5	95 ± 3
	500	60 ± 4*	62 ± 4*	100 ± 2
L-Deprenyl	100	98 ± 5	66 ± 3*	104 ± 3
	500	81 ± 3*	46 ± 2*	102 ± 6
D-Amphetamine	100	46 ± 4*	41 ± 3*	68 ± 5*
	500	37 ± 3*	35 ± 5*	64 ± 4*

Values are means ± SEM of three experiments. Synaptosomes were preincubated for 5 min at 37° with pargyline (10 μM) and diclofensine (1 μM) before incubation for 15 min at 37° in the presence or absence (controls) of the drugs. Amine content is expressed as the percent of synaptosome amine with respect to controls. Control absolute values (pmol/mg protein): 5HT 7.7 ± 0.5, DA 16.1 ± 1.1, NA 7.9 ± 0.4. * $P < 0.05$ versus controls.

Table 2. Effects of different MAO-B inhibitors and tyramine on the uptake and liberation of [^{14}C]5HT by isolated rat platelets

Drug	Concentration (μM)	Platelet [^{14}C]5 HT (% of controls) Uptake	Liberation
Lazabemide	100	67 ± 4*	101 ± 2
	500	37 ± 6*	95 ± 3
Ro 16-6491	100	59 ± 6*	99 ± 0
	500	20 ± 4*	78 ± 4*
L-Deprenyl	100	88 ± 5	99 ± 2
	500	54 ± 7*	92 ± 3
Tyramine	100	ND	75 ± 2*
	500	ND	41 ± 5*

ND = not determined. Means ± SEM of three experiments. * $P < 0.05$ versus controls. For uptake experiments platelets were preincubated in plasma for 5 min at 37° in the presence or absence (controls) of the drugs. Thereafter [^{14}C]5HT (1 μM) was added and the incubation continued for 5 min. Absolute values of [^{14}C]5HT uptake by controls (= 100%): 0.53 ± 0.01 nmol/mg protein. For liberation experiments platelets were incubated in plasma for 10 min at 37° with [^{14}C]5HT (1 μM), then washed and reincubated in modified Krebs buffer for 60 min in the presence or absence (controls) of the drugs. Values are expressed as percent of controls (control absolute values: [^{14}C]5HT 0.47 ± 0.03 nmol/mg protein).

Ro 16-6491 was also able to induce a liberation of [^{14}C]5HT from platelets preloaded with the radioactive amine, while lazabemide and L-deprenyl were not effective in this respect. Under the same experimental conditions, tyramine, taken as a typical amine releaser, induced a clear [^{14}C]5HT decrease at a concentration as low as 100 μM (Table 2).

DISCUSSION

Lazabemide and its analogue Ro 16-6491 are reversible MAO inhibitors with remarkably high potency and selectivity for MAO-B [1–5]. Besides being useful tools in basic research, e.g. in localizing the isoenzyme by specific binding in different tissues and brain areas [16–18], they might serve as adjuvants in the therapy of Parkinson's disease and other degenerative brain disorders [19, 20]. Lazabemide in particular does not potentiate the pressor effect of tyramine [21] and is able almost completely to prevent Parkinson-like MPTP-induced DA-ergic neurotoxicity [4, 21].

This *in vitro* work shows that the two structural analogues, besides their primary action on MAO-B, have additional effects on monoamine uptake at high concentrations. Compared with L-deprenyl, the irreversible MAO-B inhibitor currently used in the therapy of Parkinson's disease [22, 23], Ro 16-6491 and lazabemide are approximately four to 10 times less potent in inhibiting catecholamine (NA and DA) uptake by rat brain synaptosomes and twice as potent in inhibiting 5HT uptake by both synaptosomes and blood platelets.

With regard to their effects on amine release, each MAO-B inhibitor tested behaves differently. Lazabemide is essentially devoid of amine-releasing properties, while L-deprenyl and Ro 16-6491, which share the ability to induce DA and 5HT release at high concentrations, exhibit different potencies. Indeed, compared to Ro 16-6491, L-deprenyl is more effective in releasing DA from synaptosomes, but less effective in releasing 5HT from both synaptosomes and blood platelets. The above effects were independent of MAO inhibition, since in synaptosome experiments the enzyme was previously blocked by pargyline, while in rat platelets no MAO activity is detectable [9, and unpublished data].

The observed interference with *in vitro* amine uptake or release takes place at drug concentrations

two to three orders of magnitude higher than those sufficient to determine MAO-B inhibition [1, 2]. At high doses, MAO-B inhibitor drugs are known to lose their selectivity and inhibit the MAO-A isoenzyme. The implications of amine uptake inhibition at non-selective doses of L-deprenyl have been studied and discussed in several papers. Indeed, inhibition of tyramine uptake by L-deprenyl has been advocated as a mechanism involved, at the level of sympathetic nerve terminals, in preventing enhanced NA release and consequent pressor effects of indirectly acting sympathomimetic amines (tyramine hypertensive response) due to MAO-A inhibition [24–26]. DA uptake inhibition [6, 27, 28] and DA release by L-deprenyl [29, 30] might also play a central role, i.e. in the apparent increase in cerebral dopamine turnover induced by the drug [30, 31]. DA release by L-deprenyl from rat striatal tissue has not only been demonstrated *in vitro* with a relatively high concentration of the drug (50 μM) [30], but is also strongly suggested by studies with an *in vivo* experimental model [32, 33]. Concerning L-deprenyl, it should also be noted that this drug has been shown to be metabolized, after *in vivo* treatment, to L-metamphetamine and L-amphetamine [34–36], which may act as amine uptake inhibitors and releasers [37, 38].

The carboxamide derivatives lazabemide and Ro 16-6491 have already been known to differ from L-deprenyl in that they inhibit MAO-B reversibly, display higher potency and selectivity for this isoenzyme and, as far as is known, are not transformed into active metabolites *in vivo* [1, 4, 21]. This work shows that lazabemide in particular differs from L-deprenyl since it does not interfere with DA uptake nor induce amine release. This is consistent with the notion that in the rat lazabemide does not interfere with either brain DA level or with striatal DA release [5] and animal gross behaviour [21].

Concerning 5HT, it is unlikely that the *in vitro* uptake inhibition seen at high concentrations of lazabemide may have any clinical implication, considering the low doses of the drug envisaged for therapeutic use as MAO-B inhibitor [19, 20]. It also seems worth mentioning that 5HT uptake inhibition by lazabemide is non-competitive and the uptake of [^{3}H]lazabemide itself by rat forebrain synaptosomes it not reduced by competitive inhibitors of 5HT uptake, such as cyanopramine and paroxetine (unpublished data). This rules out the possibility that lazabemide may penetrate nerve terminals by using the 5HT transporter, which might have allowed the selective access of low doses of the drug into 5HT neurons, with consequent MAO-B inhibition in discrete serotoninergic areas.

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