Mitochondria-targeted antioxidant effects of S(-) and R(+) pramipexole

Giulia Ferrari-Toninelli1, Giuseppina Maccarinelli1, Daniela Uberti1, Erich Buerger2, Maurizio Memo1*

Abstract

Background: Pramipexole exists as two isomers. The S(-) enantiomer is a potent D3/D2 receptor agonist and is extensively used in the management of PD. In contrast, the R(+) enantiomer is virtually devoid of any of the DA agonist effects. Very limited studies are available to characterize the pharmacological spectrum of the R(+) enantiomer of pramipexole.

Results: Using differentiated SH-SY5Y neuroblastoma cells as an experimental model, here we show that S(-) and R(+) pramipexole are endowed with equipotent efficacy in preventing cell death induced by H2O2 and inhibiting mitochondrial reactive oxygen species generation. Both pramipexole enantiomers prevented mitochondrial ROS generation with a potency about ten times higher than that elicited for neuroprotection.

Conclusions: These results support the concept of both S(-) and R(+) pramipexole enantiomers as mitochondria-targeted antioxidants and suggest that the antioxidant, neuroprotective activity of these drugs is independent of both the chiral 6-propylamino group in the pramipexole molecule and the DA receptor stimulation.

Background

Parkinson’s disease (PD) is the most common neurodegenerative movement disorder. The primary cause of the disorder is the progressive loss of the pigmented dopaminergic neurons in the substantia nigra pars compacta (SNpc) accompanied by the appearance of intracytoplasmic inclusions known as Lewy bodies [1-3].

To date, the etiopathogenesis of nigral dopaminergic neuron loss in PD is unknown. However, the presence of ongoing oxidative stress as the result of compromised antioxidant defence mechanisms and generation of radical oxygen species (ROS) in the SNpc of the parkinsonian brain are considered to be important Pathogenic mechanisms [3,4]. ROS can react with cellular macromolecules through oxidation and cause the cells to undergo dysfunction and eventually lead to necrosis or apoptosis. The control of the redox environment of the cell provides an additional regulation in the signal transduction pathways which are redox sensitive. Therefore, an effective anti-parkinsonian therapy should not only alleviate the disease associated symptoms, but should also stop the progressive dopaminergic cell death in the SN.

Modification of the rate of PD progression is currently a highly debated topic. Increased oxidative stress is indeed thought to be involved in the nigral cell death which is a well established peculiar neuropathological feature of PD. These mechanisms have been proven in vitro and in animal models, but their relevance in humans remains speculative [5,6]. However, several dopamine (DA) agonists of the DA D2-receptor family (including D2 and D3 subtypes) have recently been shown to possess neuroprotective properties in different in vitro and in vivo experimental PD models [7,8]. At cellular level, independent groups have demonstrated decreased free radical production and an amelioration of DA neuronal loss following DA agonist treatment [9-17]. Interestingly, not all the neuroprotective effects were clearly mediated by DA-receptor stimulation.

Pramipexole (2-amino-4,5,6,7-tetrahydro-6-propylaminobenzathiazole) is a non-ergot DA agonist that has been successfully applied to the treatment of Parkinson’s disease. Pramipexole exhibits a high affinity for the D2 and D3 DA receptor subtypes but little or no affinity for the D1 receptor family. The neuroprotective effects elicited by this drug have directly and/or indirectly been
associated with antioxidant effects, mitochondrial stabilization or induction of the antiapoptotic Bcl-2 family [18-21]. In particular, Le et al., [18] reported that pramipexole protected DAergic MES 23.5 cell line against DA, 6-OH-DA and hydrogen peroxide (H$_2$O$_2$)-induced cytotoxicity possibly through antioxidant effects, and such neuroprotection was independent from DA receptor stimulation not being prevented by selective D$_2$ or D$_3$ antagonists. Similar results were obtained by Fujita et al., [20] and Uberti et al. [22], who demonstrated that pramipexole inhibited generation of H$_2$O$_2$-induced reactive oxygen species in PC12 cells and SH-SY5Y neuroblastoma cells, respectively, in a DA receptor independent way. Recent data also demonstrated neuroprotection by pramipexole against β-amyloid ROS generation and toxicity [19,22].

Pramipexole exists as two isomers. The S(-) enantiomer is a potent D$_2$/D$_3$ receptor agonist and is extensively used in the management of PD. In contrast, the R(+) enantiomer is virtually devoid of any of the DA agonist effects. Very limited studies are available to characterize the pharmacological spectrum of the R(+) enantiomer of pramipexole [19,22-27].

Here we show that S(-) and R(+) pramipexole are endowed with equipotent efficacy in preventing cell death induced by H$_2$O$_2$ and act as mitochondria-targeted antioxidants.

Results

Neuroprotection against H$_2$O$_2$

SH-SY5Y neuroblastoma cell lines were differentiated with 10 μM all-trans retinoic acid for 1 week to acquire a neuronal phenotype. Cells were then challenged with 1 mM H$_2$O$_2$ for 5 min then cells returned to fresh medium for additional 24 h. H$_2$O$_2$ caused a reduction in cell viability of about 70% in comparison with untreated control cells (figure 1). As shown in figure 1A, treatment of the cells with increasing concentrations of S(-) or R(+) pramipexole dose-dependently prevented the viability impairment induced by H$_2$O$_2$. The tested drugs showed equipotent efficacy with calculated IC$_{50}$ values of 8.8 ± 0.9 μM and 9.2 ± 0.6 μM for S(-) and R(+) pramipexole enantiomer, respectively. The neuroprotective effects of both pramipexole enantiomers were not prevented by preincubation of the cells with 10 μM phenoxybenzamine (data not shown), 10 μM haloperidol or 10 μM (-) sulpiride (Figure 1B). Haloperidol and sulpiride treatments did not induce cell viability modifications (data not shown).

Inhibition of mitochondrial ROS generation

The effects of pramipexole and its R(+) enantiomer have been studied in an experimental model of mitochondrial ROS generation by video-rate confocal microscopy in living neuronal cells. This model allows detection of ROS levels specifically generated in mitochondria and is based on the CM-DCF formation after laser light stimulation [28-30]. Figure 2A, upper panel, shows the results from a representative experiment. Mitochondrial ROS generation was evaluated in cells after increasing exposure to laser at different intensity. In a parallel experiment (lower panels), cells were preincubated with Vitamin E (2 μl/100 ml) for 30 min before the laser excitation. Fluorescence emission intensity was calculated as average grey level value per pixel and corrected for background. Data are reported in the graph reported in Figure 2B. The results clearly show that Vitamin E prevented laser-induced ROS generation in mitochondria of differentiated SH-SY5Y neuronal cells.

Using the same experimental paradigm, we tested the effects of different concentrations of S(-) and R(+) pramipexole in laser-induced mitochondria ROS generation. As shown in figure 3, both S(-) and R(+) pramipexole dose-dependently prevented laser-induced ROS generation in mitochondria of differentiated SH-SY5Y neuronal cells. When calculated as inhibition of ROS generation after 9% laser intensity, both drugs showed similar potency with IC$_{50}$ values of 0.91 ± 0.14 μM and 0.85 ± 0.21 μM for S(-) and R(+) pramipexole enantiomer, respectively. The prevention of mitochondrial ROS generation induced by both pramipexole enantiomers was not affected by preincubation of the cells with 10 μM phenoxybenzamine (data not shown), 10 μM haloperidol or 10 μM (-) sulpiride (Figure 4). Haloperidol and sulpiride treatments did not modify laser-induced increase in ROS production (data not shown).

Discussion

Pramipexole exists as two stereoisomers. The S(-) enantiomer is a potent D$_2$/D$_3$ receptor agonist and is extensively used in the management of PD. In contrast, the R(+) enantiomer is virtually devoid of any of the DA agonist effects. A growing number of experimental data indicate an antioxidant property of pramipexole enantiomers, evidenced by equal antioxidant efficacy toward H$_2$O$_2$ and nitric oxide [24] and equipotent efficacy in preventing viability impairment induced by H$_2$O$_2$ and mitochondrial ROS generation (present results). We found S(-) and R(+) pramipexole enantiomers relatively weak H$_2$O$_2$ scavengers, with apparent IC$_{50}$ values in the low micromolar range. Our data are consistent with previous data showing neuroprotection elicited by the S(-) and R(+) enantiomers against different neurotoxic agents [9,22-27]. In our study, S(-) and R(+) pramipexole enantiomer showed equipotent efficacy suggesting that the neuroprotective effects against H$_2$O$_2$ in differentiated SH-SY5Y neuroblastoma cells were DA receptor independent.
Although this study did not examine the precise site of action of pramipexole, several findings implicate the permeability transition pore (PTP) as a possible target of this drug [19,24,25]. Apart from binding to DA receptors, pramipexole has in fact been shown to enter and accumulate in mitochondria driven by the mitochondrial membrane potential [24]. Targeting to mitochondria has also been recently demonstrated by patch clamp studies showing inhibition of PTP by pramipexole [25]. PTP inhibition by pramipexole was further supported by experimental data obtained in functional intact mitochondria showing that this drug abolished Ca++-triggered swelling [25]. By video-rate confocal microscopy in living neuronal cells, we found that both S(-) and R (+) pramipexole enantiomers prevented laser-induced ROS generation in mitochondria of differentiated SH-SY5Y neuronal cells. Interestingly, both pramipexole enantiomers prevented mitochondrial ROS generation with a potency about ten times higher than that elicited for neuroprotection.

The apparent discrepancy between the different potencies of pramipexole enantiomers in preventing mitochondrial ROS generation (about 0.9 μM) and inhibiting H$_2$O$_2$-triggered viability impairment (about 8 μM) may be related to the different experimental models. In fact, H$_2$O$_2$ itself is not a radical but reacts with iron to form hydroxyl radicals, the most reactive oxygen species. Thus, in our experimental paradigm, ROS are generated both intra- and extracellularly and cause apoptosis by the induction of several intracellular converging...
pathways involving lipid peroxidation, protein oxidation and DNA damage. We hypothesize that accumulation of promipexole enantiomers in the mitochondria [23,24] may limit their scavenger properties to specific subcellular compartments. Although accumulation of pramipexole into mitochondria has not been definitely established, the high potency of these drugs in preventing mitochondrial ROS generation strongly suggest the mitochondria as their primary site of action.

Conclusions
These results support the concept of both S(-) and R(+) pramipexole enantiomers as mitochondria-targeted antioxidants and suggest that the antioxidant, neuroprotective activity of these drugs is independent of both the chiral 6-propylamino group in the pramipexole molecule and the DA receptor stimulation.

Methods
Cell culture
The human neuroblastoma cell line SH-SY5Y was routinely cultured in Ham’s F12 and Dulbecco modified Eagle’s medium (DMEM) in a ratio of 1:1, supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 50 μg/ml penicillin, and 100 μg/ml streptomycin and kept at 37°C in humidified 5% CO₂/95% atmosphere. For differentiation, cultured cells were treated for one week with 10 μM all trans retinoic acid. To obtain reproducible results, SH-SY5Y cells ranging from passage 18 to passage 25 were used for all the experiments.

Drug treatment
S(-) and R(+) pramipexole were dissolved in water and added to the culture media 1 h before H₂O₂ pulse or laser light stimulation. IC₅₀ values for S(-) and R(+) pramipexole enantiomer were calculated using at least 5 data points. Haloperidol and sulpiride were added to the culture media 1 h before pramipexole. Vitamin E (2 ng/100 μl) was added to the culture media 30 min before the laser excitation. All drugs were from Sigma. S(-) and R(+) pramipexole were kindly supplied by Boehringer Ingelheim GmbH, Germany.

Evaluation of cell viability
Cell viability was measured by quantitative colorimetric assay with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), showing the mitochondrial activity of living cells. Differentiated SH-SY5Y neuronal
cells in 96-well plates were challenged with H$_2$O$_2$ for 5 min, then 500 µg/ml MTT was added in each well and cells were incubated at 37°C for additional 3 h. MTT was removed, and cells were lysed with dimethyl sulfoxide. The absorbance at 595 nm was measured using a Bio-Rad 3350 microplate reader. Control cells were treated in the same way without H$_2$O$_2$, and the value of different absorbances were expressed as a percentage of control. All the drugs and reagents concentrations are to be considered as final concentrations.

Reactive oxygen species detection

SH-SY5Y neuroblastoma cells were first differentiated in neuronal-like phenotype by treatment with retinoic acid for 7 days; then, according to Koopman et al. [28], cells were loaded with 5-chloromethyl-2',7'-dichlorodihydrofluorescein (CM-H$_2$DCF) and its oxidative conversion into CM-DCF was monitored by video-rate confocal microscopy and real-time image averaging after increasing laser intensities. For mitochondria localization, cells were also loaded with Mitotracker Deep Red. Cells were then excited at increasing laser intensity and CM-DCF fluorescence intensity was selectively recorded in mitochondria. Fluorescence emission intensity was calculated as average grey level value per pixel and corrected for background.

Statistical evaluation

Results were given as mean ± standard error mean values. Statistical significance of differences was determined by one way ANOVA, followed by Bonferroni’s multiple comparison test as post-hoc analysis. A probability of less than 0.05 was considered as a significant difference.

Acknowledgements

This research was supported in part by a grant from Boehringer Igelheim.
Author details
1 Department of Biomedical Sciences and Biotechnologies and National Institute of Neuroscience, University of Brescia, Brescia, Italy. 2 Department of Central Nervous System Research, Boehringer-Ingelheim Pharma, Biberach an der Riss, Germany.

Authors’ contributions
GFT, GM and DU conducted and managed the study and analyzed the imaging data, WM drafted the manuscript. EB and MM participated in the design and coordination of the project. All authors cooperatively designed the project and discussed data interpretation. All authors participated in critical editing of the manuscript.

Received: 16 April 2009
Accepted: 5 February 2010
Published: 5 February 2010

References
1. Davie CA: A review of Parkinson’s disease. Br Med Bull 2008, 86:109-127.
2. McKee IG, Mosmann UP: Dementia with Lewy bodies and Parkinson’s disease. Parkinsonism and Related Disorders 2004, 10:S15-S18.
3. Adams JD Jr, Odunze IN: Oxygen free radicals and Parkinson’s disease. Free Radic Biol Med 1991, 10:161-169.
4. Olanow CW: A radical hypothesis for neurodegeneration. Trends Neurosci 1993, 16:439-444.
5. Schapira AH: Neuroprotection in PD - a role for dopamine agonists? Neurology 2003, 61:S34-S42.
6. Jenner P: Dopamine agonists, receptor selectivity and dyskinesia induction in Parkinson’s disease. Curr Opin Neuro 2003, 16:53-57.
7. Ferrari-Toninelli G, Bonini SA, Cenini G, Maccarinelli G, Grilli M, Uberti D, Memo M: Dopamine receptor agonists for protection and repair in Parkinson’s disease. Curr Top Med Chem 2008, 8:1089-1099.
8. Radd K, Gille G, Rausch WD: Short review on dopamine agonists: insight into clinical and research studies relevant to Parkinson’s disease. Pharmacol Rep 2005, 57(6):701-712.
9. Gassen M, Glinka Y, Pinchasi B, Youdim MB: Apomorphine is a highly potent free radical scavenger in rat brain mitochondrial fraction. Eur J Pharmacol 1996, 308(2):219-225.
10. Kondo T, Itō T, Sugita Y: Bromocriptine scavenges methamphetamine-induced hydroxyl radicals and attenuates dopamine depletion in mouse striatum. Ann NY Acad Sci 1994, 738:222-229.
11. Ogawa N, Tanaka K, Asanuma M, Kawai M, Masumizu T, Kohno M, Mori A: Bromocriptine protects mice against 6-hydroxydopamine and scavenges hydroxyl free radicals in vitro. Brain Res 1994, 657:207-213.
12. Takashima H, Tsujihata M, Kishikawa M, Freed WJ: Bromocriptine protects dopaminergic neurons from levodopa-induced toxicity by stimulating D2 receptors. Exp Neurol 1999, 159:98-104.
13. Ferger B, Teismann P, Merau J: The dopamine agonist pramipexole scavenges hydroxyl free radicals induced by striatal application of 6-hydroxydopamine in rats: an in vivo microdialysis study. Brain Res 2000, 833:216-223.
14. Uberti D, Carana T, Francisconi S, Ferrari-Toninelli G, Canonico PL, Memo M: A novel mechanism for pergolide-induced neuroprotection: inhibition of NF-kappaB nuclear translocation. Biochem Pharmacol 2004, 67:1743-1750.
15. Iida M, Miyazaki I, Tanaka K, Kabuto H, Isawa-Ichikawa E, Ogawa N: Dopamine D2 receptor-mediated antioxidant and neuroprotective effects of ropinirole, a dopamine agonist. Brain Res 1999, 838:51-59.
16. Tanaka K, Miyazaki I, Fujita N, Haque ME, Asanuma M, Ogawa N: Molecular mechanism in activation of glutathione system by ropinirole, a selective dopamine D2 agonist. Neurochem Res 2001, 26:31-36.
17. Uberti D, Bianchi I, Olvari L, Ferrari-Toninelli G, Bonini SA, Memo M: Dopaminergic agonists: possible neurorescue drugs endowed with independent and synergistic multistites of action. Neurochem Res 2007, 32:1726-1729.
18. Le WD, Jankovic J, Xie W, Appel SH: Antioxidant property of pramipexole independent of dopamine receptor activation in neuroprotection. J Neuro Transm 2000, 107:1165-73.
19. Abramova NA, Cassarino DS, Khan SM, Painter TW, Bennett JP Jr: Inhibition by R(+) or S(+) pramipexole of caspase activation and cell death induced by methylpyridinium ion or beta amyloid peptide in SH-SYSY neuroblastoma. J Neurosci Res 2002, 67:494-500.