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Pirlindole and dehydropirlindole protect rat cultured neuronal cells against oxidative stress-induced cell death through a mechanism unrelated to MAO-A inhibition

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1 It has been shown that the MAO (monoamine oxidase)-B inhibitor deprenyl (DPR, selegiline) protects some cell types against oxidative stress. By decreasing H_2O_2 production, MAO-A inhibitors could also reduce oxidative stress.

2 This study reports the effect of the MAO-A inhibitors, pirlindole (PIR), dehydropirlindole (DHP), brofaromine (BRO) and moclobemide (MCL) on primary-cultured brain cells exposed to iron-mediated toxicity. A comparison with trolox (TRO), a hydrosoluble vitamin-E analogue that protects against such an induced stress, was performed.

3 Rat hippocampal or cortical cultured cells were exposed either to 2 μM $FeSO_4$ alone or in the presence of PIR, DHP, BRO, DPR, MCL or TRO. Cell survival (lactate-dehydrogenase measurements, 16 h incubation), intracellular peroxide production (DCF-fluorescence, 1 h incubation), lipoperoxidation (TBARS-fluorescence, 6 h incubation) and mitochondrial function (MTT-test, 16 h incubation) were assessed.

4 PIR, DHP and TRO significantly protected cultures ($P<0.05$) against Fe^{2+}-induced toxicity in a concentration-dependent manner. The EC_{50s} of these compounds were 6, 12 and 19 μM, respectively, in hippocampal cells. For cortical cell cultures incubated in the presence of iron and PIR or DHP, EC_{50s} were 5 and 6 μM respectively. All Hill coefficients were close to unity. BRO, MCL and DPR were not protective in any type of culture. The IC_{50s} for the inhibition of MAO-A were 2, 2 and 0.2 μM for PIR, DHP and BRO, respectively. PIR, DHP and TRO, but not DPR, induced a significant decrease in both intracellular peroxide production and lipoperoxidation. They also improved mitochondrial function.

5 These experiments show that PIR and DHP can protect hippocampal and cortical neurons against oxidative stress at pharmacologically relevant concentrations. This protective effect seems unrelated to inhibition of MAO-A, but possibly involves free radical scavenging.
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Keywords: Monoamine oxidase; oxidative stress; pirlindole; dehydropirlindole; deprenyl; vitamin E; cell culture; neurone; iron

Abbreviations: BRO, Brofaromine; DCF, 2,7-dichloro-fluorescein diacetate; DHP, Dehydropirlindole; DPR, Deprenyl; HVA, Homovallinic acid; LDH, Lactate dehydrogenase; MAO, Monoamine oxidase; MCL, Moclobemide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; PIR, Pirlindole; TBARS, Thiobarbituric acid reactive substances; TRO, Trolox

Introduction

It is generally accepted that oxidative stress induced by free radicals and mitochondrial dysfunction is implicated in the pathophysiology of some neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Olanow, 1993; Richardson, 1993; Smith *et al*., 1995; Williams, 1995; Ebadi *et al*., 1996; Simonian & Coyle, 1996; Schapira, 1999).

Studies of brain tissue after death in AD or PD patients have provided evidence to support the involvement of oxidative stress. This includes alterations in brain iron content, alteration of the antioxidant protective systems, lipid peroxidation, oxidation of proteins and DNA (Halliwell & Gutteridge, 1992; Jenner & Olanow, 1996; Simonian & Coyle, 1996; Jellinger, 1999; Pratico & Delanty, 2000) and impaired mitochondrial function (Cohen, 1990; Schapira, 1999; Cohen, 2000; Pratico & Delanty, 2000). The nature of the free radical responsible for cell death in neurodegenerative diseases remains unknown, but data suggest the involvement of highly reactive oxygen species (ROS) such as the hydroxyl radical (OH°), nitric oxide and its derivative peroxynitrite (Jenner & Olanow, 1996). However, other ROS are produced and may also induce cell death: e.g. hydrogen peroxide (H_2O_2), which is continually produced by cellular metabolic activity, can react with iron ions through the Fenton reaction to generate the hydroxyl radical (Halliwell & Gutteridge, 1992; Smith *et al*., 1995). In nerve cells, H_2O_2 production can result in part from the catabolism of catecholamines by the monoamine oxidases (MAO, monoamine, oxygen oxidoreductase (deaminating) (flavin-contain-

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ing) EC 1.4.3.4) (Thorpe *et al*., 1987; Shih, 1991), which is present mostly in catecholaminergic neurones, but presumably also in other neuronal types (Saura *et al*., 1992; Jahng *et al*., 1997). Moreover, it has recently been shown that oxidative glutamate toxicity requires monoamine as a source of free radicals (Maher & Davis, 1996).

The MAO type B (MAO-B) inhibitor deprenyl (DPR = selegiline) has been used in the therapy of PD due to its neuroprotective and antioxidant properties (The Parkinson Study Group, 1993). It has also been clinically tested as a protective agent in AD with some significant benefit in delaying the primary outcome of disease progression (Sano *et al*., 1997).

Pirlindole (PIR, pirazidole; 1,10-trimethylene-8-methyl-1,2,3,4-tetrahydropyrazino [1,2,-a] indole hydrochloride) is a tetracyclic compound that has been characterized as a potential antidepressant drug (De Wilde *et al*., 1996; Bruhwyler *et al*., 1997; Tanghe *et al*., 1997). The main mechanism of action of PIR consists of a selective and reversible inhibition of the MAO type A (MAO-A) (Medvedev *et al*., 1992; 1996; Bruhwyler *et al*., 1997). Drugs such as PIR may help to protect neuronal cells against oxidative stress because of the MAO requirement in oxidative toxicity in non-catecholaminergic neurones and because of the ability of MAO inhibitors to decrease H_2O_2 production. Therefore, we designed our experiments to assess whether the MAO-A inhibitors PIR, its dehydro-derivative dehydropirlindole (DHP), brofaromine (BRO, 4(-7-bromo-5-methoxy-2-benzofuranyl)-piperidine hydrochloride), moclobemide (MCL, *p*-chloro-N-[2-morpholinoethyl]benzamide) and the MAO-B inhibitor, DPR, are able to promote survival of rat cultured neurones exposed to an iron-induced oxidative stress. Moreover, we compared their effect with that of trolox C (TRO, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). TRO is a water-soluble derivative of vitamin E, which is known to protect various neuronal cells against iron-induced oxidative stress (Boland *et al*., 2000). Interestingly, Vitamin E has also been shown to slow the progression of AD in patients with moderately severe impairment (Sano *et al*., 1997). However, no benefit was noted in PD patients (The Parkinson Study Group, 1993).

Methods

Hippocampal and cortical cell primary culture

Primary cell cultures were performed following the method previously described (Boland *et al*., 2000). Briefly, brain cortices and hippocampi were removed from 17–18 embryonic days Wistar rats. After having removed the meninges and isolated areas of interest, cells were dissociated enzymatically (0.25% trypsin in PBS) and then mechanically, using a narrowed Pasteur pipette. Cells were seeded (cortex: 80,000 cells per cm^2, hippocampi: 50,000 cells per cm^2) into poly-L-ornithin coated plates containing culture medium (modified Eagle's medium (GIBCO-BRL, Cat. No 11700-077) supplemented with (in mM): KCl 16, sodium bicarbonate 26, glucose 55, L-glutamine 1, pyruvate 1 and 10% foetal calf serum).

After a 4–6 h incubation period, the medium was replaced with fresh medium. Cultures were maintained in a humidified atmosphere (5% CO_2–95% air) at 37°C. All experiments were performed with cells that had been kept in culture for 7–10 days without any replacement of the culture medium.

Drugs and iron exposure

Exposure to the drugs and iron was performed as follows: 7 to 10-day-old cell cultures were washed three times with Locke's solution (in mM): NaCl 154, KCl 5.6, $CaCl_2$ 2.3, $MgCl_2$ 1.0, $NaHCO_3$ 3.6, HEPES 5 and glucose 10, pH 7.2, supplemented with 10 mg l^{-1} gentamycin) (Mattson *et al*., 1995; Blanc *et al*., 1997). The cells were then kept in Locke's solution and immediately exposed to the drugs for a 3 h pre-incubation. Finally, after pre-incubation, 2 μM $FeSO_4$ (from a 400 μM stock solution prepared extemporaneously in double-distilled sterile water), was added and incubation was prolonged for either 1 h (intracellular peroxides measurement), 6 h (lipid peroxidation assessment) or 16 h (cell death determination, mitochondrial function measurement).

Assessment of MAO activity

Enzyme activity was measured as described by Snyder & Hendley (1968) with minor modifications (Gerardy, 1994). In addition, minor changes were made in order to adapt the method to cell cultures: (1) After 3 h of incubation in Locke's solution in the presence of MAO-A inhibitors (50 mm dishes), cortical cells (four pooled dishes per condition) were washed twice with 2 ml 0.25 M sucrose iced solution then scraped in the same solution (60 μl per dish). Afterwards, they were homogenized by sonication for 6 s. Aliquots were removed for protein determination. Before use, homogenates were frozen and stored at −20°C. Storage of homogenates for several days did not significantly modify MAO-A activity. (2) The calibration curve for H_2O_2 measurements was adapted by decreasing the amounts of standard hydrogen peroxide solution.

Briefly, the incubation mixture (final volume: 3 ml) contained 300 μmoles of sodium-potassium phosphate buffer pH 7.8, 1.37 μmoles of homovallinic acid (HVA), 5.7 μmoles octopamine, 120 μg of peroxidase and 120 to 200 μl of cell homogenate. After a pre-incubation of 10 min in the presence of homogenate and peroxidase, a solution of HVA and substrate was added in order to start the reaction, and the tubes were incubated during 30 min. Pre-incubation and incubation were conducted at 37°C with constant shaking. The reaction was stopped by chilling the tubes in an ice bath for 15 min. Finally, the tubes were centrifuged at 4°C for 10 min at 15,000 × *g*. The fluorescence measurements (315 nm excitation–428 nm emission) were performed without delay on the supernatant. The calibration curve was determined by adding increasing amounts of standard hydrogen peroxide solution to a set of tubes containing phosphate buffer pH 7.8, HVA and peroxidase. Blanks contained homogenates, but no added substrate. Values were expressed as percentage of MAO-A activity of control cells.

Assessment of cell death

Cell death was assessed by the usual lactate dehydrogenase (E.C.1.1.1.27) activity assay which measures the quantity of

lactate dehydrogenase released by dying cells (Boland *et al*., 2000).

Briefly, at the end of the experiment, the extracellular medium was removed. Total releasable lactate dehydrogenase was obtained by submitting the cells to a freeze–thaw cycle. Phosphate buffer containing sodium pyruvate (0.1 mg ml^{-1}) and β-nicotinamide adenine dinucleotide (0.2 mg ml^{-1}) was added to the culture medium sample. The absorbency decrease (340 nm) was then immediately determined for 6 min (Cecil Spectrophotometer). Values were expressed as percentages of the total releasable lactate dehydrogenase.

Measurement of intracellular peroxides

The level of intracellular peroxides was quantified by fluorescence with 2,7-dichloro-fluoroscein diacetate (DCF, Molecular Probes) (Boland *et al*., 2000).

Cells were loaded for 50 min with 100 μM DCF. At the end of the incubation, cells were washed once and the relative levels of fluorescence were quantified using a fluorescence plate reader (485 nm excitation and 538 nm emission, Spectra Max Gemini XLS, Molecular Devices) (Blanc *et al*., 1997). Values were expressed as percentage of fluorescence in control cultures.

Measurement of lipid peroxidation

The fluorescence of thiobarbituric acid reactive substances (TBARS) was used as a measure of the membrane lipoperoxidation. This test estimates the level of malonyl dialdehyde precursors (Blanc *et al*., 1997).

The level of lipoperoxidation was estimated following the method previously described (Boland *et al*., 2000). Briefly, after experimental treatment (6 h of incubation), cortical cells (50 mm dishes) were washed with ice-cold phosphate buffered saline–0.5 mM dithiothreitol. Cells were scraped and the samples were sonicated; aliquots were removed for protein determination. Trichloroacetic acid (5%) and the TBARS reagent were added. The solution was incubated at 95°C for 30 min. After cooling, isobutanol was added and the samples were vigorously mixed then centrifuged at 1800 r.p.m. for 10 min. The fluorescence of the upper organic phase was quantified using a plate reader (544 nm excitation and 590 nm emission, Spectra Max Gemini XLS, Molecular Devices). Values of TBARS fluorescence were expressed as the percentage of the level in vehicle-treated control cultures.

Assessment of mitochondrial function

Cellular MTT levels measure mitochondrial redox status and function (Mosmann, 1983); they were quantified using a method similar to those described previously (Mattson *et al*., 1995). In brief, MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, Sigma) was added to the cells at a final concentration of 0.25 mg ml^{-1} and incubated 2–3 h to allow the conversion of MTT into purple formazan crystals. Then the incubating medium was removed and the cells were lysed with 100% DMSO. Absorbency (595 nm) in each well was quantified using a microplate reader (Labsystems Multiskan MS–Biolise Software). Results were expressed as percentages of the absorbency in vehicle-treated control culture wells.

List of drugs

The following drugs were generously supplied: pirlindole (Therabel Research, Brussels, Belgium), brofaromine (Novartis), moclobemide (Roche). Dehydropirlindole was a generous gift from Pr. Delarge, Laboratory of Medicinal Chemistry, University of Liège, Trolox and deprenyl were purchased from Sigma.

Data analysis

All data are presented as mean and s.d. values. Curve fitting was carried out using Kaleidagraph© software and the standard equation:

$$E = E_{max}[1 + (IC_{50}/x)^{h}]^{-1},$$

where x is the concentration of drug and h the Hill coefficient. For experiments involving multiple treatment conditions, statistical comparisons were based on ANOVA followed by Dunn's *post hoc* test for pairwise comparison; Student's t-test was used for data analysis in experiments involving comparison of a control value and one treatment value.

Results

Protective effect of pirlindole and dehydropirlindole

When hippocampal cells were exposed to an iron-induced oxidative stress, both PIR and DHP were able to significantly protect cells in a concentration-dependent manner, as shown on Figure 1a. We observed a significant decrease of cell mortality from 5 μM DHP and 10 μM PIR. The maximal protective effect was reached at 50 μM for PIR and 100 μM for DHP (78% and 92% cell protection, respectively). Estimated EC_{50s} were 6 μM for PIR and 12 μM for DHP. The Hill coefficients were 1.19 and 1.13 for PIR and DHP respectively. Contrary to DHP, PIR seems to lose its protective effect at the highest concentration (200 μM). A toxic effect of the higher concentrations (100–200 μM) of PIR was shown ($P<0.05$) by incubating hippocampal cells in the absence of the oxidative stress (Figure 1b). DHP was weakly but significantly toxic at 200 μM (Figure 1b). However, this toxicity did not affect its protective effect (Figure 1a). The protective effect of PIR and DHP was confirmed by morphological observation (data not shown).

Similar experiments were also performed on cortical cells in order to determine whether these two compounds were able to protect cells in this structure. The results (Figure 1c) showed that both PIR and DHP were able to protect cortical cells as well. The protection was concentration-dependent and was significant from the 10 μM concentration for both substances, reaching a maximum at the same concentrations as those observed for hippocampal cells with a maximal effect of 99 and 100% cell protection, respectively. The EC_{50s} were 5.2 μM and 6 μM for PIR and DHP respectively. Corresponding Hill coefficients were 1.02 and 1.35. As for hippocampal cells, we observed that higher concentrations of PIR (100 and 200 μM) were toxic compared to the 50 μM concentration.

In order to know if protective effects against oxidative stress can be generalized to other MAO inhibitors, similar

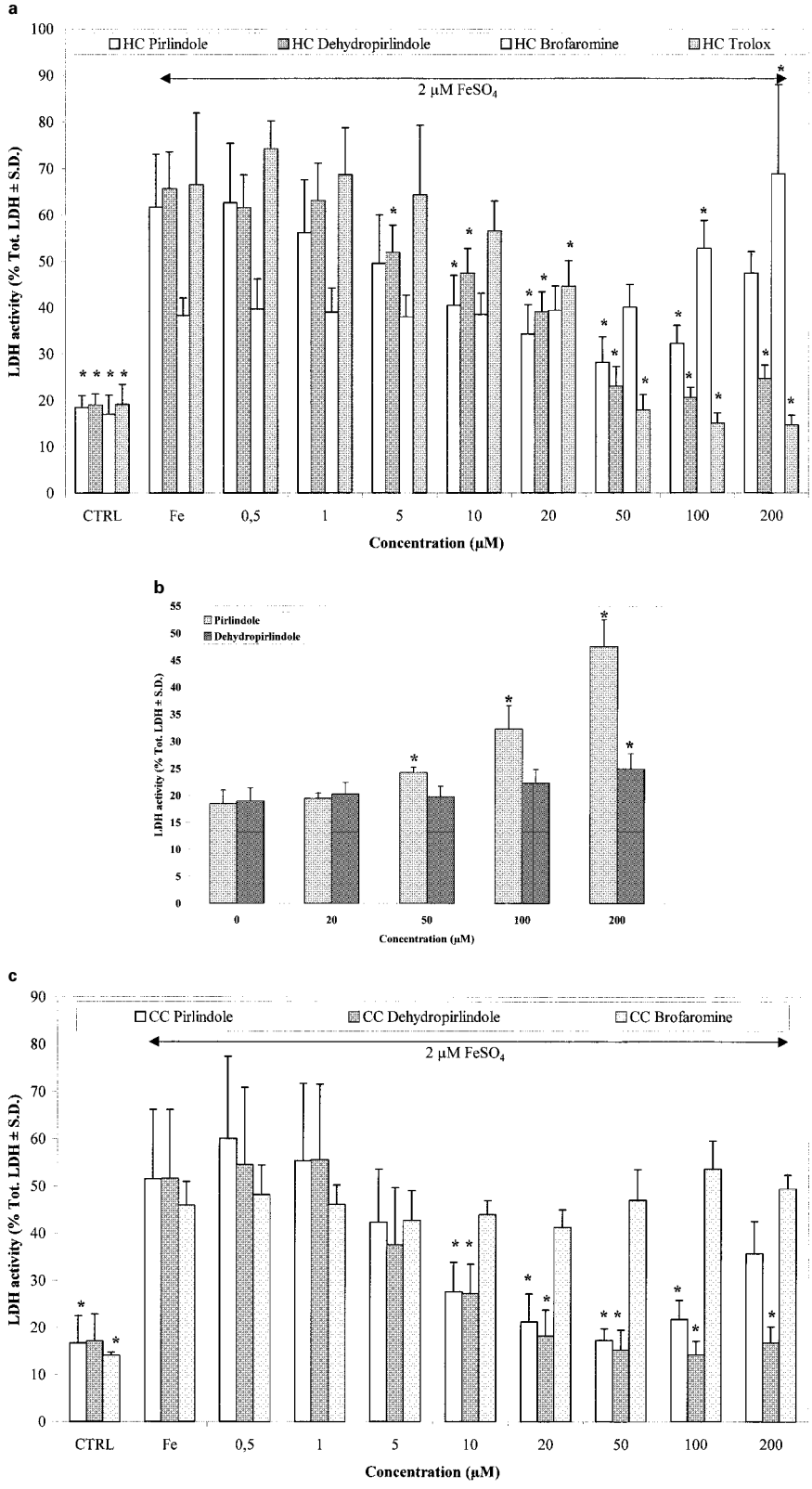

Figure 1 (a) Determination of the protective effect of pirlindole, dehydropirlindole, brofaromine and trolox. Hippocampal cells were exposed to various concentrations of drug during 16 h in the presence of 2 μM $FeSO_4$. Cell death was then assessed by the LDH assay. Statistical analysis: ANOVA analysis and Dunn's *post hoc* tests, $*P<0.05$ compared to iron-treated cells, $n=10-51$. (b) Determination of the toxicity of pirlindole and dehydropirlindole. Hippocampal cells were exposed to pirlindole or dehydropirlindole during 16 h. Cell death was then estimated. Statistical analysis: ANOVA test, $*P<0.05$ compared to control cells (0 μM), $n=4-18$. (c) Determination of the protective effect of pirlindole, dehydropirlindole and brofaromine on cortical cells. Cortical cells were treated as in a. Statistical analysis: ANOVA analysis and Dunn's *post hoc* tests, $*P<0.05$ compared to iron-treated cells, $n=8-30$.

experiments were performed using the MAO-A inhibitor BRO. Results obtained were quite different from those obtained with PIR or DHP. Indeed, as shown in Figure 1a,c, we did not observe any protective effect of BRO against iron-induced cell death. This was true for both cortical and hippocampal cells. High concentrations of BRO were toxic for hippocampal, but not cortical cultures (Figure 1a,c).

We also performed similar survival experiments on hippocampal cells exposed to iron, using MCL, another MAO-A inhibitor, or DPR, a MAO-B inhibitor known to be neuroprotective. However, neither MCL nor DPR were able to offer any protection to the cells in our experiments (data not shown).

In order to compare the potency of PIR and DHP to that of a free radical scavenger, we assessed the effect of TRO in the same experimental conditions. Figure 1a summarizes the results of these experiments. TRO protected hippocampal cells in a concentration-dependent manner. This protection was complete at a concentration of 50 μM and higher. Higher concentrations were not toxic to the cultured hippocampal cells. The estimated EC_{50} was 19 μM and the Hill coefficient was 1.2. This EC_{50} was higher than that for PIR or DHP.

MAO-A inhibition

In order to assess whether the protective effect was related to the inhibition of the MAO-A or due to another mechanism, we determined the effect of MAO inhibitors on the activity of MAO-A in cultured cortical cells. Figure 2 summarizes the results obtained. It shows, firstly, that cultured cortical cells had a significant MAO-A activity, and secondly, that PIR, DHP and BRO inhibited the enzyme in a concentration-dependent manner. The IC_{50s} were 2.3, 2 and 0.19 μM for PIR, DHP and BRO respectively. Hill coefficients were 1.32, 0.72 and 0.96. The enzyme was almost completely inhibited at 10 μM for each compound. MCL was not tested. Taken together, these results show that the protective effect of PIR and DHP was not dependent on the inhibition of MAO-A.

Mechanism(s) of protection

Modulation of production of lipid peroxides by MAO inhibitors (TBARS test) Our results suggest that the protection of cells by PIR and DHP could be due to another mechanism than the inhibition of MAO-A. We therefore determined the effect of PIR, DHP and TRO on the production of lipid peroxides when cortical cells were incubated in the presence of 2 μM iron. Incubation of cortical cells in the presence of iron induced a significant increase in lipid peroxide production (1.8 fold increase, $P<0.05$) as shown in Table 1. The use of a 50 μM concentration of PIR, DHP and TRO in the presence of iron yielded a significant inhibition in the induced lipoperoxidation ($P<0.05$). Indeed, we observed that the increase in lipid peroxide production was 33, 13 and 28% for PIR, DHP and TRO respectively with respect to control cells (100% increase = iron-induced lipoperoxidation).

Effect of MAO inhibitors on intracellular peroxides production (DCF assay) In order to assess whether the protective effect of the MAO-A inhibitors used in our experiments could be due to a modulation of the production of intracellular peroxides, we performed DCF assays on both types of cells. After 3 h of pre-incubation in the presence of 50 μM concentrations of either PIR, DHP, DPR or TRO, iron was added to the incubating medium (final concentration: 2 μM). The incubation was prolonged for 1 h. Then DCF measurements were performed. In these experimental conditions, iron induced an important increase in the production of intracellular peroxides (2.83 and 4.36 times for cortical and hippocampal cells respectively, Table 1). Although we observed a tendency for a decrease in the iron-induced peroxide production when MAO-A inhibitors were used, this effect was not significant, except for DHP in hippocampal cells (54% increase with respect to control cells, $P<0.05$). However, in both types of cells, TRO modulated the production of intracellular peroxides (28 and 56% increase, when compared to control cells for hippocampal and cortical cells respectively, $P<0.05$, iron-induced stress = 100%). DPR used in the same protocol with hippocampal cells did not modify endoperoxide production (Table 1).

Effect of MAO inhibitors on mitochondrial function (MTT assay) Mitochondria play an important role in the process of cell death. Mitochondrial redox status and function are indicators of cellular health. We therefore determined whether PIR and DHP are able to act on these mitochondrial characteristics. As shown in Table 1, iron drastically reduced mitochondrial function. PIR and DHP significantly improved mitochondrial function in iron-exposed hippocampal and cortical cells ($P<0.05$). Indeed, in hippocampal cells, PIR, DHP and TRO induced 68, 91 and 94% of mitochondrial improvement respectively. However, DPR did not improve mitochondrial function. In cortical cells, the improvement provided by PIR was close to that observed in hippocampal cells (66%). DHP completely preserved mitochondrial status. Therefore, in both types of cultured cells, PIR was significantly less efficient than DHP for improving mitochondrial function.

Discussion

Our results can be summarized as follows: PIR, DHP and BRO significantly inhibit MAO-A. In our cultures however,

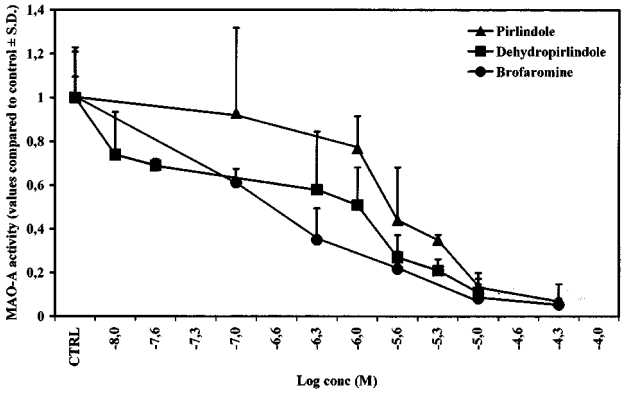

Figure 2 Measurement of the inhibition of MAO-A activity by pirlindole, dehydropirlindole or brofaromine. Cultured cortical cells were exposed during 3 h to various concentrations of MAO-A inhibitors. MAO activity was then measured. $n \geqslant 3$ except for 10 and 50 μM PIR and 10 μM DHP ($n=2$).

Table 1 Determination of the mechanism of action of PIR and DHP

	Hippocampal cells			*Cortical cells*	
	Intracellular Peroxides (DCF)	*Mitochondrial Function (MTT)*	*Lipid Peroxidation (TBARS)*	*Intracellular Peroxides (DCF)*	*Mitochondrial Function (MTT)*
Control cells	1.00±0.31*	1.00±0.21*	1.00±0.04*	1.00±0.35*	1.00±0.16*
	0% (19)	100% (52)	0% (10)	0% (18)	100% (58)
2 μM Fe^{2+}	4.36±3.02	0.31±0.17	1.83±0.34	2.83±0.67	0.18±0.01
	100% (18)	0% (42)	100% (10)	100% (18)	0% (58)
50 μM pirlindole	2.96±2.14	0.78±0.24*	1.27±0.22*	2.39±0.79	0.72±0.23*
+2 μM Fe^{2+}	58% (29)	68% (23)	33% (12)	76% (28)	66% (22)
50 μM dehydropirlindole	2.80±2.42*	0.94±0.24*	1.11±0.14*	2.20±0.81	1.12±0.19*
+2 μM Fe^{2+}	54% (29)	91% (24)	13% (14)	66% (28)	115% (18)
50 μM trolox	1.95±1.48*	0.95±0.25*	1.23±0.17*	2.03±0.86*	------
+2 μM Fe^{2+}	28% (30)	94% (26)	28% (10)	56% (28)	
50 μM deprenyl	4.03±2.37	0.33±0.12	------	------	------
+2 μM Fe^{2+}	90% (30)	3% (13)			

Experiments concerning intracellular peroxides formation, mitochondrial function and peroxidation of cellular lipids were performed (see text for details). All the data are expressed with respect to control. Statistical analysis was carried out using ANOVA and Dunn's *post hoc* tests, $*P<0.05$ compared to iron treated cells, numbers between parenthesis = number of experiments.

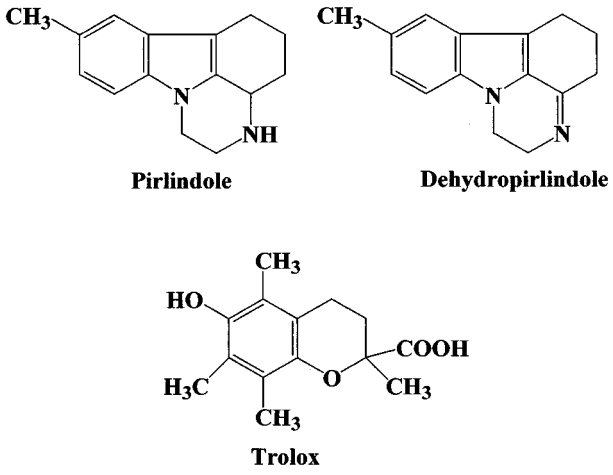

Figure 3 Structural formulas of pirlindole, dehydropirlindole and trolox.

only the two former substances can protect against iron-induced oxidative stress. Therefore the protection does not appear to be linked to the inhibition of MAO-A. We showed that the protective effect of PIR and DHP could be related to the ability of these substances to decrease production of both lipoperoxides and intracellular peroxides and to improve mitochondrial function. DPR, the MAO-B inhibitor reported to have protective effects in other cell types, was unable to protect cells or to improve intracellular peroxide production or mitochondrial function in our cultures.

PIR, DHP and BRO inhibited MAO-A activity of our cultured cortical cells in a concentration-dependent manner. The literature IC_{50} values of PIR range from 0.09 to 2 μM (Medvedev *et al*., 1992; 1996; Bruhwyler *et al*., 1998). For DHP, authors have shown that IC_{50} ranges from 0.0045 μM (Medvedev *et al*., 1996) to 0.04 μM (Gerardy & Dresse, 2002). The IC_{50} range of BRO is 0.01 μM (Da Prada *et al*., 1988; Gérardy, personal communication) to 0.1 μM (Anderson *et al*., 1991). It appears therefore that the IC_{50} values of the different MAO-A inhibitors are quite variable. This could be due to the method used for measurement of MAO-A activity or to the variation between tissues on which those measurements were performed. Indeed, the values obtained are from mitochondrial fractions of rat brain (Medvedev *et al*., 1992; 1996), human placenta (Medvedev *et al*., 1996), rat liver (Anderson *et al*., 1991) or from rat brain homogenates (Da Prada *et al*., 1988; Bruhwyler *et al*., 1998; Gerardy & Dresse, 2002). Our values of IC_{50} are generally in the range or higher to those reported in the literature. Such differences could be due either to the species used in the experiments or to the fact that we used cultured cells rather than isolated mitochondria or tissue homogenates. Indeed, in our model, contrary to the latter preparations, the drugs have to penetrate into the cell before being able to reach the enzyme that is located on the outer mitochondrial membrane. Depending on the lipophilic character of the substance, penetration into the cell is variable. Moreover, PIR, DHP and BRO are three reversible inhibitors and their action on the enzyme is tightly modulated according to the ratio between the concentration of the drug and that of the enzyme (Anderson *et al*., 1991).

To our knowledge, these data are the first demonstration that the MAO-A inhibitors, PIR and DHP, are able to protect cultured brain cells against oxidative damage. The same experiments performed on hippocampal cells with BRO, DPR or MCL show that these drugs are not able to protect cells. Numerous papers have been published either concerning antioxidative actions of the MAO-B inhibitor DPR or its beneficial use on AD or PD patients (see Introduction). However, in our model, we show that DPR does not protect against iron-induced cell death, while PIR and DHP indeed do. Therefore it seems that the slight beneficial effect observed clinically (Sano *et al*., 1997) may not be related to the ability of DPR to protect brain tissue against an oxidative stress induced by an excess of iron.

Because of the lack of effect of MCL and BRO, it is not possible to extend the protective action observed with PIR and DHP to all MAO-A inhibitors. Furthermore, it is not possible to explain the protective effect of PIR and DHP by the fact that they inhibit MAO-A. Indeed, we have tested BRO to determine both its protective aptitude and its inhibitory capacity. The drug inhibited MAO-A, similarly to PIR and DHP, but was

unable to protect cells against iron-induced cell death. This establishes that the protective effect is not linked to MAO-A inhibition. Hence, another mechanism was involved in the protection of cells. Such data are in agreement with other data on MAO inhibitors. Indeed, as explained in the introduction, it is now admitted that the protective effect of DPR is not directly linked to its ability to inhibit MAO-B. It has also been shown that lazabemide, another MAO-B inhibitor, is a potent inhibitor of oxy-radical damage, independently of its action on MAO-B (Mason *et al*., 2000).

In order to establish the mechanism involved in PIR and DHP protection, we assessed the effect of these MAO-inhibitors, DPR and TRO on lipid peroxidation, intracellular peroxide production and mitochondrial function.

We showed that the drugs could significantly limit iron-induced production of lipid peroxides. The ability to limit lipid peroxide production is almost similar for PIR and TRO. However, DHP appears to be twice more efficient. Thus, like some other MAO inhibitors, PIR and DHP can help to limit lipid peroxidation. Indeed, using a membrane-based model of oxidative stress, other authors have shown that lazabemide could inhibit lipoperoxidation in a concentration-dependent manner, and more effectively than vitamin E or DPR (Mason *et al*., 2000).

Data concerning intracellular peroxide production show a tendency to a reduction of this production; however, among the MAO inhibitors, only DHP tested on hippocampal cells exhibited a significant effect. TRO significantly limits intracellular production of peroxides in both types of cells. DPR used on hippocampal cells did not modify the production of endoperoxides. The effect of DHP on the production of intracellular peroxides may be related to its ability to limit lipid peroxide production in the early stages of the insult. Indeed, DCF measurements were performed after 1 h of incubation in the presence of iron, whereas TBARS assessment was performed after 6 h of incubation. A reduction of the production in endoperoxides might contribute to the limitation of the lipoperoxidation and cell survival effect. The mechanism implicated in the limitation of the production of endoperoxides remains to be elucidated. Indeed, the limitation could be linked either to the inhibition of the enzyme that, in turn, induces a reduction in the production of H_2O_2 or to a mechanism similar to that of TRO (free radical scavenging, see below).

Because mitochondrial status is important to cell survival and the cell death process, we assessed the effect of PIR, DHP, TRO and DRP on mitochondrial function. In the presence of iron, we observed an important decrease in MTT conversion, which also reflects the cell loss induced by oxidative stress. PIR, DHP and TRO significantly improved mitochondrial function when cells were preincubated in the presence of iron. It should be stressed that DHP was more efficient than PIR in the maintenance of mitochondrial function. In addition, DPR was unable to improve the mitochondrial status in hippocampal cells when they were exposed to iron.

As already mentioned, data obtained with DHP more closely resembled data obtained with TRO with PIR. On the one hand, the fact that PIR is slightly less efficacious than DHP could be linked to the toxicity of PIR (see Figure 1b). On the other hand, this difference could be linked to the structure of the substances. Figure 3 illustrates the structural formula of PIR, DHP and TRO. It should be noted that DHP is formed by oxidation of PIR. A first common feature to PIR, DHP and TRO is the presence of at least one methyl group attached to an aromatic ring. A second common point is the presence of the aromatic ring. The major difference between PIR and DHP is the presence of an additional double bond in DHP with respect to PIR. Therefore, by comparison with the scavening mechanism of vitamin E or TRO in the presence of free radicals, it can be hypothesized that PIR and DHP act as free radical scavengers. Hence, it is possible that, during an oxidative stress, the free radicals react with PIR and DHP, probably by taking an atom of hydrogen from the methyl group present on these molecules. The stability of the subsequent PIR and DHP radicals is presumably linked to the presence of both the aromatic cycle and double bonds. However, DHP has an additional double bond that could increase its stability and explain its higher efficacy in protecting cells against oxidative stress. Clearly, additional studies need to be performed to assess the chemical mechanism of action of these drugs.

In conclusion, PIR and DHP protect hippocampal and cortical cells against iron-mediated stress by improving cell viability and mitochondrial function and by decreasing both intracellular peroxide production and lipid peroxidation. This protective effect appears to be unrelated to the inhibition of MAO-A, but it is likely to be associated with antioxidant properties, e.g. free radical scavenging, which may be comparable to those of TRO. However, the exact mechanism involved remains to be elucidated. Because the level of MAO-A activity is quite variable among species, it is difficult to extrapolate our results to the human. However, these experiments provide an experimental support for investigating the potential benefit of using such MAO-A inhibitors in patients suffering from diseases involving oxidative stress.

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