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Antioxidant properties of PF9601N, a novel MAO-B inhibitor: assessment of its ability to interact with reactive nitrogen species

Lydia Bellik[1], Stefania Dragoni[1], Federica Pessina[1], Elisenda Sanz[2], Mercedes Unzeta[2] and Massimo Valoti[1]✉

[1]Dipartimento di Neuroscienze, Università di Siena, Italy, [2]Departamento de Bioquimica i Biologia Molecular, Universitat Autonoma de Barcelona, Spain

The novel MAO-B inhibitor PF9601N, its cytochrome P450-dependent metabolite FA72 and l-deprenyl were studied as potential peroxynitrite ($ONOO^-$) scavengers and nitric oxide synthase (NOS) inhibitors. The scavenging activity of these compounds was evaluated by measuring the oxygen consumption through peroxynitrite-mediated oxidation of both linoleic acid and brain homogenate. FA72, PF9601N and L-deprenyl caused a concentration-dependent inhibition of $ONOO^-$-induced linoleic acid oxidation with an IC_{50} value of 60.2 μM, 82.8 μM and 235.8 μM, respectively. FA72 was the most potent also in inhibiting $ONOO^-$-induced brain homogenate oxidation with an IC_{50} value of 99.4 μM, while PF9601N and L-deprenyl resulted weaker inhibitors in the same experimental model, showing an IC_{50} value of 164.8 and 112.0 μM, respectively. Furthermore, both the novel MAO-B inhibitor as well as its metabolite were able to strongly inhibit rat brain neuronal NOS (IC_{50} of 183 μM and 192 μM, respectively), while L-deprenyl at the highest concentration used (3 mM), caused only a slight decrease of the enzyme activity. Moreover, inducible NOS was strongly inhibited by FA72 only. All these results suggest that PF9601N could be a promising therapeutic agent in neurodegenerative disorders such as Parkinson's disease.

Keywords: MAO-B inhibitors, L-deprenyl, Parkinson's disease, peroxynitrite, nitric oxide

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INTRODUCTION

Oxidative stress is thought to be an important contributor to neuronal damage in neurodegenerative disorders. Different cellular components such as lipids, proteins, DNA undergo oxidative reactions; moreover, the resulting degenerative products may react with other biological macromolecules to impair cell viability and cause the loss of neurons in different brain areas. In particular, loss of the dopaminergic neurons of *substantia nigra pars compacta* may lead to the Parkinson's disease (PD). Different studies have demonstrated that oxidative stress contributes to the cascade leading to dopamine cell degeneration and is intimately linked to other components of the degenerative process, such as mitochondrial dysfunction, excitotoxicity, inflammation and nitric oxide toxicity (Schapira *et al.*, 1990; Bolaños *et al.*, 1997; Rodriguez *et al.*, 1998).

NO is a free radical species produced by different mammalian cell types and is involved in several physiological and pathological conditions. There is, indeed, considerable evidence showing that oxidative stress and oxidative damage occurring in PD might result from several actions of NO. The toxicity of NO would be mediated by reactive nitrogen species and also by the reaction of NO with superoxide to form $ONOO^-$, which not only is an oxidizing agent on its own but degrades to form hydroxyl radicals, among other radical species (Beckman *et al.*, 1990). The *substantia nigra* in PD showed increased immunoreactivity for inducible nitric oxide synthase (iNOS), presumably as a result of glial cell activation (Hunot *et al.,* 1996). Increased protein damage resulting from attack by $ONOO^-$ is found in the core of Lewy bodies as measured by 3-nitrotyrosine immunoreactivity (Duda *et al.,* 2000). Indirect evidence similarly implicates NO in the mechanisms underlying nigral cell degeneration as NOS inhibitors protect against MPTP/MPP^+-induced experimental PD in mice and monkeys (Schulz *et al.,* 1995; Hantraye *et al.*, 1996). This presumably relates to the effect of MPP^+ on mitochondrial function to initiate excitotoxic mechanisms and activate NO formation. Mice deficient for either neuronal NOS (nNOS) or iNOS (Przedborski *et al.,* 1996; Liberatore *et al.,* 1999) show reduced toxicity to MPTP. The toxic effects of NO/$ONOO^-$ can also involve damage to DNA leading to products such as 8-hydroxyguanine and 8-nitrodeoxyguanosine and increase in DNA single-strand breakage (Byun *et al.*, 1999).

In this context, the development of new molecules able to reduce the oxidative stress, thereby slowing the rate of neuronal degeneration, appears to be necessary. A new series of acetylenic and allenic derivatives of tryptamine synthesised as MAO-B inhibitors (MAOIs) have been described (Avila *et al.,* 1993; Perez *et al.*, 1999). Among these, the 5-benzyloxy derivatives have shown themselves to be highly potent and selective MAO-B inhibitors (Perez *et al.*, 1999), and *N*-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine (PF9601N) was shown

✉e-mail: valoti@unisi.it

Abbreviations: DTPA, diethylenetriaminepentaacetic acid; ER, endoplasmic reticulum; MAO-B, monoamine oxidase B; MAOIs, MAO-B inhibitors; MPP, 1-methyl-4-phenylpyrinidinum; MPTP, 1-methyl-4-phenyl-1,2,3,6-tertrahydropyridine; NOS, nitric oxide synthase; 6-OH DA, 6-hydroxydopamine; PD, Parkinson's disease.

to have a neuroprotective effect in several experimental models of PD. PF9601N protects nigrostriatal dopamine neurons against MPTP neurotoxicity in C57BL/6 adult mice (Perez *et al.,* 2003) and protects rat nigral neurons after 6-hydroxydopamine striatal lesion (Cutillas *et al.,* 2002). It has also been demonstrated that PF9601N enhances the duration of L-DOPA-induced contralateral turning of 6-OH DA-lesioned rats (Prat *et al.*, 2000) and prevents cell death induced by complex I inhibition and ER stress, two of the suggested underlying mechanisms of neurodegeneration in PD (Sanz *et al.,* 2008; 2009). Finally, antioxidant properties of PF9601N have been demonstrated *in vitro* and by its neuroprotective effect observed in SHSY5Y dopaminergic cells damaged with dopamine (Sanz *et al.,* 2004).

On the base of the above observations, the aim of this study was to investigate the protective effects of PF9601N and its cytochrome P450-dependent metabolite FA-72 (Dragoni *et al.*, 2007) on NO system, both by measuring their effects on $ONOO^-$-mediated oxidation and the influence on NOS activity. The same experiments were performed with the MAO-B inhibitor L-deprenyl, used with L-DOPA in the therapy of PD (Knoll, 1986).

MATERIALS AND METHODS

Chemicals. Linoleic acid and human recombinant nitric oxide synthase were obtained from Sigma (St. Louis, MO, USA). *N*-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine or PF9601N, and 5-benzyloxy-indol)methylamine or FA72 were synthesized in our laboratory. L-(-)-*N*,α-dimethyl-*N*-2-propynylphenethyl-amine hydrochloride (L-deprenyl-HCl) was a gift from Chinoin Chemical Works (Budapest, Hungary). All other chemicals and solvents were of the highest grade available and were obtained from common commercial sources.

Peroxynitrite synthesis. Peroxynitrite was synthesized in a quenched flow reactor and stored in 1.5 M NaOH at –70 °C, as previously described by Beckmann *et al.* (1994). Its concentration was determined spectrophotometrically by measuring absorbance at 302 nm ($\varepsilon = 1670\ M^{-1} \cdot cm^{-1}$) as described by Kooy *et al.* (1997).

Effect of MAO inhibitors on peroxynitrite-mediated oxidation of linoleic acid and brain homogenate. Peroxynitrite scavenging activity was assayed by following $ONOO^-$-promoted oxidation of linoleic acid through oxygen consumption measured with a Clark oxygen electrode (Patel & Darley-Usmar, 1996). The electrode chamber was filled with 33 mM linoleic acid in 50 mM phosphate buffer, pH 7.4, containing 100 μM DTPA, with variable concentrations (10–1000 μM) of MAO-B inhibitors (MAOIs) or FA72 in 3 ml final volume, and oxygen consumption was measured at 37 °C for 3 min. The reaction was started by adding 500 μM peroxynitrite to the assay mixture and oxygen consumption was monitored for 10 min.

In a second series of experiments linoleic acid was replaced by brain homogenate at a final protein concentration of 1 mg/ml. All experiments were performed in triplicate and controls were obtained by measuring linoleic acid or brain homogenate oxidation in the presence of $ONOO^-$ alone. All solutions were made using high purity deionized water.

Spectrum changes of MAO inhibitors in the presence of peroxynitrite. The reaction of the tested compounds and $ONOO^-$ was followed spectrophotometrically at 500–220 nm with a double beam UV-visible spectrophotometer (Shimadzu UV-1601). The reaction mixture contained 50 mM phosphate buffer, pH 7.4, 100 μM MAOIs and 100 μM $ONOO^-$ added under vigorous stirring. The spectra were recorded every 60 s. A blank cuvette contained reaction mixture without $ONOO^-$.

nNOS preparations. Rats were fasted overnight prior to the sacrifice by CO_2 asphyxia. Brains were quickly removed and freed from cerebral vessels. Each sample was processed individually. The tissues were chopped and suspended in five volumes of 20 mM Hepes buffer, pH 7.2 containing 320 mM sucrose, 1 mM EDTA, 1 mM DTT, 10 μg/ml leupeptine, 10 μg/ml trypsine soybean inhibitor, 2 μg/ml aprotinine and 0.1 mM phenylmethylsulfonyl fluoride, and homogenized as reported by Knowles and Salter (1998). The homogenate was centrifuged at 120 000 × *g* for 30 min at 4 °C (Chen *et al.,* 1997) and supernatant was batch-treated with Dowex 50w to remove the endogenous arginine.

Nitric oxide production was measured using a spectrophotometric method by following the oxidation of oxyhemoglobin to methemoglobin at 37 °C in a reaction medium containing 50 mM phosphate buffer, pH 7.4, 250 μM $CaCl_2$, 100 μM NADPH, 10 μM dithiothreitol, 1.0 mg submitochondrial protein/ml and 25 μM oxyhemoglobin (expressed per heme group). The reaction was started by adding 20 μM L-arginine. Kinetics were followed at λ 401–421 nm ($\varepsilon = 77200\ M^{-1} \cdot cm^{-1}$). The same assay was used to study the effects of the tested compounds on iNOS.

RESULTS

Scavenger properties of MAOIs

To study the protective effect of MAOIs on peroxynitrite ($ONOO^-$)-induced lipid peroxidation brain homogenates or linoleic acid were treated with $ONOO^-$ in combination with different concentrations of PF9601N, FA72 and L-deprenyl. The reaction of peroxynitrite with linoleic acid is rather complex and leads to the formation of oxidised and nitrated products (O'Donnel *et al.,* 1999) that contribute to oxygen consumption. In a similar manner $^{\bullet}NO$-derived reactive species (e.g., peroxynitrite anion, nitrogen dioxide radical) react with lipids containing unsaturated fatty acids to generate nitrated species and oxygenated species (Lima *et al.,* 2003).

Preliminary experiments showed that the oxygen consumption in the presence of either linoleic acid or brain homogenate was linear in respect to the concentration of $ONOO^-$ in the range of 50–500 μM; 500 μM was the concentration that gave the highest oxidation rate.

As shown in Fig. 1, when the studied compounds were present in the incubation medium, a concentration-dependent inhibition of oxygen consumption was observed for both linoleic acid and brain homogenate. PF9601N and its cytochrome P450 (CYP)-dependent metabolite FA72 showed the highest inhibitory effects on the $ONOO^-$-induced linoleic acid oxidation (Fig. 1) with the IC_{50} values of 82.8 ± 2.0 μM and 60 ± 1.2 μM, respectively, values about 3-fold lower than that observed for L-deprenyl (235.8 ± 6.0 μM). When the experiments were performed using brain homogenate the observed antioxidant activity of L-deprenyl was poorer, with the IC_{50} value one order of magnitude higher than those for PF9601N and FA72 (see Table 1).

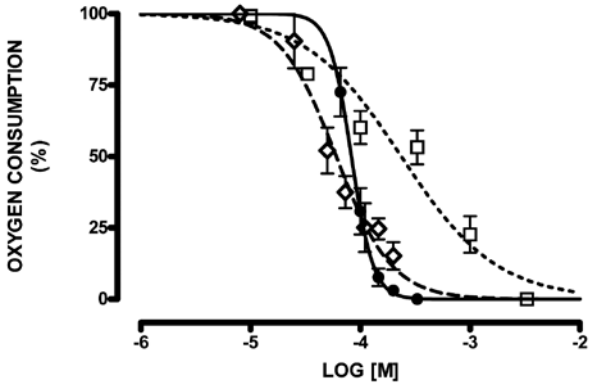

Figure 1. Effects of MAOIs on linoleic acid oxidation promoted by ONOO⁻.
Inhibition of oxygen consumption in presence of 33 mM linoleic acid at different concentrations (10^{-5} M–3.0×10^{-3} M) of (□) L-deprenyl, (●) PF9601N, or (◇) FA72 in buffer at pH 7.4. Reaction was started by adding 500 μM ONOO⁻ to assay mixture. Data are reported as percentage of control value. Data are means ± SEM of three independent experiments.

In order to clarify if the compounds were chain-reaction breakers, the compounds were added to the reaction mixture containing linoleic acid 3 min after ONOO⁻ addition. In these experiments, all the compounds showed weaker antioxidant properties, and at the highest concentrations used PF9601N and FA72 (1 mM) and L-deprenyl (3 mM) only showed a non-significant inhibitory effect (not shown).

UV-Visible spectrophotometric analysis

The reaction of MAOI compounds and FA72 with 100 μM ONOO⁻ was monitored by UV-visible analysis. When 100 μM PF9601N, FA72 or L-deprenyl were incubated in phosphate buffer, pH 7.4, (control conditions), no spectral changes were recorded during the 30 min period of observation. However, when the spectra were recorded after the addition of 100 μM ONOO⁻, a time-dependent change of the spectra was observed for all compounds studied. In particular, an increase in the absorbance between 350 nm and 280 nm and a decrease in the absorbance at λ 250–230 nm was observed.

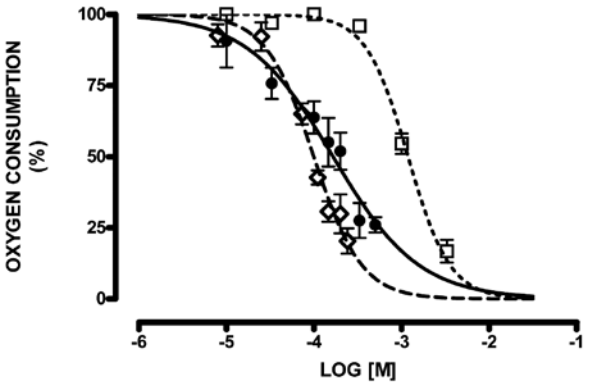

Figure 2. Effects of MAOIs on brain homogenate oxidation promoted by ONOO⁻.
Inhibition of oxygen consumption in presence of 1 mg/ml brain homogenate at different concentrations (10^{-5} M – 3.0×10^{-3} M) of (□) L-deprenyl, (●) PF9601N, or (◇) FA72 in buffer at pH 7.4. Reaction was started by adding 500 μM ONOO⁻ to assay mixture. Data are reported as percentage of control value. Data are means ± SEM of % of three independent experiments.

Effect of MAOIs on NOS activity

The *in vitro* inhibitory properties of the three MAOIs were examined on constitutive rat brain NOS. Enzyme preparations showed a K_m towards arginine and a V_{max} ($K_m = 1562\pm0.2$ μM V_{max} 0.34 ± 0.01 nmol/min per mg protein) similar to those reported by other authors. In our experimental conditions the NO formation rate was linear up to 15 min of the reaction. In those conditions both PF9601N and its metabolite FA-72 caused a concentration-dependent inhibition of nNOS with similar IC_{50} values of 183 μM and 192 μM, respectively. L-Deprenyl was less active, producing only approx. 20% inhibition at the highest concentration tested of 1 mM (Fig. 3).

L-Deprenyl presented a similar behaviour also when incubated in the presence of iNOS. It should be observed, however, that only FA72 was able to inhibit iNOS with an IC_{50} similar to that for nNOS, while its parent compound caused only a weak inhibition (see Table 1).

DISCUSSION

In this study it was demonstrated that all the compounds tested were able to inhibit peroxynitrite-mediated oxidation of both linoleic acid and brain homogenate, although with a different potency. Furthermore, this property seems to be related to a direct reaction with peroxynitrite more than to the free radical scavenging property. In fact when the compounds were added to the assay mixture 3 min after NOO⁻ addition, a time during which the linoleic acid oxidation rate increased and all NO disappeared, only a weak inhibition of oxygen consumption was detected. Moreover, the direct interaction of MAO inhibitors with NOO⁻ was confirmed by the changes of the UV-visible spectral properties occurring when

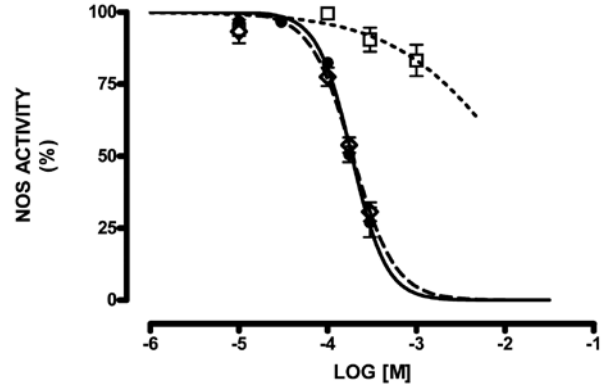

Figure 3. Concentration-dependent inhibition of brain nitric oxide synthase by compounds tested.
Partially purified rat brain nNOS (1 mg/ml proteins) was incubated in presence of different concentrations (10^{-5} M – 10^{-3} M) of (□) L-deprenyl, (●) PF9601N or (◇) FA72 in phosphate buffer, pH 7.4, containing 250 μM $CaCl_2$, 10 μM DTT and 100 μM NADPH. The reaction was started by adding 20 μM arginine to assay solution. NO production was followed spectrophotometrically by measuring oxidation of haemoglobin to methemoglobin at λ 401–421 nm. Data are reported as percentage of control value and are means ± SEM of at least four independent experiments.

Table 1. Effects of tested compounds on NO system

Compounds	Peroxynitrite inhibition		NOS inhibition	
	Linoleic acid oxidation IC_{50} (μM)	Brain homogenate oxidation IC_{50} (μM)	neuronal NOS IC_{50} (μM)	inducible NOS IC_{50} (μM)
PF9601N	82.8±2.0	164.8±1.8	187±1.1	≥10^3 (20%)*
FA72	60.2±1.2	99.4±1.5	192±1.0	274±3.8
L-Deprenyl	235.8±6.0	1120±5.2	≥10^3 (20%)*	≥10^3 (20%)*

*In brackets is reported the percentage of inhibition at compounds concentration ≥ 10^3 μM

the compounds were incubated in the presence of $ONOO^-$ alone; those changes indicated the formation of new compounds. In a previous study we demonstrated that an arylpropargylamine derivative, structurally related to MAO inhibitors, reacted with peroxynitrite to form a nitramine derivative which was rapidly deaminated to the corresponding 1-phenylpropargyl alcohol (Dragoni *et al.*, 2007). A similar chemical behaviour was observed for guanosine which is modified to 8-hydroxydeoxyguanosine in the presence of peroxynitrite (Inoue & Kawanishi, 1995). Similar reactions could be hypothesized also for FA72, PF9601N and L-deprenyl; in fact, the nitrogen present in the propargylamine and/or the indole moiety of PF9601N and its metabolite could react with NOO^-. The presence of two reactive nitrogen atoms in PF9601N and FA72 could explain their higher potency to react with peroxynitrite as compared to that observed for L-deprenyl. Further studies are needed to identify the pathways of the reactions and their products.

In a similar manner the indole derivatives PF9601N and FA72 were more efficient than L-deprenyl in inhibiting the rat brain NOS. PF9601N and FA72 showed a similar IC_{50} value, confirming that the indole ring plays an important role in the reactivity of the molecule. Furthermore, the presence of indole was also important for iNOS inhibition. However, the presence of the propargyl moiety decreased the inhibitory effect as observed for L-deprenyl and PF9601N.

Many pieces of indirect evidence such as the pivotal role of NOS in the neurotoxicity of MPTP, the higher resistance to MPTP neurotoxicity in nNOS-deficient mice, the overexpression of nNOS in basal ganglia and in the circulating neutrophils of PD patients as well as the increase of protein tyrosine nitration (for review see Zhou & Zhu, 2009) support the role of NO in the pathogenesis of PD lesions. In this context the evidence that PF9601N had neuroprotective effect towards MPTP toxicity at a lower concentration than L-deprenyl had, in both *in vivo* and *in vitro* experimental models (Perez *et al.,* 1996; Perez & Unzeta, 2003), could be ascribed not only to its more effective MAO-B inhibition and its antiapoptotic activity (Sanz, 2008) but also to its interaction with NOS and NOO. Although this effect is observed at a higher concentration than is MAO-B inhibition, it is noticeable that also the CYP-dependent metabolite FA72 is able to interact with the NO system. These observations and the fact that the CYP-dependent metabolism is active also in the brain (Dragoni *et al.,* 2003; Marini *et al.,* 2007) might account for PF9601N being a better neuroprotective compound than L-deprenyl.

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