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Synthesis and Antioxidant Activity Evaluation of Novel Antiparkinsonian Agents, Aminoadamantane Derivatives of Nitroxyl Free Radical

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Abstract—Two new analogues of the antiparkinsonian drug 1-aminoadamantane: 4-(1-adamantylamino)-2,2,6,6-tetramethylpiperidine-1-oxyl and 4-(1-adamantylammonio)-1-hydroxy-2,2,6,6-tetramethylpiperidinium dihydrochloride have been synthesized. Their antioxidant activity towards reactive oxygen species (ROS: $^{\bullet}OH$ and $O_2^{\bullet-}$) have been evaluated in three test systems. The compound with nitroxide substituent displays higher anti-oxidative capacity than those containing hydroxylamine. The in vivo study of ROS-involving 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)—rat model of induced parkinsonism was undertaken to ascertain the neuroprotective ability of the novel synthesized compounds—antioxidants. The data clearly shows that the nitroxide free radical moiety of the molecule is necessary for their neuroprotective action on dopaminergic neurons under the applied conditions of deep oxidative stress caused by the neurotoxin (MPTP). The new synthesized analogues may find application in treatment of parkinsonian syndromes, either to block or to reduce the ROS-mediated neuronal damage and death.

Introduction

Amantadine (1-aminoadamantane) is in use for the symptomatic treatment of Parkinson's disease (PD) for more than 30 years,[1–3] but the complete mechanism of its diverse action remains still elusive.[3] 1-adamantanamine (AA) is a dopaminergic, noradrenergic and serotonergic substance with neuroprotective properties.[2,4–6] AA is known to increase dopamine (DA) synthesis, its release and uptake in the striatum, which is consistent with its amphetamine-like action.[6–8] AA was found to act as blocker of brain monoaminoxidase A and as non-competitive *N*-methyl-D-aspartate (NMDA)-receptor antagonist thereby influencing the dopamine transmission and the glutaminergic brain system as well.[5,7,9] Notably, NMDA antagonism plays an important role in the symptomatological antiparkinsonian activity of AA.

Recently, it is known and widely accepted that the activation of the dopaminergic and glutaminergic systems in PD and dopamine/glutamate interactions, yields the production of reactive oxygen species (ROS), such as hydroxyl radical ($^{\bullet}OH$), superoxide ($O_2^{\bullet-}$) and H_2O_2. Consequently, ROS are in excess compared to the antioxidant capacity of neural cells, thereby produce oxidative stress (OS), damage and cells death.[9–14] Hence, this recent knowledge that brain cells death in PD is mediated by oxidative mechanism involving ROS, provides the basis for the considering antioxidant therapy as protective and symptomatical treatment.[11,13,15] Notably, it has been reported that AA per se did not act as scavenger or quencher of ROS,[16] which may limit its complete therapeutic potential in situ, under the conditions of the deep OS in PD.[11]

Our concern over the all already established actions of AA remained above, clearly showed that it would be possible to envisage its improvement, by introduction of the antioxidatively acting nitroxyl moiety[17,18] at position one of AA which could increase its scavenging ability towards ROS. We have recently demonstrated that the presence of piperidine aromatic, free radical moiety of nitroxyl can enhance the antioxidant capacity of biologically active substances.[19–21]

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We have synthesized two novel amantadine (AA, **2**) derivatives of nitroxyl (**1**): 4-(1-adamantylamino)-2,2,6,6-tetramethylpiperidine-1-oxyl (AAN, **4**) and 4-(1-adamantyl-ammonio)-1-hydroxy-2,2,6,6-tetramethylpiperidinium dihydrochloride (AAN, **5**).

The antioxidant potency of AANs (**4** and **5**) has been examined by several ROS-generating model reactions and compared with those of parent AA (**2**).

In this communication, we report on antioxidant activity of the novel synthesized AANs towards ROS. Due to the observed inhibition of DA oxidative degradation, the antioxidant functions of AANs can be also prominent. The data presented here is providing an obligative chemical basis for the comparative test investigations of the antiparkinsonian activity of AANs (**4**, **5**) and AA (**2**) in vivo model, in the MPTP-treated animals (rats). This rat model of parkinsonism[22] is exhibiting either the critical role of OS-damage to vulnerable dopamine neurons and multiple factors contributing to the parkinsonian phenotypes.[22–25] In the present study we have examined the effect of AA(2) and AANs (**4** and **5**) pretreatments for their possible actions against MPTP-induced insults on rat dopaminergic neurons. The results clearly showed the importance of the free radical nitroxide moiety of AAN for improving the endogenous 'antioxidant reserve' of neural cells before MPTP actions and suggest that the AANs administration may prove a valuable way for treatment of PD which should be of considerable pharmacologic interest.

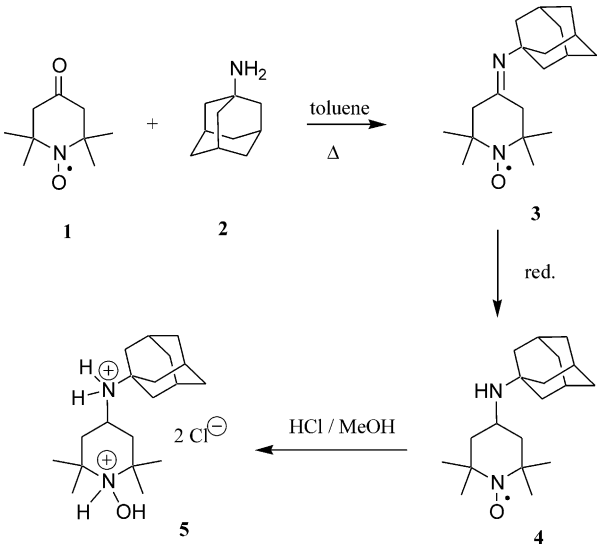
Results and Discussion

Chemistry

Synthesis of the 1-adamantanamine(amantadine) derivatives of nitroxyl (AANs; **4** and **5**) is shown in Scheme 1. Schiff base (**3**) was chosen for ease of the carbonyl group dehydration of 2,2,6,6-tetramethylpiperidon-4-oxyl-1 (**1**) with amino group of 1-adamantanamine (AA, **2**). Briefly, the stepwise addition of ten equivalents of **1** to one equivalent of **2** in anhydrous toluene was performed and the reaction mixture was refluxed to obtain corresponding nitroxide imine (**3**). The nitroxide imine (**3**) was transformed to the aminonitroxide (**4**) by its reduction with sodium cyanoborohydride.

The addition of 2.1 equivalents of concentrated hydrochloric acid to one equivalent of **4** in dry methanol yield a hydroxylamine and oxoammonium salt as a result of one-electron oxido-reduction of the nitroxyl group of **4**. The oxoammonium salt was transformed to hydroxylamine dihydrochloride (**5**) by oxidation of methanol to methanal. The product (**5**) was stable non-radical compound that was not affected by oxidation.

The products **4** and **5** (Scheme 1) were purified by column chromatography and recrystallization.



Scheme 1.

Antioxidant evaluations

Initial experiments using the technique of pulse radiolysis showed the obvious antioxidant reactivity of AA(2), AAN(4) and AAN(5) towards hydroxyl radical (•OH) (Fig. 1). The rate constants of the reactions of •OH with AA and AANs (Table 1) were determined by the competition method[26] and thiocyanate (SCN^-) was used as the competing scavenger. While interesting, it must be noted that a good scavenger for determining spectrophotometrically the rate constant with •OH by competitive kinetic methods should satisfy following main conditions: (i) the yield of the measured product of reaction with •OH should be the same as the yield of •OH radicals and (ii) this product should display absorption band in the region, where the sample does not absorb. Thiocyanate has important advantages over other potential OH scavengers: precisely known rate constant ($k_{\bullet OH} = 1.1\times10^{10}$ M^{-1} s^{-1})[26] and reaction mechanisms,[27] as well as high extinction coefficient of the product. SCN^- reacts with •OH via a transient adduct, forming •SCN radicals that instantly react with

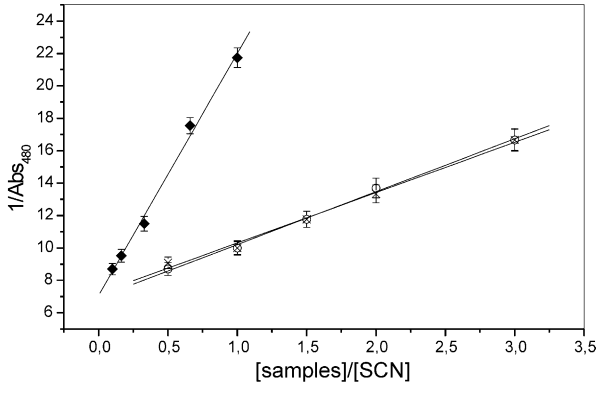


Figure 1. Comparative reactivity of AA (**2**), AAN (**4**) and AAN (**5**)* towards •OH**. *(X), AA (**2**); (◆), AAN (**4**); (○), AAN (**5**). ** •OH have been generated in pulse radiolysis. Samples containing either a mixture of the test substance and SCN^- (competing scavenger) or the scavenger (SCN^-) alone were made up in 50 mM phosphate buffer (pH 7.4) with 50 μM DTPA as a chelating agent. The measurement were carried out as described under Experimental. The data represent the mean (±) SD of five determinations.

Table 1. Rate constants of the reactions of the test substances and 2-deoxyribose (DR) as biological model target with •OH[a]

Samples	Rate constant, $k_{\text{samples}+\bullet\text{OH}}$ (M^{-1} s^{-1})
AA, **2**	5.20×10^{9}
AAN, **4**	2.32×10^{10}
AAN, **5**	4.84×10^{9}
DR	2.0×10^{9}

Competing scavenger: thiocyanate (SCN^-) $k_{\bullet\text{OH}+\text{SCN}^-}=1.1\times10^{10}$ M^{-1} s^{-1}.[26]

Experimental conditions as described in Experimental.

The data represent the mean SD (±) of five determinations.

[a]The results were based on pulse radiolysis competition data.

thiocyanate ion to give $(SCN)_2^{-\bullet}$ which absorbs at 480 nm ($\varepsilon=7.6\times10^3$ M^{-1} s^{-1}).[27] Thus, to compete with SCN^-, AA and AANs should react with •(SCN) with the rate constant not lower than 10^9 M^{-1} s^{-1}. Notably, there is no information available on the reactivity of •SCN towards nitroxide moiety. Thus, even if the absolute values of the rate constants (Table 1) may be to certain extent influenced by some systematic errors, in our opinion choosing thiocyanate as a reference scavenger allow us to minimise the possible errors. As can be seen (Fig. 1, Table 1), the greater the slope the higher the rate. Notably, the apparent activity of parent AA (**2**) towards •OH was about two times higher than that of 2-deoxyribose (DR), a common biological model target which is frequently used for assays detecting OH action.[28] The •OH-scavenging ability of AAN (**5**) containing hydroxylamine moiety (Scheme 1) was similar to that exerted by parent AA (**2**). It is worth mentioning that the displayed antioxidant activity of AAN (**4**) was significantly higher (Fig. 1) and it proved to be effective as •OH scavenger revealing the highest efficiency, with $k_{\bullet\text{OH}}=2.32\times10^{10}$ M^{-1} s^{-1} (Table 1). According to these observations, the structural feature was shown to play an important role in the antioxidant potency of the tested substances, namely the presence of the nitroxyl free radical moiety (**1**) at position one of the parent AA (**2**), which appears to enhance significantly the activity of the resulting derivative (AAN, **4**) towards •OH.

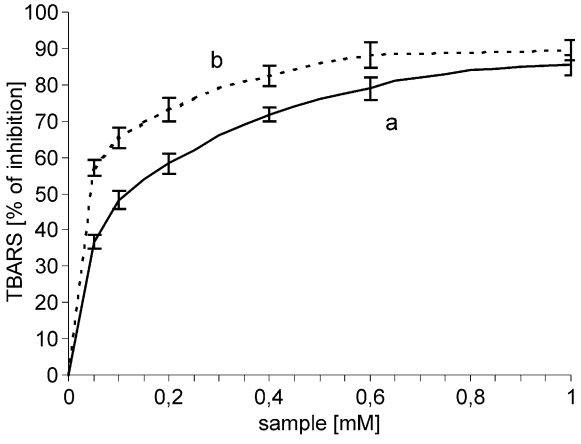


Figure 2. Antioxidative action of AA (**2**) (a) and AAN (**4**) (b) in the oxidative degradation of model biological target (DR) caused by •OH generated in iron- and ascorbate-driven Fenton system. The reaction mixture contained 0.7 mM DR, 0.7 mM H_2O_2, 20 μM $FeSO_4$, 0.1 mM EDTA and the test compound at the indicated concentration. The reaction was started by addition of 0.1 mM ascorbate, in 1 mL of 20 mM potassium phosphate buffer, pH 7.0. Experiments were carried out as described under Experimental. The data represent the mean (±SD) of three experiments done in triplicate.

The effect of •OH on biological molecules differs according to the site of its production.[11,13] It is commonly assumed that when •OH is produced by irradiation, it reacts with solutes as a function of their rate constants, which are almost diffusion limited.[11,26] However, the biological damage caused by a wide variety of ROS-generating factors in CNS is dependent on H_2O_2 and iron and therefore it has been frequently attributed to •OH radicals generated in the Fenton reaction.[11,29,30] In view of the above described results (Fig. 1, Table 1), showing that AANs are capable of scavenging of •OH radicals, it was of particular interest to test their ability to inhibit the •OH-induced chain oxidation of the common model biological target (DR)[28] in iron-driven Fenton system thereby acting as chain-breaking antioxidants. The simple and convenient deoxyribose assay[28,31] was applied in the present study (see Experimental) to study relative protective effect of AANs against Fenton based generation of •OH radicals.

Figure 2 shows the effect of varying concentration (0.05–1.0 mM) of AA (**2**) and AA (**4**), respectively, on the •OH-induced degradation of DR (0.7 mM), as detected by the TBA assay. These test substances inhibited DR chain oxidation by •OH in a concentration-dependent manner. At the concentration of 1.0 mM AA (**2**) and AAN (**4**) decreased the chain oxidation of DR by 85% (AA) and 89% (AAN, **4**) respectively. In addition, another noteworthy fact was that the AAN (**5**) does not inhibit it under the same experimental conditions. It is worth to note, that the highest concentrations of all tested substances are at the level just necessary to observe an antioxidant effect in the model solutions and about 10 times lower than can be achieved in vivo. The results presented in this work (Fig. 2) show the two major features of inhibition of DR degradation in Fenton system, caused by AAN (**4**): (i) a major impact of the oxidation state of AAN (nitroxide moiety versus amine) and (ii) and increase of protection with increasing AAN concentration. These lines of evidence, including the lack of protection afforded by AAN (**5**) support the hypothesis[32] that the observed protective effect may be attributed to oxidation of reduced iron, and its maintaining in the oxidized form thereby blocking the •OH generation in Fenton reaction.

The ability of AANs (**4** and **5**) to act as superoxide anion ($O_2^{\bullet-}$) scavengers was tested and compared in the superoxide-generating HX/XO system and measured by the reduction degree of the detector (NBT).[33] The antioxidant activity of AANs towards $O_2^{\bullet-}$ (Fig. 3) was manifested in a dose-dependent manner, in the concentration range of the test substances between 0.25 and 2.0 mM. It is noteworthy that the activity of AAN (**4**) towards $O_2^{\bullet-}$ was about two times higher than those of AAN (**5**) at 2.0 mM (Fig. 3). Moreover, the rate of HX oxidation to uric acid by XO, in the presence of the investigated compound was not changed (not

shown) indicating that AANs are neither substrates nor inhibitors of XO. Therefore, it is obvious that the tested AANs are active as superoxide scavengers and antioxidants.

Regarding the recently disputed role of amantadine (AA, **2**) as an indirect modulator of DA- transmission,[8] iron and H_2O_2[11,29,30] in the DA oxidative degradation,[11,29,30] it was of particular interest to test and to compare the apparent scavenging reactivities of AA and AANs towards •OH generated by Fenton's reagent (Fe^{2+}/H_2O_2), in the presence of DA. Optical spectrum of the products of DA oxidation taken 5 min after the start of the Fenton reaction, in the absence of test substance (control) is shown in Figure 4a. As can be seen, the formation of two peaks at 368 and 600 nm which can be related to the formed molecular products of DA

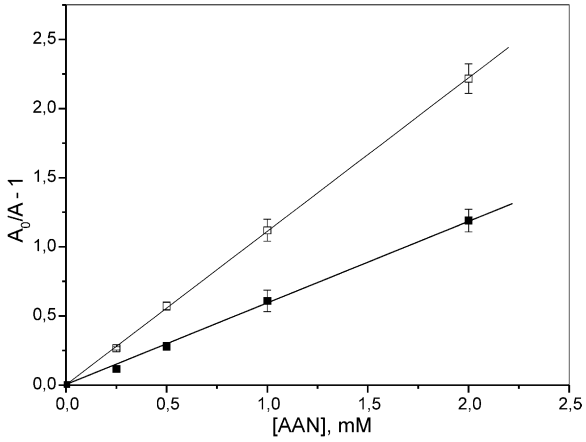


Figure 3. Superoxide anion scavenging ability* of AAN (**4**) and AAN (**5**)**. *Incubation mixture contained 0.5 mM HX, 1.0 mM NBT, 10 mU XO and the test compound at the indicated concentration in 1 mL of 20 mM potassium phosphate buffer, pH 7.0. The extent of superoxide-induced NBT reduction in the presence (A) or in the absence (A_o) of the tested substances was measured by determining the absorbance variations at 560 nm for 15 min incubation time. The data represent the mean (±SD) of three determinations. ** (□), AAN (**4**); (■), AAN (**5**).

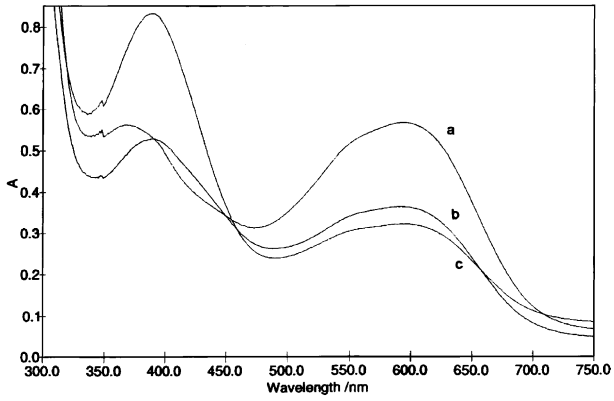


Figure 4. Antioxidative action of AA (**2**) and AAN (**4**) in the oxidative degradation of neurotransmitter (DA) caused by •OH generated in Fenton reaction. Incubation mixture contained 3.0 mM DA, 0.25 mM $FeSO_4$ and 0.8 mM H_2O_2 in 1 mL of 0.1 M KCl without or in the presence of 3.0 mM AAN (**5**) (a), 3.0 mM AA (**2**) (b) or AAN (**4**) (c), respectively. The procedures were as described under Experimental.

reaction with •OH was evident. The value of the latter wavelength is the absorption maximum value for the melanochrome(s) formed into the system.[34] However, the complete identification of the reaction products requires more extensive analysis and was not attempted in these test studies. In the presence of an equivalent molar concentration of DA and AA (**2**), the peaks intensity at 368 and 600 nm was significantly suppressed (Fig. 4b). This observation agrees with the suggestion that Fe^{2+} is rapidly oxidized to Fe^{3+}, which might be removed from the solutions by its 'inclusion' into the melanochrome. The presence of AAN (**4**) instead of AA (**2**) (Fig. 4c) led to marked changes of the absorbance profiles as compared with control (scan 4a) and parent AA (scan 4b). The appearance of a peak at 390 nm characteristic for DA-*o*-quinone formation,[34] which can coordinate Fe^{3+}[30] and the significant decrease at 600 nm, comparable to those caused by AA (**2**), was evident. On the contrary, the presence of an equivalent molar concentration of AAN (**5**) in the control mixture did not change markedly the absorbance profiles of DA oxidation as shown in Figure 4a. Briefly, the data shown in Figure 4 reveal for the first time that amantadine (AA, **2**) by itself could affect significantly the oxidative degradation of DA caused by •OH. While the presence of free radical moiety of nitroxyl (**1**) at position one of amantadine molecule (AAN, **4**) appears to influence significantly the oxidation of DA caused by Fenton's reagent generating •OH, the hydroxylamine moiety does not. These results corroborated an antioxidative mechanism involving a major role for nitroxide derivatives of AA (AAN, **4**).

Thus, the scavenging ability of AANs towards ROS instances far greater than that excerted by AA, establishing that the presence of nitroxide moiety is an essential 'driving force' for the enhancement of the antioxidant activity of parent AA. These events per se confirm the hypothesis[29,34,35] that melanin precursors (melanochromes) can be generated from DA in vivo by Fenton systems and suggest a possible protective role of AA and AANs, in non-enzymatic mechanism of neuromelanin formation and iron-induced degradation of substantia nigra, observed before in rat brain.[36]

Mechanism underlying the antioxidant effects of AANs

The mechanistic pathways that may predict the antioxidant activity of AANs towards ROS (•OH, $O_2^{\bullet-}$, H_2O_2) are outlined in Scheme 2. The sequence of reactions postulated follows those for AAN, **4**.

We consider the one-electron fast radical-radical reaction of AAN with •OH via an intermediate leading to the short-living oxoammonium cation, which is in keeping with the recently reported kinetics and mechanisms of •OH reactions with nitroxides and their hydroxylamines.[17] The nitroxide/oxoammonium redox couple may retain the antioxidant capacity of AAN molecule by switching back and forth between themselves in $O_2^{\bullet-}$-dismutation, SOD-mimic reaction, analogously to that recently suggested for other nitroxide derivatives.[21,37] This offers a considerable advantage of

AANs application as efficient $^{\bullet}OH$ and $O_2^{\bullet-}$- detoxifying, recyclable antioxidants maintaining the diverse biological activities of the AA moiety of their molecules (see Introduction).

Additionally, the results presented in this work focused on the capacity of AANs to act as effective chain-breaking antioxidants. Furthermore, nitroxides were shown to oxidize reduced metals, such as iron[38] and such oxidation can preempt the Fenton reaction thereby prevent the formation of secondary $^{\bullet}OH$ radicals[37] (Scheme 2). This does not rule out that AANs possess a $^{\bullet}OH$ scavenging ability, because the substance which is able to sequester iron could also serve as a scavenger.[30] An alternative mechanism (not shown) involves the possible comproportionation of the resulting hydroxylamine (AAN, **5**) with the oxoammonium cation yielding two nitroxide radicals, as previously proposed for other test system.[37] Thus, through a such 'flip-flop' between the **4** and **5** which could coexist in the system, their level might be continuously replenished (Scheme 2).

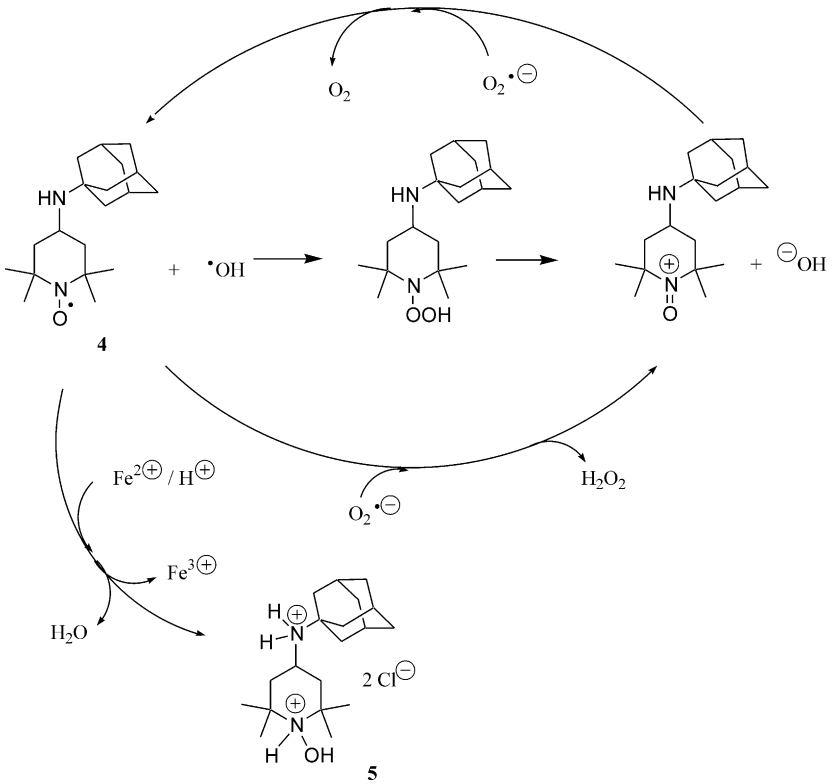
In other words, AANs being nitroxide derivatives may act as mild oxidants and chain-breaking antioxidants,[17–21] protecting biological targets of ROS and operating in a stoichiometric manner just as for direct scavenging reactions of $^{\bullet}OH$ and $O_2^{\bullet-}$, respectively. Generally speaking, a single molecular mechanism cannot account for the observed antioxidative potential of the new AANs as compared with those of parent AA. Thus, the mechanisms considered above may not exhaust the list of all possible antioxidant actions of AANs in vivo.

Considering the results of our model investigations, we can be reasonably confident, that whatever their exact mechanisms of anti-oxidative actions are, the application of the AANs may be useful under the conditions of ROS mediated acute oxidative stress in brain which is thought to be implicated in the pathogenesis of Parkinson's disease.[10–13,25]

Evaluation of the in vivo effects of AA or AANs pretreatment in MPTP-induced parkinsonism in rat brain

The purpose of the performed study was to determine and to compare the effects of AA (**2**) and AAN (**4** and **5**) pretreatments of rats for their possible neuroprotective action against MPTP-induced insults on dopaminergic neurons.[22–25] Because of the suggested similarity between PD and MPTP model, it was widely accepted that similar 'scenario' may underlie the neurodegenerative process in PD[22–25,39] and the free oxygen radicals/mechanisms involved in MPTP toxicity might be involved in the pathogenesis of PD.[40]

The present investigations are in line with a recent 'concept of neuroprotective treatment strategies in Parkinson syndrome'.[11–13,15,41,42] Notably, although there is selective symptomatic treatment in PD,[43] as yet no drugs are of proven neuroprotective value.[41,42,44] In this study, the MPTP-rat model of induced neurotoxicity, including the commonly applied antiparkinsonian drug, deprenyl, MAO-B inhibitor preventing the enzymatic conversion of MPTP to the toxic analogue MPP^+[43] was employed in conjunction with a sensitive TH-immunochemistry (see Experimental). Notably, tyrosine



Scheme 2.

hydroxylase (TH) is a rate-limiting enzyme for catecholamine (DA) synthesis and it is an excellent marker for dopaminergic systems in mammalian brains.[45]

Dopaminergic neurons with the TH antibody were easily detectable in the representative microphotographs of TH-immunostaining in the rat substantia nigra, shown in Figures 5–8. In the saline-treated control group (Fig. 5A) a uniform, weakly TH-immunoreactivity was observed throughout the intact nigrostriatal area, where neuronal cell bodies and fibres were weakly stained. These results are relatively consistent with a recent report of Nakahara et al.[46] The active phase of degeneration began at 20-min postinjection and continued up to 2 days. At very early stage of the acute MPTP administration (20 min), the histopatologic examination revealed a shrinkage of SN-neurocytes with strong cytoplasmic immunoreactivity against TH and only few fibres with scanty TH-reaction (Fig. 5B). Thus, recent reports described the appearance of TH-positive neurons after MPTP treatment in rat models,[47] although the experimental settings differed from ours. 2 h later (Fig. 5C) the TH-cytoplasmic reactivity of neurocytes was significant and fibres were inconspicious. When an attempt was made to decrease the observed damages (Fig. 5B and C), the single dose of deprenyl received 5 min after the MPTP administration was not sufficient to profoundly impair the system (c.f. Fig. 5B and E), where the medium cytoplasmic TH-reaction in both cytoplasm and fibres of neurocytes was observed. 2 h later, in the presence of deprenyl, the TH-positivity in cytoplasm becomes granular and more intensive along cell membrane (Fig. 5E). During this period, there was observed the TH-positivity of fibres suggesting that MPTP per se can cause a loss in TH without necessarily destroying the neuron.

The pre-treatment of animals with AA (see Experimental) resulted in the increase of shrunken TH-immunoreactive neurons (Fig. 6A), which are considered to be catecholaminergic.[7] This observation converges with the recent hypothesis that AA treatment can enhance the dopamine synthesis and neurotransmission and it can increase the release of DA in the synaptic cleft.[7,8] Nevertheless, a question then arises: how can the effects per se of AA on TH-immunoreactivity of SN-neurocytes be explained in the line of its known multiple pharmacodynamic actions? Thus, it would be restrictive

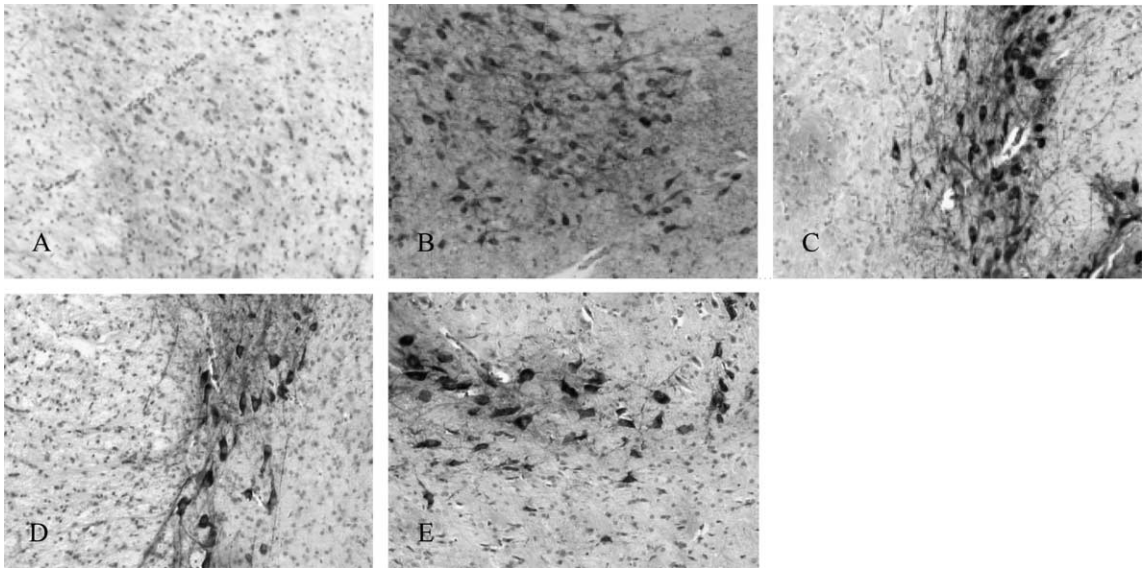


Figure 5. The striatal TH-immunostaining in the SN of saline (A) and MPTP-treated rats, 20 min (B) and 2 h (C) after acute treatment with MPTP alone or after the combination treatment of MPTP and DEP at the same time intervals: 20 min (D) and 2 h (E), respectively. Experimental procedures and conditions as described under Experimental. The microphotographs are representative of at least three experiments performed on different days and animals. Magnification = ×200.

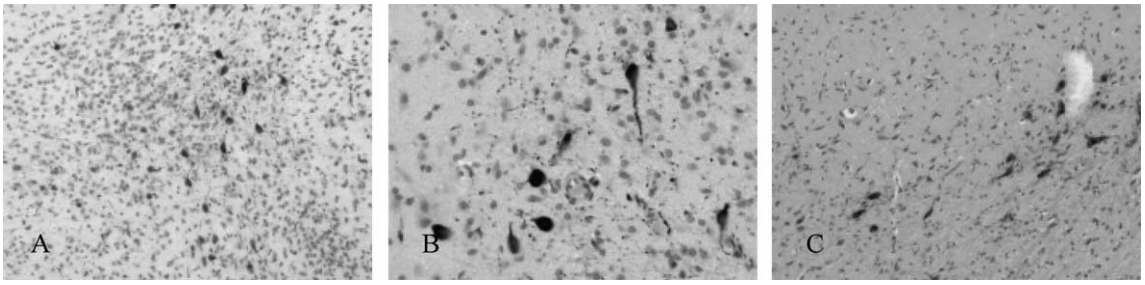


Figure 6. Effect of AA (**2**) pretreatment of rats on the striatal TH-immunostaining before (A) and after 20-min (B) or 2-h time intervals (C) following the acute MPTP and DEP administrations to the pretreated animals. Experimental procedures and conditions as described under Experimental. The microphotographs are representative of at least three experiments performed on different days and animals. Magnification = ×200.

to limit AA activity (Fig. 6A) to its NMDA-receptor antagonist property[14] and the biochemical mechanisms of AA action in the intact brain cells remain to be investigated and clarified. 20 min after the MPTP and DEP administration, the histopathologic evaluation (Fig. 6B) revealed the degeneration and loss of dendrites, a characteristic early indicator of toxin- induced neurodegradation in rat brain SN-catecholamine neurons, irrespective of how this is initiated.[48] It was accompanied by increase of shrunken TH-immunoreactive neurocytes with shrunken nuclei. Unexpectedly, the analysis of the pathological events 2 h later revealed the 'recovery' process, not observed in the brains of the non-pretreated animals: that is, strong TH-cytoplasmic reactivity, scanty TH-reaction of fibers and apparent decrease of the dendritic arbor damage (c.f. Figs. 5E and 6C).

In the AAN (**4**)-pretreated group, the TH-immunoreactivity before MPTP and DEP treatment (Fig. 7A) was comparable to those observed in AA-pretreated group (Fig. 6A). After 25 min of MPTP and DEP actions, the significant shrunkage of TH-reactive neurocytes and partial desintegration of fibers were evident (Fig. 7B). When comparing the TH-immunoreactive nigral neurons of the control group of animals (Fig. 5A) to those in the striatum of the AAN (**4**)-pretreated group 2 h after MPTP and DEP administration (Fig. 7C), the findings were much similar. As can be seen, the immunoreactive fiber densities came back close to the control striatum level and the presence of weakly stained TH-positive neurons was evident. However, the question whether these neurocytes (Fig. 7C) are functional, remains to be answered as a next step of in vivo and in vitro investigations.

As shown in Fig. 8, the pretreatment of rats with AAN (**5**) did markedly modify the TH-immunoreactivity of neurocytes (Fig. 8A) when compared with those of the control group (Fig. 5A). The treatment with MPTP and DEP magnified the damages of the rat dopaminergic neurons during 20 min–2 h time (Fig. 8B and C) and no recovery was observed.

Briefly, it can be concluded with great certainty that the test system applied at this stage of our investigations cannot comprise the whole scale of all the possible biologic scenarios, concerning the neuro-protective abilities of AANs and AA against the deleterious effects of the MPTP-induced cascade of events. It has to be emphasized here, that what is clear by now is: the pretreatment of animals with AAN (**4**) and administration of selective symptomatic drug, deprenyl,[43] at very early stage after MPTP-administration, are indispensable to achieve effective neuroprotection, whatever its mechanisms turn out to be. However, by definition,[49] neuroprotection is an effect that may result in salvage, recovery or regeneration of the nervous system, its cells, structure and function. Within this context, the present study shows the neuroprotective ability of AAN (**4**). Moreover, the results rule out the possibility of simple antioxidative-neuroprotective mechanisms of AANs, based only on a nitroxide moiety-ROS scavenging reactions, because the importance of the adamantyl group in neuroprotection became evident and its possible interactions with intracellular signal transduction pathways[46] remain to be indicated.

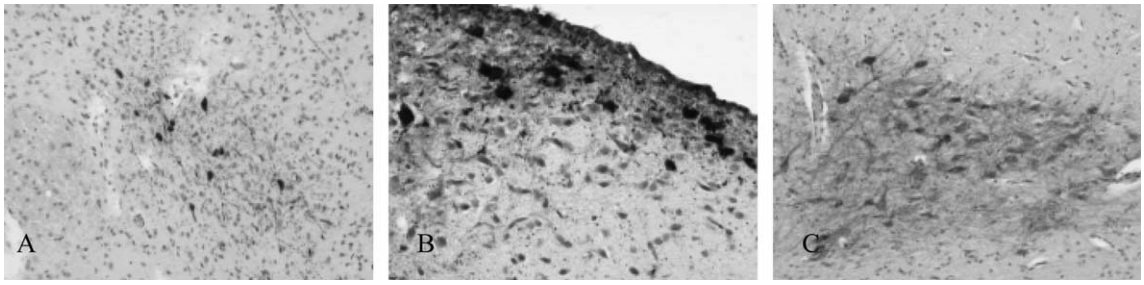


Figure 7. Effect of AAN (**4**) pretreatment of rats on the striatal TH-immunostaining before (A) and after 20-min (B) or 2-h (C) time intervals following the acute MPTP and DEP administrations to the pretreated animals. Experimental procedures and conditions as described under Experimental. The microphotographs are representative of at least three experiments performed on different days and animals. Magnification = ×200.

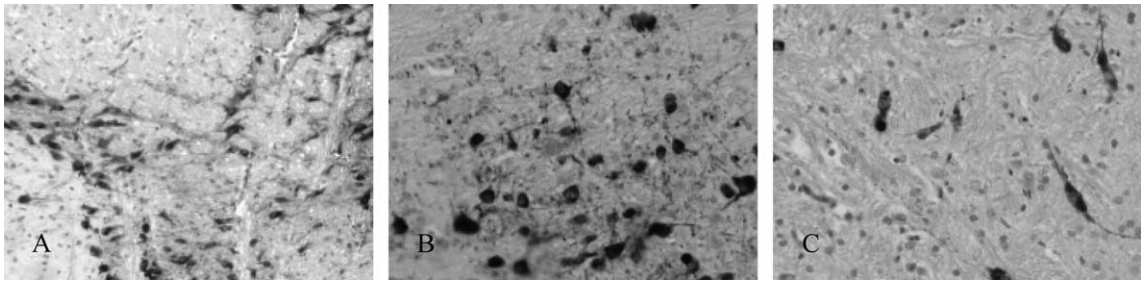


Figure 8. Effect of AAN (**5**) pretreatment of rats on the striatal TH-immunostaining before (A) and after 20-min (B) or 2-h (C) time intervals following the acute MPTP and DEP administrations to the pretreated animals. Experimental procedures and conditions as described under Experimental. The microphotographs are representative of at least three experiments performed on different days and animals. Magnification = ×200.

Conclusions

In our ongoing research efforts to improve or develop new treatment strategies for Parkinson's disease (PD), we developed a simple and efficient method to synthesize aminoadamantane derivatives of stable nitroxide radicals, named AANs, through conjugation of the aminoadamantane to nitroxyl radical. It was consistent with our concept[11,13] to create multifunctional, antioxidative acting antiparkinsonian substances: (i) containing two different, biologically important active parts of the therapeutically known compounds in correct stoichiometry; (ii) without marked changes of their chemical structures, so as to achieve the complete therapeutic effect of the resulting derivatives in situ.

The two new analogues, AAN **4** and **5**, have been synthesized in excellent yields and their capacity to act as antioxidants of potential neuropharmacological applications was tested in three ROS-generating systems: pulse radiolysis, HX/XO and iron- and ascorbate-driven Fenton. The derivative AAN **4**, which is a prototype of the novel class of nitroxide-based amantadines showed broad antioxidant activities. It was able to scavenge efficiently $^{\bullet}OH$ and superoxide anion ($O_2^{\bullet-}$) in dose-dependent manner as well as to reduce the oxidative degradation of biological targets (DR and DA), thereby acting as effective chain-breaking antioxidant. These findings were discussed from a mechanistic standpoint as well in terms of potential implications of the hydroxylamine- containing AAN **5**, which can coexist in the ROS-generating systems during the antioxidant reactions of AAN **4**.

To investigate the in vivo neuroprotective ability of AANs, the pharmacologic test including MPTP-induced parkinsonism of rat model was used, where the toxic damage of DA-secreting neurons by ROS-mediated actions was presumed.[22–25,40] The data clearly show that the nitroxide free radical moiety of AAN molecule is necessary for neuroprotection of dopaminergic neurons, but the role of adamantane moiety could not be neglected which remains to be clarified in a next step of biochemical work.

It seems reasonable to suggest that the antioxidative and neuroprotective properties of the stable free radicals, adamantane-nitroxide derivatives, should be of particular pharmacologic interest in the development of the 'new concept of neuroprotective treatment strategies',[13,41–44] where the use of AANs alone or in 'combinative therapy' may be an important way to improve PD treatment in the near future.

Experimental

All chemicals of analytical grade used for the synthesis of AAN were purchased from Fluka (Buchs, Switzerland). Amantadine hydrochloride (AA), 2-deoxyribose (DR), ascorbic acid, thiobarbituric acid (TBA), trichloroacetic acid (TCA), dopamine hydrochloride (DA), nitroblue tetrazolium (NBT) and other reagents of analytical grade were purchased from Sigma-Aldrich (Schnelldorf, Germany). Deprenyl, (*R*)-(−)-*N*-α-dimethyl-*N*-(2-propynyl)phenylethylamine was purchased from Tocris Cookson Ltd. (UK). Ferrous sulfate and potassium chloride were purchased from Merck (Darmstadt, Germany).

^{13}C NMR spectra were recorded on 62.9 MHz (for carbon) Brücker DPX 250 Avance spectrometer and values were reported in ppm. Spectrophotometric measurements were performed on a Cary (Varian) 1E spectrophotometer. The column chromatography was performed with silica gel 60 Å and thin layer chromatography (TLC) was performed on precoated silica gel plates (Merck, Germany). Elemental analyses (C, H, N) were performed at PAN Laboratories (Łódź, Poland) and are within 0.4% of the theoretical values. Melting points were determined on a Baethius apparatus with microscope device. Pulse radiolysis experiments were conducted using the linear electron accelerator with spectrophotometric detection (ELU-6, Russia) delivering nanosecond pulses of 6 MeV electrons (17 ns duration) for the experiments with a competing scavenger thiocyanate (SCN^-).

4-(1-Adamantylamino)-2,2,6,6-tetramethylpiperidine-1-oxyl (AAN 4). A solution 2,2,6,6-tetramethylpiperidon-4-oxyl-1, **1** (4-oxo-TEMPO) (0.17 g, 10 mmol), 1-aminoadamantane, **2** (1.51 g, 10 mmol) and 1-aminoadamantane hydrochloride **2**/HCl (0.188 g, 1 mmol) in anhydrous toluene (100 mL) was refluxed in a flask equipped with a Dean-Stark water separator in argon atmosphere. After the separation of the calculated amount of water (2 h), the solution was concentrated under reduced pressure (first water pump, second oil pump). The remaining Schiff base **3** was dissolved in dry THF (50 mL), the mixture was cooled at 5 °C and then 10-g molecular sieves (fresh activate; 300 °C, vacuum line was introduced at 50 °C). The reaction mixture was microwaved for 15 min at 25 °C. To this solution was added 0.5 mmol sodium cyanoborohydride by cannula over 5 min. The reaction was monitored by TLC.

Then the solution was added slowly to a stirred suspension of $NaBH_3CN$ (0.124 g, 2 mmol) and $NaBH_4$ (0.378 g, 10 mmol) and the mixture was stirred at 25 °C for 24 h. After 4 days, the brown reaction mixture was washed with 5 mL of 25% sodium hydroxide and 20 mL of saturated sodium bicarbonate and evaporated to dryness which resulted in red-brown oil **2**.

To the mixture were added 50 mL methanol, 1 mL CF_3COOH and 10 mL water. The crude hydroxylamine/nitroxide solution was reoxidized with oxygen and cooper(II) acetate (2 mg). Then the reaction mixture was evaporated to dryness and extracted with diethyl ether (3×50 mL). The extract was dried ($MgSO_4$), filtered and concentrated. The purification by column chromatography provided the product of nitroxide **4** in 78% yield (TLC: 9:1 *n*-hexane/ethyl acetate, molybdenum stain, $R_f = 0.48$). MS (FAB pos., $C_{19}H_{33}N_2O$):

M + H = 305). The crude **4** was recrystallized from a mixture of diethyl ether and *n*-hexane (0.23 g, 75.5%) as orange crystals: mp 68–69.5 °C; The analytical sample **4** was obtained by flash chromatography on silica gel (Merck 60). Anal. ($C_{19}H_{33}N_2O$, 304.48) C,H,N.

4-(1-Adamantylammonio)-1-hydroxy-2,2,6,6-tetramethylpiperidinium dihydrochloride (AAN, 5). The nitroxide compound **4** (0.122 g, 4 mmol) was dissolved in mixture of MeOH (20 mL) and 1 mL (0.444 g HCl, 12 mmol) hydrochloric acid (37 wt.% in water). The orange colour of nitroxide disappeared (1 h). The reaction mixture was shaken for 2 h at room temperature. The mixture was evaporated to dryness and dried under reduced pressure (oil pump). The solid was dissolved in absolute EtOH and diluted with dry diethyl ether to start crystallization. Dihydrochloride **5** ($C_{19}H_{36}Cl_2N_2O$) was purified by recrystallization. The crystalline dihydrochloride **5** was filtered off, washed with diethyl ether and dried (0.78 g, 51%) as off-white crystals; mp > 300 °C. Anal. ($C_{19}H_{36}Cl_2N_2O$, 379.41) C, H, N.

^{13}C NMR (CF_3COOD); (**C**H_3–) 14.7; 20.9, (**C**H_2–) 28.0; 28.5; 30.7; 31.0; 31.4; 33.7; 40.2; 48.8 (**C**H–) 25.8; 28.9; 51.8; 52.9, (**C**) 61.4; 71.6.

MPTP(1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). This reagent was synthesized according Mabic et al.[50] with some modifications. 1-methyl-4-piperidone was twice distilled from CaH_2 under reduced pressure and its conversion to MPTP was performed.[51] The obtained solution was heated at 90 °C for 4 h with concentrated HCl (fuming 37%), then the solvent was evaporated under vacuum and the obtained crude product was crystallized from dry ethanol as described before.[52] This procedure gave MPTP in 78% yield (mp 248–250 °C) as hydrochloride salt. The identity of MPTP was confirmed by ^{13}C NMR (D_2O) measurements (ppm: 136.4, 132.5, 127.1, 126.7, 123.3, 113.6, 50.3, 48.7, 40.1, 21.9).

Evaluation of the antioxidative effects

The rate constants of the reaction of •OH with the investigated substances have been determined in pulse radiolysis experiments with a competing scavenger (SCN^-). Samples, containing either a mixture of the substance studied and SCN^- or the scavenger alone (SCN^-) were made up in 50 mM phosphate buffer (pH 7.0) with 50 μM DTPA as a chelating agent. Samples were pulse-irradiated in gas-tight, rectangular quartz cells of both electron and optical part of 1 cm. Subsequent changes in absorbance at 480 nm (λ_{max} of $SCN_2)^{-\bullet}$ were followed at nano- and microsecond time scales. Each recorded trace was an average 4–16 single pulses and five such traces were recorded for every sample (50 Gy average dose per pulse). N_2O was used for the efficient removal of hydrated electrons and doubling of the •OH yield. The rate constants of the reaction of generated •OH with the investigated substances (Scheme 1) and DR have been determined by competition with SCN^- and calculated using equation:

$$\frac{1}{A_S} = \frac{1}{A_0} + \frac{k_S[\text{sample}]}{A_0 k_{SCN^-}[SCN^-]}$$

where A_0 is absorbance at 480 nm in the absence of sample (1 mM SCN^-); A_s—absorbance at 480 nm in the presence of sample and 1 mM SCN^-; k_{SCN^-}—the rate constant of •OH with SCN^- (1.1×10^{10} $M^{-1}s^{-1}$);[26] k_S—the rate constant of •OH with the investigated sample. The values of k_S were calculated from the slope of the plot $1/A_S$ versus [sample]/[SCN^-]. The experiments were done at ambient temperature (22 ± 2 °C) and millipore—quality water (resistivity of about 17 MΩ) was used throughout.

A mixture of Fe^{2+} and H_2O_2 is known as Fenton's reagent and gains its reactivity from the formation of •OH radicals. The ability of scavenging hydroxyl radical (•OH) was measured by studying the competition between 2-deoxyribose (DR) and the test compounds for •OH generated from the ascorbate-driven Fe^{2+}/H_2O_2 system, as described before,[31] with minor modifications. The reactions mixture contained 0.7 mM DR, 0.7 mM H_2O_2, 20 μM $FeSO_4$, 100 μM EDTA and the test compound at various concentration, in 1 mL of 20 mM phosphate buffer, pH 7.0. The reaction was started by the addition of 100 μM ascorbate. The mixture was incubated at 37 °C for 60 min and the extent of DR degradation and thiobarbituric acid reactive substances (TBARS) formation by •OH was measured with the TBA method: 1 mL TBA (1% w/v in 50 mM NaOH) and 1 mL trichloroacetic acid (TCA, 2.8% w/v) were added to each sample at the end of incubation. The tubes were heated at 100 °C for 15 min and after cooling the absorbance at 532 nm was measured. The extent of inhibition of TBARS formation was calculated according to the equation:

$$\%\ \text{inhibition} = 100 - [(\Delta A/\Delta A_o) \times 100]$$

where ΔA and ΔA_o are the absorbances in the presence or in the absence of scavenger, respectively.

Superoxide ($O_2^{\bullet-}$) scavenging ability was measured in a hypoxanthine (HX)/xanthine oxidase (XO) superoxide-generating system using nitroblue tetrazolium salt (NBT) as a detector molecule.[33] Incubation mixture contained 0.5 mM HX, 1.0 mM NBT, 10 mU XO and the scavenger ranging from 0.25 to 2.0 mM, respectively, in 1 mL of 20 mM phosphate buffer, pH 7.0. The apparent ability of the used HX/XO system to generate a flux of 2.8–3.0 μM $O_2^{\bullet-}$ min^{-1} was determined using cytochrome C as detector molecule instead of NBT.[33] Neither AA (**2**) nor AANs (**4**, **5**) inhibited XO activity as measured by a rise in absorbance for urate formation at 295 nm, in reaction mixtures without NBT.

The extent of reduction of NBT in the absence (A_o) and in the presence (A) of various concentrations of AAN (**4**) and AAN (**5**), respectively, was determined by measuring the absorbance variations at 560 nm for 15 min incubation time.

To test and to compare the antioxidant activity and capacity of AA (**2**) and AANs (**4**, **5**) to influence the oxidative degradation of the neurotransmitter (dopamine, DA) by $^{\bullet}OH$ generated by Fenton system,[29] the following incubation mixture was used: 3.0 mM DA, 0.25 mM $FeSO_4$ and 0.8 mM H_2O_2, in 1 mL of 0.1 M KCl, without (control) or in the presence of 3.0 mM of the test substance, respectively. The reaction was started by addition of H_2O_2. The oxidation of DA was monitored spectrophotometrically by recording rapid scans every 30 s in the 300–700 nm region. The reaction was completed in 5 min and the subsequent slow decay of the formed products was observed over 1 h incubation time (not shown).

Animals and experimental procedures in vivo

The experiments were carried out in compliance with the Animal Protection Bill published in Poland's Official Current Legislation Gazette (No. 111, 1997, item 724) and according to the NIH guide for the Care of Laboratory Animals. 80 female Buffalo rats weighing 170–200 g, approximately 3 months old (Central Animal Farm, MU, Wroclaw, Poland), were housed on groups of 5–10 under standarized conditions (temperature 22 °C, 12L: 12D cycle), with free access to standard granular diet containing 24% of crude protein and water.

The experimental protocols were approved by the appropriate institutional Governmental Agency.

In this study, rats were subjected to the following six treatments:

1. MPTP (40 mg/kg, s.c.);
2. MPTP (40 mg/kg, s.c.) and after 5-min single injection (ip) of DEP (2.5 mg/kg);
3. Adamantane (AA) pretreatment (2 days, 40 mg/kg daily, ip), 1 h after the last dose-MPTP (40 mg/kg, s.c.) and after 5-min single injection of DEP (2.5 mg/kg, ip);
4. AAN (**4**) pretreatment (2 days, 40 mg/kg daily, ip), 1 h after the last dose-MPTP (40 mg/kg, s.c.) and after 5-min single injection of DEP (2.5 mg/kg);
5. AAN (**5**) pretreatment (2 days, 40 mg/kg daily, ip), 1 h after the last dose-MPTP (40 mg/kg, s.c.) and after 5-min single injection of DEP (2.5 mg/kg);
6. all control subjects received injections of saline (1.0 mL/kg, ip) at the appropriate time instead of the tested substances.

Animals were sacrified by decapitation at the appropriate time of experiments (20 min, 1, 2, 24 and 48 h after MPTP (Group 1), MPTP and DEP (Groups 2–5) or saline administrations (Group 6), respectively. The brains were rapidly dissected, rinsed twice with cold PBS, dried on blotting paper and halved in its sagital plane. One half was weighted and fixed in 10% buffered formalin. These fixed tissues were embedded in paraffin and representative coronal sections (6 μm thick) were obtained. Brain sections were immunostained for tyrosine hydroxylase (TH) using ARK method (DAKO ARK, Animal Research Kits: K 3954 and 3955). The paraffin sections were deparaffinized, rinsed twice (10 min) with water and cooked in 0.01 M sodium citrate (pH 6.0) for 8 min at 350 Watt in microwave device. The samples were rinsed again (10 min) with water. The biotinylated ex tempore antibody (Tyrosine hydroxylase mouse monoclonal antibody, NCL-TH, Novocastra) was applied for 15 min. The sections rinsed with PBS twice (10 min) and Streptavidin-Peroxidase was added for the next incubation (15 min). Then samples were washed with PBS (twice) and treated with DAB+-Chromogen. The sections were rinsed with water twice for at least 5 min each time, counterstained with hematoxylin for 1 min, and covered with coverlips for the histologic examination.

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