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Nitroindazole compounds inhibit the oxidative activation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin to neurotoxic pyridinium cations by human monoamine oxidase (MAO)

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Abstract
Monoamine oxidase (MAO) B is a mitochondrial enzyme selectively involved in the oxidative activation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin to toxic pyridinium cations producing Parkinsonism in animal models. Various synthesized 5-nitroindazoles, 6-nitroindazole and the neuroprotectant 7-nitroindazole were examined as inhibitors of MAO and as antioxidants and radical scavengers. The oxidation of MPTP by human MAO-B and mitochondria was assessed by HPLC. Simple nitroindazoles inhibited MPTP oxidation to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) and 1-methyl-4-phenylpyridinium (MPP^+) in a competitive and reversible manner. 5-Nitroindazole ($IC_{50} = 0.99$ μM, $K_i = 0.102$ μM) and 6-nitroindazole ($IC_{50} = 2.5$ μM) were better inhibitors of human MAO-B than 7-nitroindazole ($IC_{50} = 27.8$ μM). 6-Nitroindazole also inhibited MAO-A. Nitroindazole isomers were good hydroxyl radical ($OH^\bullet$) scavengers, with 5-nitro-, 6-nitro- and 7-nitroindazole showing similar activity ($k \sim 10^{10}\ M^{-1}\ s^{-1}$). Neuroprotective actions of nitroindazoles (7-nitroindazole) could be linked to their MAO-inhibitory and antiradical properties besides inhibition on nitric oxide synthase (NOS). 5-Nitro- and 6-nitroindazole, previously reported as weak NOS inhibitors, were better inhibitors of human MAO-B and more active against MPTP neurotoxin oxidation (lower $MPDP^+$ and MPP^+ levels) than 7-nitroindazole and acted as good radical scavengers and could be potential neuroprotective agents in addition to MAO-B inhibitors.

Keywords: *Monoamine oxidase inhibition, MPTP neurotoxin, nitroindazoles, antioxidants, hydroxyl radical scavengers, neuroprotection, Parkinson's disease*

Introduction

Monoamine oxidase (MAO) is a flavoenzyme located at the outer membranes of mitochondria in the human brain and peripheral tissues and catalyses the oxidative deamination of neurotransmitters and vasoactive dietary and xenobiotic amines, including dopamine, serotonin, norephinephrine, tyramine, tryptamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin. MAO appears as two isozymes, MAO-A and B, distinguished by substrate and inhibitor selectivity [1,2]. MAO isozymes play an important role in the central nervous system and peripheral organs [3], with MAO-A being involved in psychiatric conditions and depression and MAO-B implicated in neurological disorders and diseases [1,2,4–6]. The oxidation of biogenic amines and neurotransmitters by MAO results in the production of hydrogen peroxide (H_2O_2) and oxygen radicals which represent risk factors for cell oxidative injury [7–9] and therefore inhibition of MAO may offer protection against oxidative stress [2,9].

MAO bioactivates xenobiotic amines such as MPTP to toxic metabolites [10–13]. MPTP is a parkinsonism-inducing neurotoxin in humans that was discovered as a contaminant of synthetic heroin

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[14]. Following injection, MPTP easily crosses the blood–brain barrier and is preferentially metabolized by MAO-B present in glial cells to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) (Figure 1). This enzymatic metabolite is subsequently oxidized to 1-methyl-4-phenylpyridinium (MPP^+), which is selectively uptaken by dopaminergic cells, producing inhibition of complex I of mitochondria, energy depletion, oxidative stress and cell death [10,15]. $MPDP^+/MPP^+$ causes an overflow of dopamine and induces oxidative stress through the generation of free radicals such as $OH^\bullet$, superoxide and $NO^\bullet$, implicated in dopaminergic neurotoxicity [16–18]. These biochemical features of MPP^+ toxicity may be somehow reversed by antioxidants [19]. MPTP produces neurotoxicity in humans and monkeys and it is currently used to generate Parkinson's disease (PD) in a mouse model of this disease [20]. Inhibition of MAO-B may afford neuroprotection throughout a diminished activation of toxins such as MPTP or related substances and/or also by a reduced production of reactive oxygen species and aldehydes [7,8]. Identification of novel MAO inhibitors is a current subject of interest in drug discovery [1,2], as both antidepressants [4,21] and neuroprotectants [2,22]. MAO inhibitors may also exhibit potential pharmacological effects related with the addictive properties and the lowest incidence of Parkinson's disease (PD) in smokers [23–27].

Indazole compounds exhibit a number of biological and pharmacological activities [28–30]. 7-Nitroindazole inhibits neuronal nitric oxide synthase (nNOS) [31,32] and is a neuroprotectant against MPTP-induced parkinsonism in animal models [33–36]. Inhibition of nNOS may decrease nitric oxide ($NO^\bullet$), peroxynitrite and neuronal oxidative stress, affording neuroprotection [19]. However, 7-nitroindazole was also reported as an inhibitor of MAO and this latter effect could account for its neuroprotective actions [2,28,29,34,37–42]. Moreover, 7-nitroindazole may offer its protection by antioxidant and radical scavenging actions [18]. In this regard, this research studies the inhibitory effects of a number of nitroindazoles on MPTP and kynuramine oxidation by human MAO enzymes and examines their activities as antioxidants and free radical scavengers. Simple nitroindazoles, particularly 5-nitro- and 6-nitroindazole, were good inhibitors of the oxidation of MPTP neurotoxin and reduced the formation of neurotoxicant pyridinium cations both in human MAO protein fractions and mitochondria. Remarkably, they were better inhibitors of human MAO-B than the neuroprotectant 7-nitroindazole. The three nitroindazoles were also good hydroxyl radical ($OH^\bullet$) scavengers and exhibited similar reaction rates. Then, 5- and 6-nitroindazole which are both radical scavengers and potent MAO-B inhibitors might offer potential neuroprotection against MPTP oxidative activation as occurs with 7-nitroindazole, but with the particularity of the absence of involvement of NOS enzyme inhibition since they appear to lack significant inhibition over this latter enzyme [43]. In the future, *in vivo* studies are needed to confirm if these compounds are indeed neuroprotective agents in addition to MAO-B inhibitors.

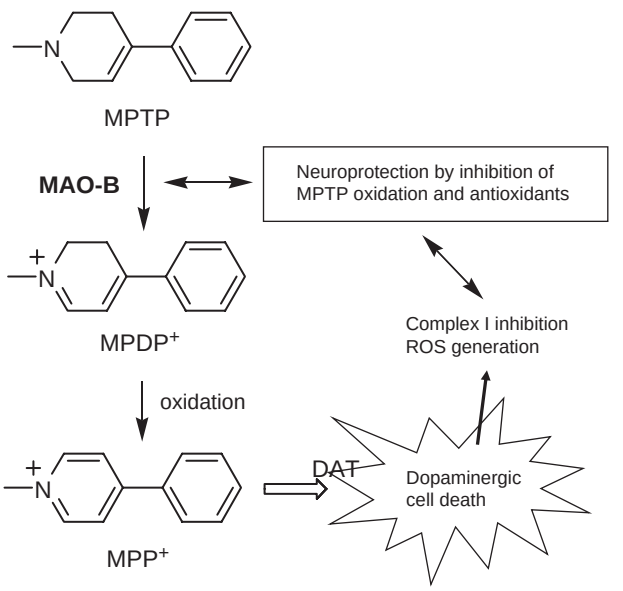

Figure 1. Oxidative bioactivation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxin by MAO-B in the brain to $MPDP^+$, which is chemically or enzymatically oxidized to MPP^+, a direct-acting metabolite, producing dopaminergic cell death through mitochondrial complex I inhibition and ROS generation. MAO inhibitors reduce neurotoxin activation and antioxidants reduce ROS, being protective agents in this system.

Material and methods

Recombinant human monoamine oxidase A and B were obtained from Gentest BD biosciences (Woburn, MA). Enzymes were expressed in insect cells from MAO-A and MAO-B cDNA using a baculovirus expression system and were prepared as membrane protein fractions. Pooled human liver mitochondria subcellular fraction was obtained from Xenotech (Lenexa, KA). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) hydrochloride (caution: MPTP is a neurotoxin and should be handled with appropriate precautions), 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) perchlorate, 1-methyl-4-phenylpyridinium (MPP^+) iodide, kynuramine, 4-hydroxyquinoline, *R*-deprenyl and clorgyline were from Sigma Chemical Co (St. Louis, MO). A number of novel 5-nitroindazole derivatives (2–10) (Figure 3) were synthesized in the laboratory as mentioned elsewhere [44,45]. Briefly, the pairs of compounds 2/9 and 7/11 were prepared from 1-ethoxycarbonyl-5-nitroindazol-3-ol by alkylation with the required alkyl halide, followed by removal of 1-protecting group

with KOH/EtOH [44]. 1-Substituted indazol-3-ols 5 and 6 were prepared from 5-nitroindazol-3-ol by alkylation with the required alkyl halide in NaOH/H_2O [44]. 1-Substituted indazol-3-ols 4 and 8 were prepared from the corresponding indazolium-3-olates by treatment with refluxing 36% aq. hydrochloric acid [45]. Compounds 3 and 10 could be prepared by methylation [44] of 4 or by thermal decomposition of 1,1-dimethy-5-nitroindazolium-3-olate [45] followed in both cases by chromatographic separation. 5-Nitroindazole 1, 6-nitroindazole 12 and 7-nitroindazole 13 were purchased from Sigma. HPLC grade acetonitrile, methanol and dimethyl sulphoxide (DMSO) were from Scharlau (Spain) and dichloromethane from Merck (Germany).

Monoamine oxidase (MAO-A and B) assay and inhibition

MAO activity was determined following the dehydrogenation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to give 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) and 1-methyl-4-phenylpyridinium (MPP^+) and by deamination of kynuramine to give 4-hydroxyquinoline [11,12,46] and analysed by RP-HPLC-DAD (MS). A reaction mixture (0.2 ml, final volume) containing membrane protein fractions of the enzyme (recombinant human MAO-A or -B) (0.01–0.05 mg/ml) in 100 mM potassium phosphate buffer (pH 7.4) were added with MPTP (0.3 mM) or kynuramine (0.25 mM) as substrates and incubated at 37°C for 40 min. On the other hand, reaction mixtures (0.2 ml, final volume) containing human liver mitochondria (0.075 mg/ml protein) in 100 mM potassium phosphate buffer (pH 7.4) were added with MPTP (0.3 mM) and incubated at 37°C for 40 min. The enzymatic reaction was stopped by addition of 2N NaOH (75 μl) and 70% perchloric acid (25 μl) and subsequently centrifuged (9000 × g, 5°C) and 20 μl of the supernatant injected into the HPLC. To perform inhibition assays, aliquots containing nitroindazole compounds (0–50 μM), clorgyline (1 μM) or *R*-deprenyl (1 μM) were added to reaction mixtures containing MPTP or kynuramine and MAO or mitochondria in 100 mM potassium phosphate buffer (pH 7.4) and the reaction incubated and processed as above. Incubation reactions were performed at least in duplicate.

Enzyme kinetics and mechanism of inhibition were assessed by analysing the corresponding Michaelis-Menten curves by fitting reaction velocity vs substrate concentration (0–600 μM) to non-linear regression analysis (Graphpad Prism 4.0) and by double reciprocal Lineweaver-Burk plots obtained at different concentrations of substrates and inhibitors. Reaction velocity (v) was determined as the nmol of products ($MPDP^+$, MPP^+ or 4-hydroxyquinoline) per min and mg of protein. To determine MAO-binding reversibility, MAO-B (0.2 mg/ml) in 100 mM phosphate buffer (pH 7.4) was preincubated (37°C, 40 min) with or without 5-nitroindazole (24 μM) as inhibitor in vivaspin tubes (Vivascience, Aubagne, France) and the mixture was centrifuged (12000 xg) for 20 min, 5°C, to pellet membrane proteins, washed twice with 100 mM phosphate (pH 7.4)–5% DMSO and finally the pellet resuspended in 100 mM phosphate buffer (pH 7.4), added with MPTP (300 μM) and used for enzyme activity. MAO activity of those preincubated with 5-nitroindazole were compared with corresponding controls without inhibitor.

Antioxidant activity and activity as hydroxyl radical ($OH^\bullet$) scavengers

The ABTS assay developed by Re et al. [47] was used to measure total antioxidant activity of nitroindazoles (concentration 0–20 μM into the assay) against the radical $ABTS^{\bullet+}$ (decreasing absorbance at 734 nm) [48]. To measure the activity of nitroindazoles as hydroxyl radical ($OH^\bullet$) scavengers, hydroxyl radicals ($OH^\bullet$) were generated in a test tube by Fenton reaction in presence of benzoate [49,50] and its hydroxylation products: 4-hydroxybenzoate and 3-hydroxybenzoate, measured by RP-HPLC (254 nm). Hydroxylation was determined in the presence of increasing concentrations of nitroindazoles and the reaction was performed in eppendorf tubes (1 ml, final volume) containing by this order, $FeSO_4$ (50 μM), EDTA (30 μM), sodium benzoate (150 μM), nitroindazole (0–100 μM, added from aqueous stock solutions with the corresponding dilutions prepared in the complete absence of organic solvents) and phosphate buffer 5 mM (pH 7.2). The reaction was initiated by the addition of H_2O_2 (500 μM) and subsequently the mixture incubated at 37°C for 60 min in a water bath. Then, the mixtures were frozen until analysed by HPLC in the same day to determine hydroxylation products of benzoic acid. Reaction rate k ($M^{-1}\ s^{-1}$) of nitroindazole with $OH^\bullet$ was measured as previously by competition kinetic and using a reaction rate k of $OH^\bullet$ with benzoate of 3.3×10^9 $M^{-1}\ s^{-1}$ [50].

RP-HPLC chromatographic analysis and mass spectrometry

The analysis of the enzymatic reaction products: $MPDP^+$, MPP^+ and 4-hydroxyquinoline was performed by RP-HPLC with *uv-DAD* and fluorescence detection using an HPLC 1050 (Agilent, Santa Clara, CA, USA) with a Diode Array Detector (DAD) and a 1046A-fluorescence detector. A 150 mm × 3.9 mm, 4 μm, Nova-pak C18 column (Waters, Milford, MA) was used for chromatographic separation. Chromatographic conditions were: 50 mM ammonium phosphate buffer (pH 3) (buffer A) and 20% of A in acetonitrile (buffer B). Gradient programmed from

0% (100% A) to 32% B in 8 min and 90% B at 15 min. The flow rate was 1 ml/min, the column temperature was 40°C and the injection volume was 20 μl. Absorbance detection was set at 355 nm for analysis of $MPDP^+$, 280 nm for analysis of MPP^+ and 320 nm for analysis of 4-hydroxyquinoline. A response curve of area vs concentration was constructed to calculate the concentration of each compound. Identification of reaction products was done by UV (DAD spectra) and fluorescence. Confirmation of the identity of the reaction products (4-hydroxyquinoline, $MPDP^+$ and MPP^+) was performed with HPLC-ESI-mass spectrometry as previously [11,46]. The analysis of hydroxylation products of benzoic acid (4-hydroxybenzoic acid and 3-hydroxybenzoic acid) in the Fenton reaction was carried out using the same HPLC method and eluents with the compounds identified by coelution with standards and by HPLC-MS (ESI negative ionization) and quantified by UV (254 nm).

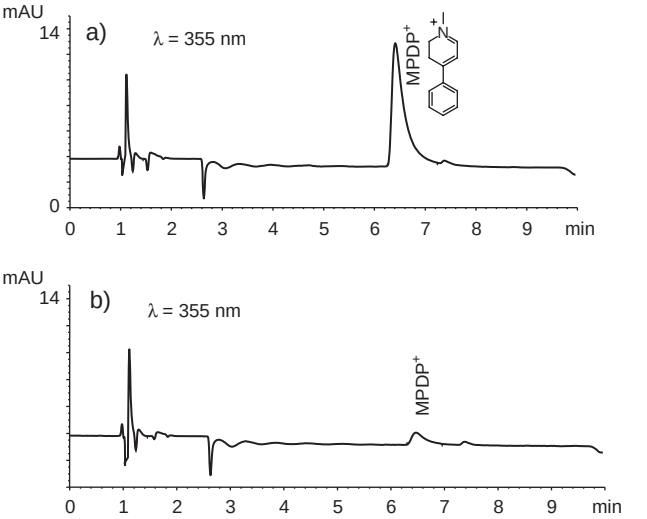

Figure 2. HPLC chromatograms ($\lambda = 355$ nm) of the reaction media of MPTP oxidation (300 μM) by MAO-B (0.05 mg/ml) in the absence (control) (A) or in the presence (B) of 5-nitroindazole 1 (5 μM). Incubation at 37°C for 40 min. $MPDP^+$ is 1-methyl-4-phenyl-2,3-dihydropyridinium. MPP^+ (1-methyl-4-phenylpyridinium) appeared at 280 nm and was inhibited in the same manner in the presence of 5-nitroindazole.

Results

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is metabolically oxidized (α-carbon oxidation) by MAO enzymes to give 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) and hydrogen peroxide. In a further step, $MPDP^+$ is readily oxidized to 1-methyl-4-phenylpyridinium (MPP^+), which is a directly-acting neurotoxic substance (Figure 1). This sequence of events produces neurotoxicity through mitochondrial inhibition and oxidative stress in dopaminergic neurons and is currently used to generate Parkinsonism in animal models. The oxidation of MPTP neurotoxin by human MAO-B to give 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) as a primary metabolite and 1-methyl-4-phenylpyridinium (MPP^+) in a lower proportion (MPP^+ comes from spontaneous oxidation of $MPDP^+$) was determined by HPLC (Figure 2). The activity of MAO-B was measured in presence of a number of nitroindazole compounds (Figure 3) and some of them reduced the formation of $MPDP^+$ (nmol of $MPDP^+$ produced/min × mg of membrane protein) (and MPP^+) in the media. The corresponding inhibitions obtained at 15 μM are summarized in Table I. Thus, isomeric 5-, 6- and 7-nitroindazoles 1, 12 and 13 showed appreciable inhibition on human MAO-B over the oxidation of MPTP. Despite that simple 5-nitroindazole 1 afforded a high degree of inhibition, most of their synthesised derivatives (i.e. 4–11) did not improve inhibition of the parent compound. Nevertheless, the nitroindazoles 2 and 3 afforded appreciable reduction of MAO-B activity. Inhibitory results were given as $MPDP^+$ that is the enzymatic metabolite, however the formation of MPP^+ into assays was always proportional to that of $MPDP^+$ (direct precursor) and its inhibition in the presence of nitroindazoles followed the same pattern.

The activity of MAO-B was subsequently studied in the presence of increasing concentrations of inhibitors 1, 12 and 13 (Figure 4A). Thus, 5-nitroindazole 1 was the strongest inhibitor on the oxidation of MPTP, giving an IC_{50} of 0.99 ± 0.2 μM, followed by 6-nitroindazole 12 with IC_{50} of 2.52 ± 0.7 μM, whereas 7-nitroindazole 13 inhibited the oxidation of MPTP in a much lower degree reaching an IC_{50} of 27.8 ± 2.1 μM. A kinetic study on the inhibition by 5-nitroindazole 1 over human MAO-B showed that this nitroindazole is a competitive inhibitor (Figure 4B) of MAO-B with an apparent K_i of 0.102 μM. Preincubations of MAO-B protein with 1 and further determination of the activity compared with controls preincubated without this inhibitor showed that 1 was a reversible inhibitor since a high percentage (> 95%) of activity was recovered in the assays. By assuming that they are competitive inhibitors [51], IC_{50} values of 6-nitroindazole and 7-nitroindazole were used to estimate K_i values of 0.67 and 7.4 μM for 12 and 13, respectively. In order to evaluate the selectivity of nitroindazoles as MAO-B inhibitors, the inhibition over human MAO-A (kynuramine oxidation) was assessed. Nitroindazoles 1 and 13 weakly inhibited MAO-A (12 and 22% inhibition at 15 μM, respectively), whereas 6-nitroindazole 12 appeared to inhibit this isozyme with an IC_{50} of 7 μM. Therefore, among the nitroindazoles, 5-nitroindazole 1 appeared to be the most selective inhibitor of human MAO-B. On the other hand, separate experiments using kynuramine as substrate confirmed the inhibition of 1 and 12 on MAO-B with

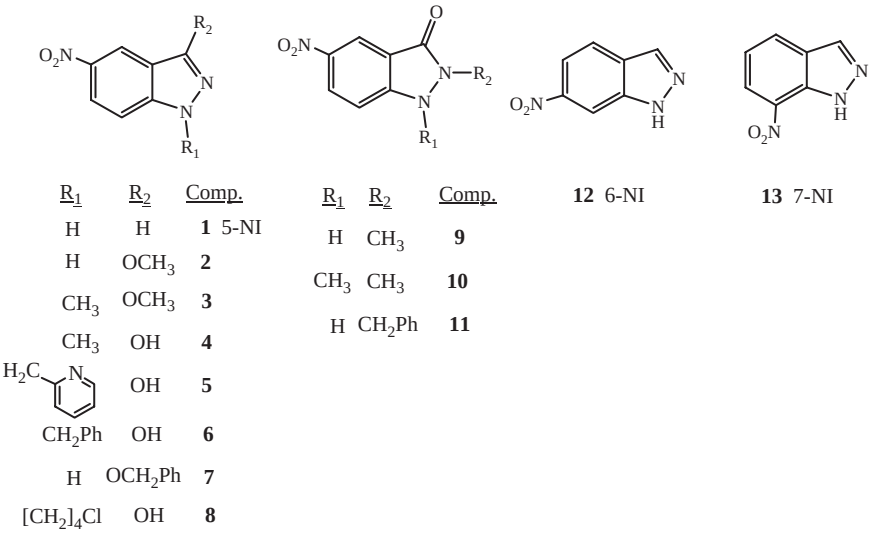

Figure 3. Synthesized 5-nitroindazole compounds and 6-nitro- and 7-nitroindazole positional isomers evaluated as inhibitors of MAO enzymes.

IC_{50} of 2.5 and 6.8 μM, respectively, whereas 13 was a weak inhibitor with IC_{50} higher than 50 μM.

Mitochondria membranes are involved in the metabolism of biogenic amines and neurotransmitters as well as in the oxidative bioactivation of MPTP neurotoxin. The inhibition of nitroindazoles on the oxidation of MPTP neurotoxin by human liver mitochondria which contain MAO enzymes in addition to other mitochondrial enzymes was subsequently investigated. Both 5-nitroindazole 1 and 6-nitroindazole 12 significantly inhibited the oxidation of MPTP to $MPDP^+$ and MPP^+ by human mitochondria at a low concentration of 5 μM, whereas 7-nitroindazole 13 was a weak inhibitor up to 15 μM of nitroindazole (Figure 5). The pattern of inhibition was generally similar for both metabolites $MPDP^+$ and MPP^+. At the same concentration (15 μM), the formation of $MPDP^+$ plus MPP^+ metabolites in the presence of 1 and 12 was up to 4.7- and 2.8-times lower, respectively, than that of 13. These results agree well with those obtained with human MAO-B and show that 5- and 6-nitroindazole inhibited MAO-B in mitochondria subcellular fractions and reduced the formation of neurotoxicants $MPDP^+$ and MPP^+ pyridinium cations produced from MPTP neurotoxin. When using selective inhibitors of MAO, formation of $MPDP^+$ and MPP^+ by mitochondria was highly inhibited (83%) by using deprenyl (1 μM), a selective inhibitor of MAO-B. In contrast, clorgyline, a selective inhibitor of MAO-A, was not able to significantly inhibit oxidation of MPTP (less than 7% inhibition at 1 μM). Then, MAO-B was the main isozyme involved in the oxidation of MPTP in human mitochondria and it was inhibited by 5-nitroindazole 1 and 6-nitroindazole 12.

Previous results have suggested that 7-nitroindazole 13 acts as a potent antioxidant and radical scavenger, likely contributing to its neuroprotective action [18]. In this research, the total antioxidant activity of 1, 12 and 13 was evaluated in the ABTS assay and none of these indazoles (0–20 μM in the

Table I. MAO-B inhibition on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) oxidation to $MPDP^+$ by a number of nitroindazoles (Figure 3), including the isomeric 5-nitro-, 6-nitro- and 7-nitroindazoles.

Nitroindazole	MAO-B inhibition (%) (nitroindazole, 15 μM)	IC_{50} (μM)	K_i (μM)
1	98.5	0.99±0.2	0.102
2	51.8	–	–
3	33.8	–	–
4	6.3	–	–
5	7.4	–	–
6	<3	–	–
7	8.8	–	–
8	<3	–	–
9	9.5	–	–
10	9.2	–	–
11	<3	–	–
12	89.1	2.52±0.7	0.67
13	35.0	27.8±2.1	7.4

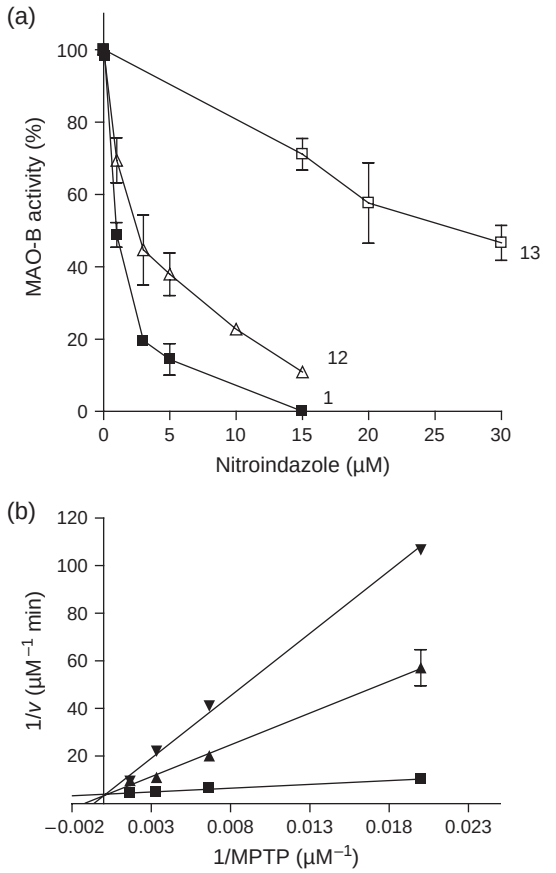

Figure 4. (A) MAO-B activity (%) as nmol of $MPDP^+$ produced/min mg of protein and inhibition in presence of 5-nitro- (■), 6-nitro- (△) and 7-nitroindazole (□). Average activity of controls in absence of inhibitor was 6.07 nmol $MPDP^+$/min mg prot. MPP^+ was inhibited following the same pattern as $MPDP^+$; (B) Lineweaver-Burk plot of MAO-B activity: control (■) and in presence of 5-nitroindazole (▲, 0.7 μM and ▼, 1.5 μM) as an inhibitor.

assay) exhibited significant antioxidant activity of elimination of $ABTS^{\bullet+}$ when compared to Trolox or ascorbic acid. Subsequently, they were evaluated as hydroxyl radical ($OH^\bullet$) scavengers in a Fenton system (Fe^{2+}, H_2O_2). Nitroindazoles 1, 12 and 13 inhibited the hydroxylation of benzoate competitively and were good scavengers of $OH^\bullet$ (Figure 6), as measured by the reduction of benzoate hydroxylation to 4-OH-benzoate and 3-OH-benzoate determined by HPLC in the presence of $OH^\bullet$ generated in the Fenton reaction [50]. In the reaction, OH-indazoles were detected as the main reaction products, indicating that nitroindazole compounds were direct scavengers and reacted with $OH^\bullet$. The calculated IC_{50} values (concentration of nitroindazole inhibiting benzoate hydroxylation by 50%) were 41.4, 39.1 and 42.9 μM for nitroindazoles 1, 12 and 13, respectively. The rate constants were calculated by competition kinetics [52] by using the rate constant of benzoate with $OH^\bullet$ ($3.3 \times 10^9\ M^{-1}\ s^{-1}$) and were 1.26×10^{10}, 1.4×10^{10} and $1.4 \times 10^{10}\ M^{-1}\ s^{-1}$ for 1, 12 and 13, respectively. Then, these results showed that the three nitroindazoles were good hydroxyl radical scavengers and exhibited similar reaction rates.

Discussion

The results presented above have shown that isomeric 5-nitroindazole 1, 6-nitroindazole 12 and 7-nitroindazole 13 inhibited human MAO-B in a different degree, with 1 being the most selective and potent inhibitor. An attempt to improve the inhibitory potency of 1 by synthesizing a number of derivatives failed, although the compounds 2 and 3 gave appreciable inhibition. 5-Nitroindazoles 4–11 containing polar and/or large substituents were poor inhibitors of MAO-B over the oxidation of MPTP. Among simple nitroindazoles, only 12 slightly inhibited MAO-A. The primary role of MAO in biological systems lies in the metabolism of amines and the regulation of neurotransmitter levels and intracellular amine stores [2]. These biological implications are of pharmacological interest and MAO isozymes are targets for antidepressant (MAO-A inhibitors) and neuroprotectant (MAO-B inhibitors) drugs [2,4,21]. The oxidation of biogenic amines by MAO results in the production of potentially toxic hydrogen peroxide, oxygen radicals, ammonia and aldehydes that represent risk factors for cell oxidative injury [7,8]. In addition, MAO bioactivates toxic xenobiotic amines such as MPTP [10–12,28,53,54] (Figure 1). A convenient use of selective MAO-inhibiting drugs may result in biological protection against both oxidative stress and toxicants [2,7,8,53]. Indazole derivatives exhibit many biological and pharmacological activities, including enzyme inhibition [30,43] and 7-nitroindazole is a selective inhibitor of nNOS [31,32], being a neuroprotective agent against neurotoxicity of MPTP (MPP^+) in rodents and baboons [35,36]. At present, its mechanism of action is not known although it may be linked to nNOS inhibition [31–33] by decreasing $NO^\bullet$, peroxynitrite and neuronal oxidative stress. However, 7-nitroindazole is also an inhibitor of MAO and this action could account, at least in part, for its neuroprotective actions against MPTP [28,37]. Castagnoli et al. [28] and Boireau et al. [34] found up to a 55% decrease in the concentrations of MPP^+ neurotoxin in the striatum of mice treated with MPTP plus 7-nitroindazole. This effect was attributed to a reduced conversion of MPTP to $MPDP^+$/MPP^+ by MAO-B (see Figure 1). Inhibition of MAO-B by 7-nitroindazole produced similar qualitative effects to known MAO-B inhibitors such as pargyline and *R*-deprenyl and different from Nω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS [15,38,39,42,53]. Alternatively, 7-nitroindazole may

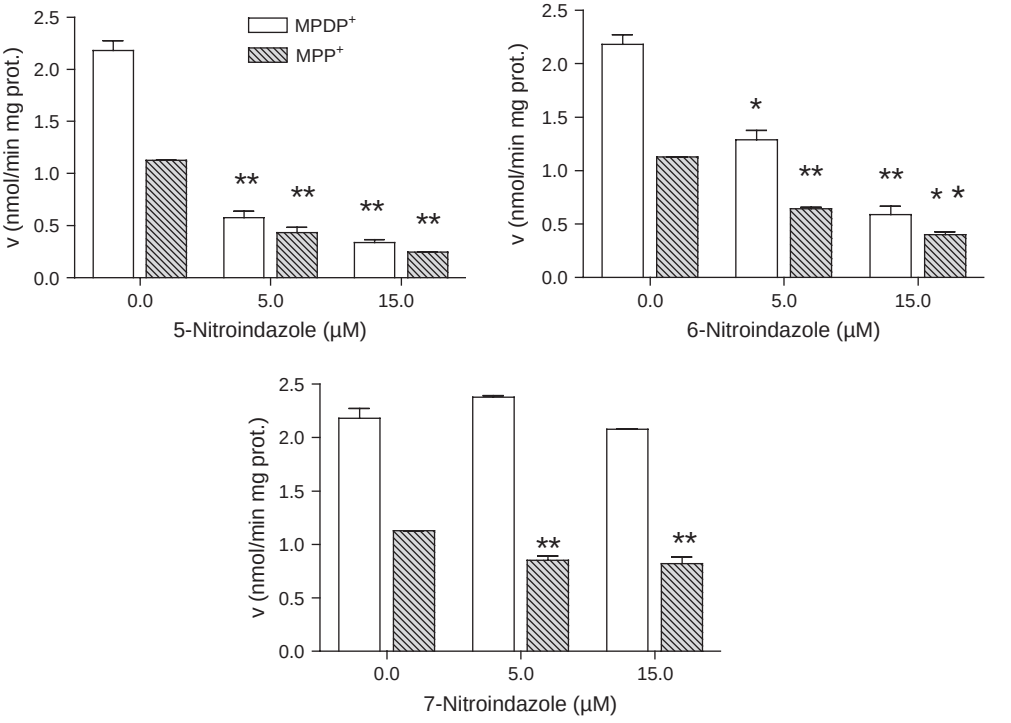

Figure 5. Oxidative activation of MPTP neurotoxin to $MPDP^+$ and MPP^+ pyridinium toxicants (nmol/min mg prot.) by human liver mitochondria in the absence (control) and in the presence of nitroindazoles (5, 15 μM). Results are significantly different from control (* $p < 0.05$; ** $p < 0.01$).

afford neuroprotection by mechanisms different from MAO inhibition, such as reduction of $NO^\bullet$ and antioxidant effects since it also protects against MPP^+ toxicity [18,19,55,56]. Further investigations are needed to clarify this matter.

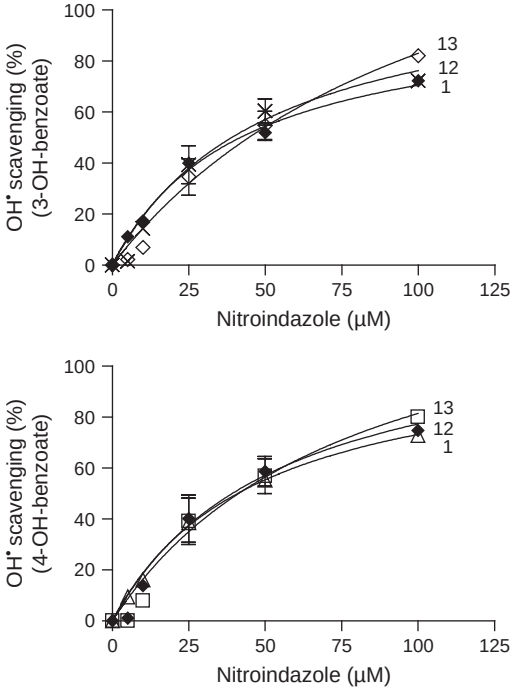

Figure 6. Activity as hydroxyl radical ($OH^\bullet$) scavengers of nitroindazoles measured as a percentage (%) of inhibition of the formation of 3-hydroxy- and 4-hydroxybenzoate from benzoate and the $OH^\bullet$ generated in the Fenton reaction ($Fe^{2+} + H_2O_2$) in the presence of increasing concentrations of 5-nitroindazole (1), 6-nitroindazole (12) and 7-nitroindazole (13).

Among nitroindazoles of Figure 3, only 5-nitroindazoles 1, 2 and 3 and the isomeric nitroindazoles 12 and 13, were able to inhibit MPTP oxidation by human MAO-B. Among them, 5-nitroindazole 1 followed by 6-nitroindazole 12 gave the highest inhibition and acted as a reversible and competitive inhibitor. The active site of human MAO-B is a flat cavity (420 $Å^3$) lined by a number of aromatic and aliphatic amino acids, providing a highly hydrophobic environment with an adjacent smaller hydrophobic 'entrance cavity' (290 $Å^3$) [57]. Then, it appears that hydrophobic nitroindazoles which are small and planar heterocycles would fit well within the active site of MAO-B and they showed higher inhibition. In contrast, the presence of polar (OH) and/or larger substituents decreased inhibition. The presence of nitro substituents in positions 5- or 6- of heterocyclic ring would favour better interactions since it produced a substantial increase of the inhibitory potency (e.g. 1, 2, 3, 12 vs 7-nitroindazole 13). Differences occur depending on the sources and tissues of MAO and substrates [28,58], but these results generally agree with others [28,29], although contradictory results regarding the lack or instead weak inhibition of MAO-B by 7-nitroindazole can be attributed to differences in animals, mitochondrial preparations or the substrates used as the affinity could be different [18,33].

The inhibitory potency on MAO-B and the reduction of MPTP oxidation by 5-nitroindazole, 6-nitroindazole and compound 2 were much higher than that of 7-nitroindazole. Although the neuroprotective effects of 7-nitroindazole are known, its mechanism of action remains unknown. It is still unclear whether it arises from nNOS inhibition, reduction of $NO^{\bullet}$ and oxidative stress or from MAO-inhibition and reduction of MPP^+ specie (ultimate neurotoxicant) or results from both mechanisms [28,34,35,37,59]. 5-Nitro-, 6-nitro and 7-nitroindazole appear to inhibit nNOS from bovine brain [31] in a reversible manner with IC_{50} values of 1.15 mM, 40 μM and 2.5 μM, respectively [32]. However, in contrast to 7-nitroindazole, the other isomeric nitroindazoles appear to lack inhibition of nNOS *in vivo* [43]. Compared to 7-nitroindazole, both 5-nitro and 6-nitroindazole are weak inhibitors of nNOS [32] but strong inhibitors of human MAO-B, with 6-nitroindazole showing inhibition on MAO-A. Then, it could be of further interest to evaluate a purported neuroprotection in the MPTP-mouse model of PD of 5-nitro and 6-nitroindazole vs 7-nitroindazole in order to shed new light on the relative role of MAO and nNOS. Indeed, if 7-nitroindazole exerts neuroprotection against MPTP mainly throughout MAO inhibition, the results obtained here suggest that compounds 2, 3 and particularly 5- and 6-nitroindazole (1 and 12) might become equally or perhaps even better neuroprotectants (at lower doses) than 7-nitroindazole 13 itself, provided that nitroindazoles follow similar absorption and metabolic patterns. These results could potentially lead to new agents with strengthened inhibition on MAO-B and neuroprotective actions.

Oxidative stress and oxygen radicals are involved in PD [9,60]. In the MPTP model of PD, MPTP crosses the blood–brain barrier and it is enzymatically oxidized by MAO-B in the glial cells with the formation of $MPDP^+$ toxin as well as H_2O_2 and oxygen radicals. $MPDP^+$ intermediate is chemically unstable and readily participates in redox reactions, being subsequently oxidized presumably by a non-enzymatic or another unknown process [12,54] to MPP^+, which is the ultimate toxin selectively uptaken by dopaminergic cells and accumulated in mitochondria (Figure 1). MPP^+ inhibits electron transport in mitochondria and generates oxidative stress through dopamine eflux and dopamine auto-oxidation as well as enzymatic oxidation by MAO [16,17]. These events involve the formation of cytotoxic oxygen radicals playing an important role in neurotoxicity and dopaminergic cell death. Biological actions of 7-nitroindazole 13 as neuroprotectant could arise from its ability to act as a potent radical scavenger of $OH^{\bullet}$ or to protect against iron-induced neurotoxicity [18,61]. The results obtained above showed that 13 was a good scavenger of hydroxyl radical ($OH^{\bullet}$) showing a reaction rate of the same order than other well-known $OH^{\bullet}$ scavengers such as DMSO or melatonin, an indole with good antioxidant and radical scavenging properties [48,62]. The three isomeric nitroindazoles 1, 12 and 13, gave similar IC_{50} and rate constants against $OH^{\bullet}$ generated in the Fenton reaction and did not exhibit antioxidant activity in the ABTS assay. In a previous study [18], 13 was an effective scavenger of $OH^{\bullet}$ produced *in vivo* by MPTP toxin in mice. Then, nitroindazoles 1 and 12 might work in a similar way against $OH^{\bullet}$ as they exhibit a similar reaction rate. However, $OH^{\bullet}$ also reacts with many organic compounds in good rates and $OH^{\bullet}$ formed in biological systems from catalytic metals and H_2O_2 rapidly reacts with biomolecules at the site of formation. Then, for an effective protection, scavengers should be present in very high concentrations to compete with biomolecules [49] or alternatively modify the antioxidant systems. On the other hand, it should not be ruled out that nitroindazoles could make oxygen radicals in other situations.

In conclusion, simple nitroindazoles 1, 12, 2, 13 and 3, by this order, showed good inhibitory properties on the oxidation (bioactivation) of MPTP neurotoxin to $MPDP^+$ (and MPP^+) by human MAO-B under a competitive and reversible type of inhibition. This inhibition was also produced for the oxidation of MPTP by human mitochondria. The same compounds were good $OH^{\bullet}$ scavengers but did not exhibit appreciable antioxidant activity in the ABTS assay. 7-Nitroindazole is a neuroprotectant agent and therapeutic strategies using this or related compounds may offer potential for the development of novel neuroprotective agents and therapies for PD [63]. In this regard, results in this research showed that isomeric 5- and 6-nitroindazoles offer differential singularities with respect to 7-nitroindazole owing to their relatively higher inhibitory potency on human MAO-B while exhibiting little effect on nNOS [43] and at the same time showing similar good radical scavenging properties. As they are good MAO-B inhibitors on MPTP oxidation, 5- and 6-nitroindazole might act as neuroprotectants against MPTP neurotoxin and this hypothesis may be worthy of testing in the mouse model of PD and also in other systems not involving MPTP. Additionally, these results could be useful to get new and most potent MAO-B inhibitors.

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