Polish Journal of Pharmacology
Pol. J. Pharmacol., 2003, 55, 389–400
ISSN 1230-6002

ANTIOXIDANT PROPERTIES OF NEWLY SYNTHESIZED N-PROPARGYLAMINE DERIVATIVES OF NITROXYL: A COMPARISON WITH DEPRENYL

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Antioxidant properties of newly synthesized N-propargylamine derivatives of nitroxyl: a comparison with deprenyl. A. KOCHMAN, J. SKOLIMOWSKI, L. GĘBICKA, D. METODIEWA. Pol. J. Pharmacol., 2003, 55, 389–400.

In our search for novel, low-toxic, cell-penetrable and neuroprotective antioxidants, we have designed a number of novel N-propargylamine derivatives of nitroxyl, named "JSAKs". The reactivity and antioxidative potency of two selected JSAKs and their parent nitroxyl against reactive oxygen species (ROS) were examined *in vitro*, in a cell-free γ-radiolysis and in model Fenton-type reaction systems and compared with those of deprenyl, the investigated member of adjunct therapies in clinical neurology. The efficiency of JSAKs to suppress the oxidative degradation of a model target (deoxyribose), deprenyl and dopamine, caused by hydroxyl radical (•OH) was also investigated. The data demonstrated that the novel compounds, JSAKs, can act as promising antioxidants and protectors of targets against ROS toxicity, thus, providing a sound chemical basis for further comparative investigations of their activity *in vivo*. The findings were discussed from a mechanistic point of view as well as in terms of the structure-dependent, comprehensive properties of JSAKs as dual-function compounds: antioxidants and anti-apoptotic propargylamines. The novel class of N-propargylamine nitroxyls, JSAKs, may have potential implications for the experimental therapies of Parkinson's disease, where ROS mediate deleterious effects, because these compounds have an ability to either block or reduce the progression of neurotoxic cascade of brain damage.

Key words*: oxygen radicals, novel propargylamines, deprenyl, reactivity, mechanisms*

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Abbreviations: DEP – *deprenyl,* ESR – *electron spin resonance,* FAB – *fast atom bombardment,* IR – *infra red,* JSAK – *novel N-propargylamine derivatives of nitroxyl,* NMR – *nuclear magnetic resonance,* ROS – *reactive oxygen species*

INTRODUCTION

Neurodegenerative processes are generally characterized by selective destruction of neuronal populations in the brain tissue and neuronal death. In Parkinson's disease (PD), where a loss of dopaminergic neurons in the midbrain has been observed, the cells are killed due to oxidative stress wherein reactive oxygen species (ROS) are generated in excess [16, 18, 19, 21, 24, 29, 32, 37, 42]. The surviving neurons of the nigrostriatal pathway exhibit an increased dopamine (DA) turnover [21] and its enzymatic and/or nonenzymatic oxidation may play a key role in the generation of ROS ($^{\bullet}OH$, $O_2^{\bullet-}$, H_2O_2) in the iron-rich basal ganglia [4, 7, 36, 43]. ROS and DA quinone/semiquinone radicals in the striatum may cause the increased lipid peroxidation [23], altered iron metabolism [4, 9, 26, 44] and retrograde degeneration of nigrostriatal neurons. Once formed, oxygen radicals can abstract hydrogen from DNA forming DNA-radicals, that leads to DNA fragmentation, activation of protective mechanisms, NAD and ATP depletion and apoptosis [1, 4, 15, 19, 24, 32, 37].

The oxidative pathogenesis of PD remains still largely enigmatic, but all recent findings give evidence that ROS neurotoxicity can play an important pathogenic role leading to neuronal degeneration and neuronal apoptotic death and that the dopaminergic neurons of the midbrain may become unable to effectively protect themselves [41]. Thus, the neuroproprotection, defined as an effect which results in salvage, recovery or regeneration of the nervous system, its cells, structure and function, remains an important goal of the experimental therapeutics in PD for new millennium [10, 30, 41, 48]. However, respective symptomatic, antioxidative and anti-parkinsonian capacities of the applied substances should be fundamental to the "concept of neuroprotective treatment strategies" [5, 8, 19, 25, 30, 32]. Notably, to date almost all well established chemotherapeutic approaches in PD have been focused mainly on DA replacement or replenishment, either by its precursors, agonists or enzyme(s) inhibitors [5, 8, 10, 22].

MAO-B inhibition is already a well-established neuroprotective therapy in PD. The selelective MAO-B inhibitor, deprenyl (DEP) (selegiline, N-methyl-N-propargyl amphetamine) has been shown to protect neurons but it is still uncertain whether DEP is neuroprotective *per se* or exerts its mild antiparkinsonian effect by enhancing the dopaminergic neurotransmission [12]. It has been suggested that DEP provides neuroprotection through multiple mechanisms, probably including its antioxidative action against ROS and blockade of neuronal apoptosis [11, 35, 45, 46]. DEP needs metabolic conversion to be anti-apoptotic but the resulting "effective metabolite" is still unknown [45]. The well-identified metabolites of DEP, amphetamine and methamphetamine [2], are potentially neurotoxic and may contribute to so-called undesirable "selegiline-related amphetamine-like side effect", which is not related to its MAO-B blocking action and can limit significantly the therapeutic results [13, 22, 45].

Thus, although a definite conclusion from well-performed modern clinical studies of the neuroprotective action of DEP in PD treatment is still lacking, the present knowledge clearly suggests the necessity of further search for and development of compounds that would reduce oxidative stress, thereby slowing the rate of neuronal degeneration caused by ROS and illness progression [10, 19, 22, 24, 30].

Antioxidants with widely varying chemical structures have been investigated as potential therapeutic agents in neural disorders [12, 15, 16, 19, 32, 40]. Recently, considerable attention has been given to the stable nitroxide free radicals (nitroxyls) because of the unique properties they possess as blood-brain barrier permeable antioxidants, which can protect brain tissue by terminating radical-chain reactions, oxidizing deleterious iron ions and by removal of superoxide acting as SOD-mimics [30, 31, 33, 34, 38, 51]. All these abilities of nitroxyls seem to be relevant to the good protection of the nigrostriatal dopaminergic neurons against ROS actions. On the other hand, all recent findings discussed above clearly show that the neuroprotective effect is attributable to the presence of the propargyl group in the molecule of the therapeutics, which has been previously shown to be also essential for the anti-apoptotic function of the established neuroprotective anti-parkinsonian drugs,

and their action as MAO-B inhibitors [5, 25, 28, 35, 39, 45, 50].

As a part of our studies on the chemical, biochemical and neuroprotective characteristics of newly synthesized antioxidants with potential neuropharmacological applications, we have developed a number of N-propargylamine derivatives of nitroxyls, named "JSAKs" (abbreviation derived from names of **J**anusz **S**kolimowski[1] and **A**gata **K**ochman[2], who synthesized[1] and tested[2] these substances). These novel compounds possess the molecular structure which allows for exerting the synergistic dual effect: suppressing the action of ROS (nitroxyl moiety), and reducing the catabolism of DA by inhibition of MAO-B (propargylamine moiety). Therefore, these compounds are able to share a common site of action with DEP but are devoid of "amphetaminergic" effects. In the present study, we have examined the reactivity and antioxidant potency of two selected JSAKs, our chosen candidates for drugs, and compared it with those of DEP and parent nitroxyl. The efficiency of JSAKs to protect a model target (deoxyribose, DR), DEP and DA from the oxidative degradation was also examined and compared.

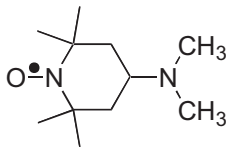
MATERIALS and METHODS

Chemicals

(R)-(–)-Deprenyl hydrochloride, (R)-N-(–)-dimethyl-N-2-propynylbenzeneethanoamine (DEP) (Sch. 1D) was purchased from Tocris Cookson Ltd (Bristol, UK). All chemicals of analytical grade used for synthesis of the novel derivatives (this work) were purchased from Fluka (Buchs, Switzerland). D-2-deoxyribose (DR), ascorbic acid, thiobarbituric acid (TBA), trichloroacetic acid (TCA), lactic peroxidase (LPO, RZ of 0.8), dopamine hydrochloride (DA) and other reagents of analytical grade were from Sigma-Aldrich (Schnelldorf, Germany). All the other reagents of analytical grade were obtained from the Polish Reagent Company (P.O.CH., Poland). Iron (II) sulfate and potassium chloride were purchased from Merck (*pro analysi* quality).

Synthesis

Synthesis of parent nitroxyl, Nx-640 (4-dimethylamino-2,2,6,6-tetramethylpiperidine-1-oxyl, 4-dimethylamino-TEMPO) (Sch. 1A). A solution of 2,2,6,6-tetramethyl-4-piperidinon-1-oxyl (0.10 mol) in a mixture of dimethylamine (0.80 mol), dimethylamine hydrochloride (0.10 mol), 3 Å molecular sieves (5 g) and methanol (absolute, 100 ml) was cooled to 5°C. Then, sodium cyanoborohydride (0.08 mol) was added over 20 min at the same temperature. After the mixture had been stirred at ambient temperature for 60 h, it was filtered, washed with methanol and evaporated to dryness. To this mixture, 20 ml of concentrated sodium carbonate solution, 25 ml of diethyl ether were added and the mixture was stirred. After 5 min, the reaction mix-



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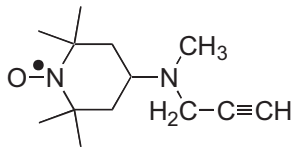

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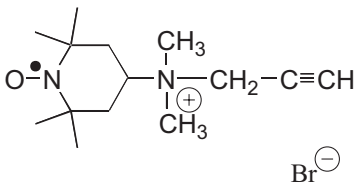

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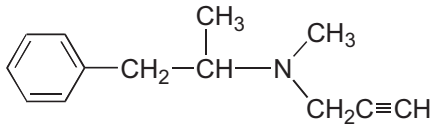


D

Scheme 1. Chemical structures of the investigated substances. A – Nx-640, 4-(dimethylamino)-2,2,6,6-tetramethylpiperidine-1-oxyl, 4-dimethylamino-TEMPO; B – JSAK-648, 4-(N-methyl-N-propargylamino)-2,2,6,6-tetramethylpiperidine-1-oxyl ; 4-N-methyl-N-propargylamino-TEMPO; C – JSAK-641, 4-[(N,N-dimethyl)-propargyl]ammonio-2,2,6,6-tetramethyl-piperidine-1-oxyl bromide; D – DEP, deprenyl, (R)-(–)-N-α-dimethyl-N-2-propynylbenzeneethanomine, selegiline, N-methyl-N-propargyl amphetamine

ture was filtered through Celite (10 g), the Celite was washed with 10 ml of diethyl ether, and volatiles were removed *in vacuo* to give a red viscous oil which slowly crystallized. The red oil was distilled under reduced pressure (0.1 mm Hg, Kugelrohr apparatus). Purification by flash column chromatography (neutral aluminium oxide 90 active – Merck, 25 mm column, n-hexane/dichloromethane ratio 1:9) afforded 15.5 g of pure 4-dimethylamino-TEMPO as a red viscous oil which slowly crystallized (78%) overnight as red solid substance. The nitroxide molecular weight ($C_{11}H_{23}N_2O$) was estimated at 199.32. The identity of Nx-640 (Sch. 1A) was confirmed by mass spectroscopy, IR, ESR spectra and determination of melting point (45–48°C).

Synthesis of JSAK-648 (4-(N-methyl-N-propargylamino)-2,2,6,6-tetramethylpiperidine-1-oxyl, [4-(N-methyl-N-propargylamino)-TEMPO]) (Sch. 1B) was performed in a similar manner as described in (a). The identity of JSAK-648 (molecular weight for $C_{13}H_{23}N_2O$-223.34) was also confirmed by mass spectroscopy and (FAB) ^{13}C NMR spectroscopy ($CF_3COOD/CDCl_3$-oxoammonium salts), ESR and IR spectra and its chemical purity was established by elemental analysis.

Synthesis of JSAK-641 (4-[(N,N-dimethyl)-propargyl]ammonio-2,2,6,6-tetramethylpiperidin-1-oxyl bromide) (Sch. 1C). The propargyl ammonium salt was prepared by adding 4-(dimethylamino)-2,2,6,6-tetramethylpiperidine-1-oxyl (4-dimethylamino-TEMPO) to an ether solution (THF) of propargyl bromide at 10°C. The cooling bath was removed and the mixture was stirred for 30 h at room temperature. Red solid substance slowly crystallized from the solution. The salt was filtered. The sample was purified further by recrystallization from methanol. Melting point was 211–212°C, and TLC (ethyl acetate) with molybdenum staining yielded R_f = 0.48. The identity of JSAK-641 (molecular weight for $C_{14}H_{26}BrN_2O$ = 318.28) was confirmed by mass spectroscopy and (FAB) ^{13}C NMR spectroscopy ($CF_3COOD/CDCl_3$-oxoammonium salt/hydroxylamine), ESR and IR spectra and its chemical purity was established by elemental analysis.

Reactivity towards hydroxyl radicals and superoxide

The rate constants of the reaction of $^{\bullet}OH$ and $O_2^{\bullet -}$ with the investigated substances (Sch. 1, A–D) were determined in pulse radiolysis experiments with spectrophotometric detection. These experiments were carried out with ELU-6 linear accelerator (Eksma, Russia) delivering nanosecond pulses of 6 MeV electrons (7 ns duration for experiments with a competing scavenger thiocyanate, SCN^-). Samples, containing either a mixture of the studied substance and SCN^- or the scavenger alone (SCN^-) were dissolved in 50 mM phosphate buffer (pH 7.4) supplemented with 50 μM DTPA as a chelating agent. Samples were pulse-irradiated in gas-tight, rectangular quartz cells with both electron and optical part measuring 1 cm. The changes in absorbance at 480 nm (λ_{max} of SCN^-) were followed at nano- and millisecond time scales. Each recorded trace was an average of 4–16 single pulses and 5 such traces were recorded for every sample (10 Gy average dose per pulse). N_2O was used for the efficient removal of hydrated electrons and doubling of the $^{\bullet}OH$ yield. The rate constants of the reaction of the generated $^{\bullet}OH$ with the investigated substances (Sch. 1, A–D) and DR were 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 reaction of $^{\bullet}OH$ with SCN^- ($1.1 \times 10^{10}\ M^{-1}s^{-1}$) [6]; k_S – the rate constant of reaction of $^{\bullet}OH$ 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 conducted at ambient temperature (22 ± 2°C) and Millipore – quality water (resistivity of about 17 MΩ) was used throughout.

Superoxide radical anion ($O_2^{\bullet -}$) was generated by pulse irradiation of O_2-saturated buffered solutions of the investigated substances in the presence of $^{\bullet}OH$-scavenger (0.1 M sodium formate) and the kinetic measurements of its reactions were performed as described earlier [14].

Protective efficiency of JSAKs against ROS action

The capacity of JSAKs to protect a model target (deoxyribose, DR) against ROS action was assayed according to Turiak et al. [47] in Fenton reaction. Briefly, the final concentrations of all reactants in 1 ml volume were as follows: potassium phosphate buffer (20 mM, pH 7.4), extemporaneously pre-

pared ferrous sulfate (20.0 μM), EDTA disodium salt dihydrate (0.1 mM), DR (0.7 mM), hydrogen peroxide (0.7 mM). The reaction was started by addition of ascorbate (0.1 mM). The investigated substance, in the concentration range between 0.05 mM –1.0 mM, was added immediately before ascorbate. EDTA disodium salt dihydrate and ferrous sulfate were premixed. The reaction mixtures were incubated at 37°C for 100 min.

Protective efficiency of JSAKs against DEP oxidation by hydroxyl radicals

The N_2O-bubbled buffered solutions of DR or DEP (0.10 to 1.0 mM, respectively, 50 mM potassium phosphate buffer, pH 7.4), without or in the presence of 0.6 mM of the investigated substances, were exposed to steady-state irradiation with ^{60}Co source to produce hydroxyl radicals, with radiation rate of 213 Gy min^{-1} as measured by ionization dosimetry. N_2O was bubled prior to irradiation to produce $^{\bullet}OH$ with a yield (G) of 5.4 (2.5 kGy final dose, scattering effect < 1%). This exposure resulted in cumulative $^{\bullet}OH$ production up to 1.35 mM.

DR and DEP oxidation assay

DR and DEP oxidation was monitored by reaction with TBA according to the method of Winterbourn [49]. One milliliter of 1% (w/v) TBA dissolved in 0.05 M NaOH and 1 ml of 2.8%. TCA were added to the samples and the reaction mixtures were heated at 102°C for 20 min. Absorbance at 532 nm were read against a blank containing both reagents and an apropriate concentrations of DR or DEP, respectively. The oxidation of targets was expressed as concentration of TBARS (thiobarbituric acid–reactive substances) using an absorbance coefficient of 153,000 $M^{-1}cm^{-1}$.

Effects of JSAKs on the DA oxidation

The peroxidative one-electron oxidation of DA (0.10 mM), in the absence or in the presence of the investigated substances (0.10–0.40 mM), was started by adding hydrogen peroxide (0.16 mM) to the reaction mixture containing LPO (60.0 nM, 20 mM potassium phosphate buffer, pH 7.4). The reaction was monitored by following time-dependent spectral changes in the 220–700 nm region and measured every 30 s with a Cary-1E (Varian) spectrophotometer using rapid scan program [44]. The reaction was completed in 3 min and subsequent spectral changes were observed over 1 h incubation time.

The non-enzymatic oxidation of DA (3.0 mM) without or in the presence of equal amounts of the investigated substances was carried out in 1 ml volumes containing KCl (0.10 M), ferrous sulfate (0.25 mM) and hydrogen peroxide (0.80 mM) [26]. Ferrous sulfate was premixed with the investigated substance and the reaction was started by addition of hydrogen peroxide. 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 decay of the formed products was observed over 1h incubation time.

RESULTS

Comparative reactivity of JSAKs against $^{\bullet}OH$ and $O_2^{\bullet -}$

As shown in Figure 1, the reactivity of JSAKs, parent nitroxyl (Nx-640), DEP and DR towards $^{\bullet}OH$ differed significantly. The investigated substances can be arranged in the following order in terms of their $^{\bullet}OH$-scavenging ability: Nx-640 > JSAK-641 > DEP > JSAK-648 (Sch. 1). The rate constants of the respective reactions were calculated using competition kinetic method and listed in Table 1. It was evident that the greater the slope (Fig. 1), the higher the rate (Tab. 1).

The best scavengers of $^{\bullet}OH$, Nx-640 and JSAK-641, were examined for their reactivity to-

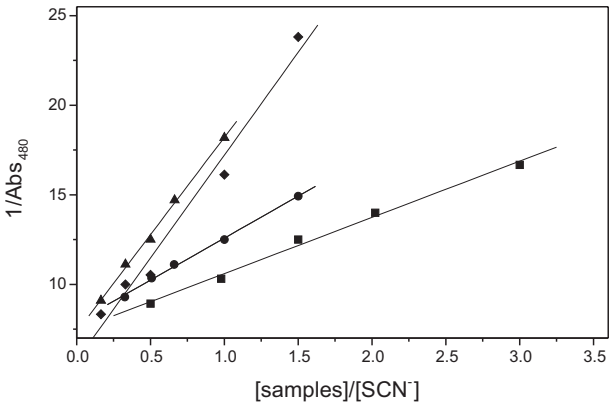


Fig. 1. Comparison of the reactivity* of Nx-640 (▲), JSAK-641 (◆), DEP (●) and JSAK-648 (■) towards hydroxyl radical ($^{\bullet}OH$). *The measurements were carried out in pulse radiolysis experiments as described in Materials and Methods and the data represent the mean ± SD of five determinations

Table 1. Rate constants of the reactions of the investigated substances and DR (model target) with •OH, based on pulse radiolysis competition data

Samples	Rate constant, $k_{samples + \bullet OH}$ ($M^{-1}s^{-1}$)
DR	2.0×10^9
DEP	7.2×10^9
Nx-640	1.77×10^{10}
JSAK-641	1.65×10^{10}
JSAK-648	5.1×10^9

Competing scavenger: thiocyanate (SCN^-), $k_{\bullet OH+SCN^-} = 1.1 \times 1010\ M^{-1}s^{-1}$ [6]. Experimental conditions are described in Materials and Methods

wards superoxide ($O_2^{\bullet -}$) (Fig. 2). Their concentration-dependent, pseudo-first order rates constant of 5.3×10^6 and $1.4 \times 10^6\ M^{-1}s^{-1}$, respectively, were calculated from the slopes of the curves shown in Figure 2.

Comparative protective efficiency of JSAKs against oxidative degradation of a model target (DR) and DEP

The protective ability of JSAKs, 641 or 648, Nx-640 to suppress the oxidative degradation of the biologically relevant model target (DR), caused by •OH generated in iron- and ascorbate-driven Fenton reaction, was also examined and compared with DEP action under the same experimental conditions. As indicated in Figure 3, the amount of TBARS formed by the oxidative degradation of DR (0.70 mM) was strongly related to the concentrations of the investigated substances in the reaction mixtures. These inhibitory effects were concentration-dependent in the concentration range between 0.05–1.0 mM (Fig. 3) and for higher concentrations a saturation effect was registered (not shown). In terms of protective efficacy, the examined substances can be arranged in the following order: ISAK-641 > JSAK-648 > Nx-640. Notably, at the concentration of 1.0 mM, JSAK-641 suppressed the oxidative degradation of DR (0.70 mM) by 75% and Nx-640- by 42% (Fig. 3). Surprisingly, the degradation of DR to TBARS was increased by 30–38% when DEP (0.40–1.0 mM) was present in the reaction mixures instead of nitroxyls. Thus, a new feature of DEP oxidation by •OH was discovered, i.e. degradation of its molecule by •OH to TBARS (aldehyde-like products). Notably, the reactions of JSAKs or Nx-640 with •OH in the absence of DR in the reaction mixtures did not result in a measurable TBARS formation.

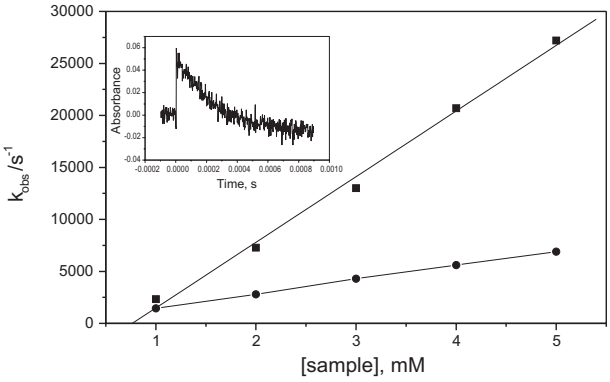


Fig. 2. The pseudo-first order rate constants (k_{obs}/s^{-1})* for the reaction of superoxide ($O_2^{\bullet -}$) versus the concentrations of Nx-640 (■) and JSAK-641 (●). Inset: typical time trace of the reaction of Nx-640 (2.0 mM) with $O_2^{\bullet -}$ (135.0 μM) generated radiolytically. *The measurements were carried out in pulse radiolysis experiments as described in Materials and Methods and the data represent the mean ± SD of five determinations

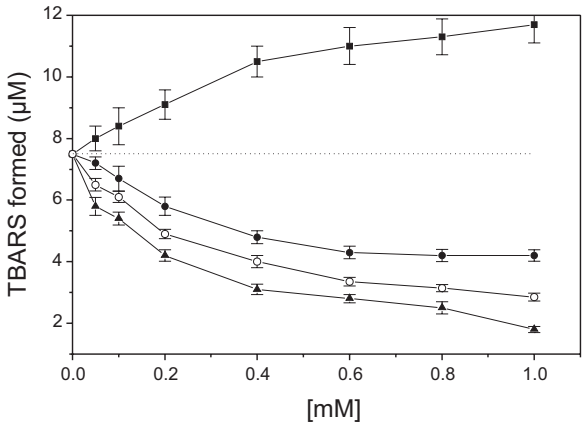


Fig. 3. Oxidative degradation of deoxyribose (DR)* in the Fenton system in the presence of increasing concentrations of deprenyl (DEP) (■), Nx-640 (●), JSAK-641 (▲) and JSAK-648 (○). *The solutions containing DR (0.7 mM) were exposed to the •OH formed in Fenton system. The procedures and measurements of TBARS were as described in Materials and Methods. The data represent the mean ± SD of three sets of experiments

In view of the above evidences (Fig. 1 and 3, Tab. 1), some other experiments were conducted: (i) to exclude the possibility that DEP *per se* might interfere with the color development in Fenton reaction mixtures and (ii) to access whether the presence of JSAKs or Nx-640 in solutions containing DEP, can protect it from the oxidative degradation caused by •OH. The exposure of N_2O-bubbled buf-

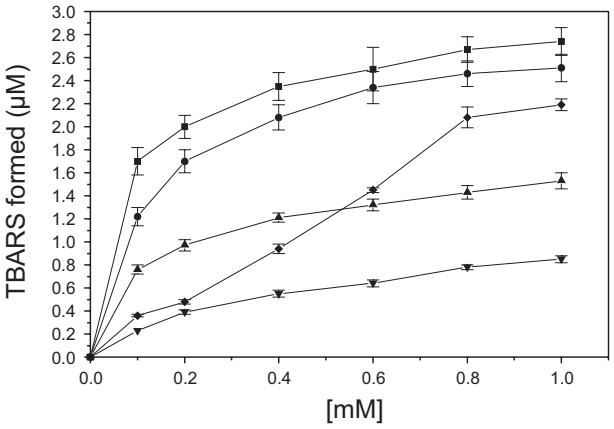


Fig. 4. Concentration-dependent degradation of deprenyl (DEP)* by hydroxyl radical (•OH) and inhibitory effects of JSAKs or Nx-640 presence into solutions**. *The •OH radicals were generated radiolytically and the procedures and measurements of TBARS were as described in Materials and Methods. The data represent the mean ± SD of three sets of experiments. **(●) DR only; (■) DEP only; (◆) DEP containing JSAK-648 (0.6 mM), (▲) DEP containing JSAK-641 (0.6 mM); (▼) DEP containing Nx-640 (0.6 mM)

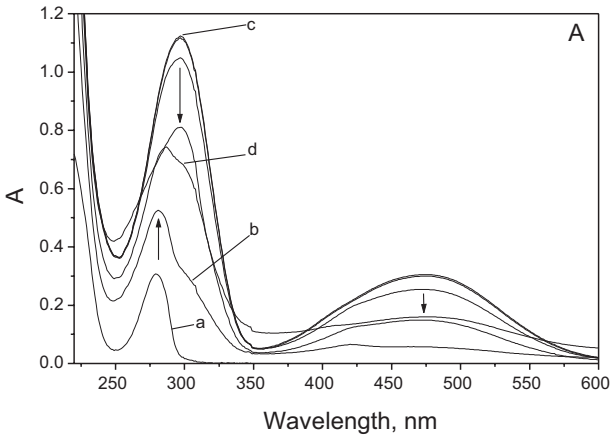


Fig. 5. Time-course of the spectral changes in the peroxidative oxidation of DA. Scans: a – DA; b – immediately (5 s) after the start of the reaction by addition of hydrogen peroxide ; b – c and d – after 3 and 10 min from the start of the reaction, respectively. The procedures were as described in Materials and Methods

fered solutions containing only DEP in physiologically relevant concentration range from 0.05 to 1.0 mM, to radiolytically generated •OH (1.35 mM), resulted in concentration-dependent TBARS formation from DEP, which was comparable to those formed from DR under the same experimental conditions (Fig. 4). As can be seen in Figure 4, the presence of 0.60 mM of JSAKs (641 or 648, respectively) or Nx-640 provided clear protection of DEP against •OH thereby indicating that they can act not only as competitive •OH scavengers (Tab. 1), but also as promising chain reaction-breaking antioxidants. Moreover, the molecule with the lowest constant rate (Tab. 1), JSAK-648, was the least protective against the oxidative degradation of DEP caused by •OH, at the concentrations of the solutes of 0.6 mM and higher (Fig. 4).

Comparative effects of JSAKs on one-electron oxidation of DA and melanochrome formation

Optical spectrum of DA (Fig. 5a) taken immediately (5 s) after the start of its peroxidative oxidation (scan b) and every 30 s for 3 min (scans b–c) showed an increase in the UV region (at 300 nm) and at 475 nm, corresponding to dopaminechrome [44]. The reaction rate of the aminochrome formation was determined to be 0.7 μMs^{-1} (molecular extinction coefficient = 3058.0 $dm^3\ mol^{-1}cm^{-1}$), from the build-up kinetics at 475 nm and the aminochrome formation was completed after 3 min (yield 93.3 μM). The subsequent spectral changes characteristic of polymerization decay reactions of aminochrome (scan c) occurred very slowly (scan d) and the process was completed over 1 h incubation time (not shown). However, the complete identification of the transients requires more extensive analysis and was not attempted here. Essentially the same results were obtained when the same experiment was repeated in the presence of JSAKs (641 or 648, respectively), Nx-640 or DEP, in the range from 0.10 to 0.40 mM (Fig. 5). The patterns, the lifetime of the transients, rate constant of the aminochrome formation and its yield did not show any significant changes. These results showed that JSAKs, Nx-640 or DEP could not accelerate or increase the formation of toxic o-quinones of DA thereby indicating that they are unable to undergo one-electron transfer reactions either with the continuously formed semiquinone radicals and its quinones.

The effects of JSAKs, Nx-640 or DEP presence on the DA oxidation by •OH generated in the model Fenton-type reaction system [26], are depicted in Figure 6. In the absence of the investigated substances (Fig. 6a), 5 min after the start of the reaction, the formation of two spectral species from DA (3.0 mM) absorbing at 600 nm and 390 nm and corresponding to melanochrome and o-dopaminequinone [26, 43] was completed. The omission

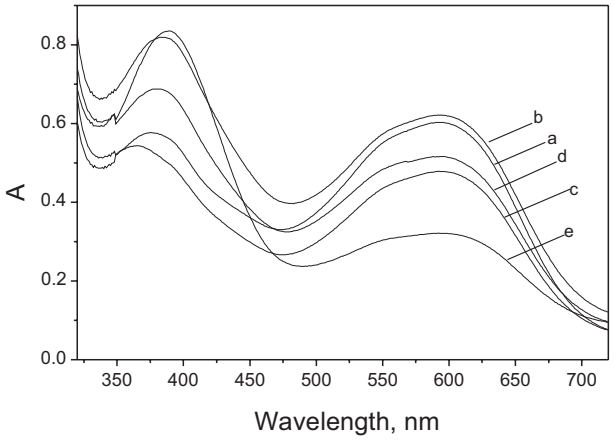


Fig. 6. Oxidation of DA (3.0 mM) in the presence of Fe(II) ions and hydrogen peroxide (a) and in the presence of the equal amounts of nitroxide (Nx-640) (b), DEP (c), JSAK-648 (d) and JSAK-641 (e). The procedures were as described in Materials and Methods

of H_2O_2 from this reaction mixture resulted in the slow formation (1 h) of only one broad peak at 510 nm (not shown), attributable to dopaminequinone-coordinated iron ions, $Fe(LH)_2^+$ [26]. The presence of Nx-640 (3.0 mM) in the complete reaction mixture caused a significant increase in absorbance at 380 nm, whereas the peak intensity at 600 nm was essentially unchanged (Fig. 6b). The presence of DEP (3.0 mM) instead of Nx-640 in the reaction mixture led to up to 25% decrease in the absorption intensity at 600 nm and shift of the absorption maximum from 380 to 350 nm (Fig. 6c). Figure 6d illustrates the mild inhibiting effect (about 15%) of JSAK-648 (3.0 mM) on the melanochrome formation in the system, correlated to the 2-fold lower absorption values at 380 nm, as compared with Nx-640 (Fig. 6b). When JSAK-641 (3.0 mM) was present in the reaction mixture, it caused a 50% decrease in the melanochrome formation accompanied by a peak at 390 nm with intensity value comparable to that of Nx-640 at 380 nm (Fig. 6e).

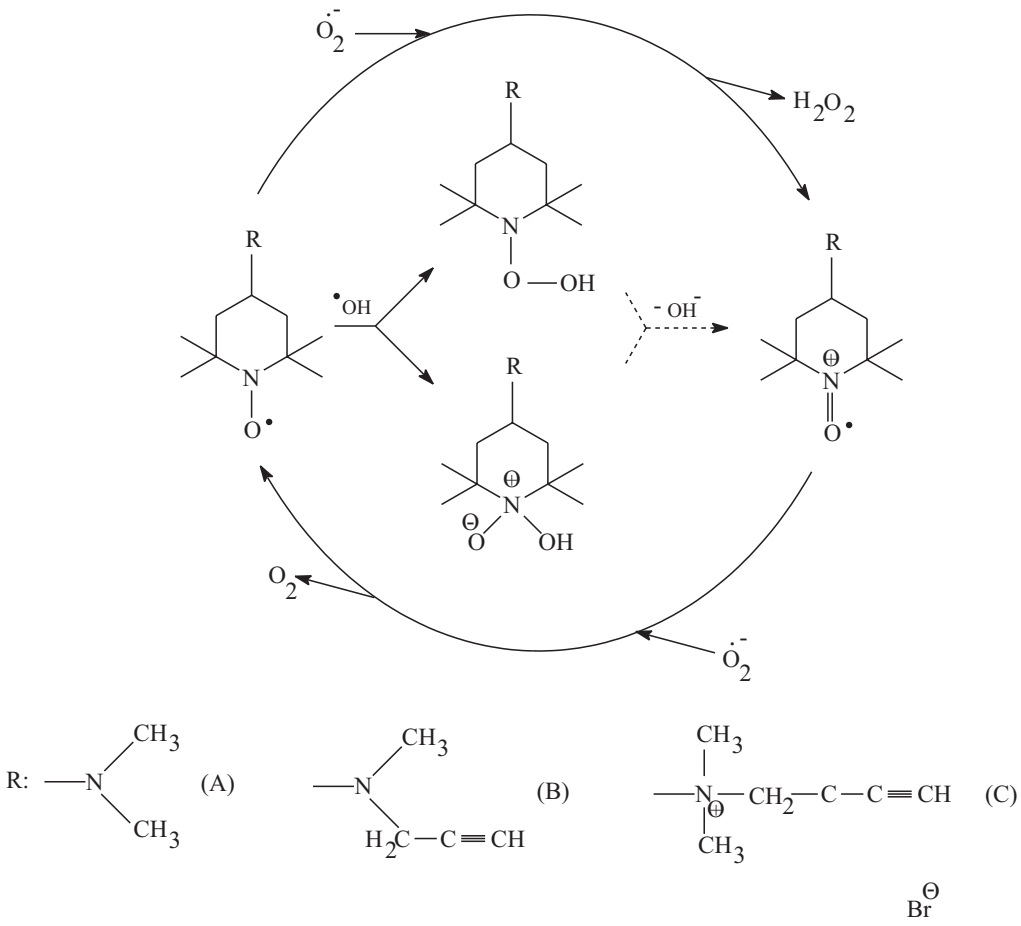
Thus, as shown in Figure 6, the investigated substances (Sch. 1) protected DA against $^{\bullet}OH$ action resulting in its melanization and their protective efficiency can be arranged in the following order: JSAK-641 > DEP > JSAK-648 > Nx-640.

DISCUSSION

An increasing number of recent reports suggest the involvement of oxidative stress in PD, where the increased formation of ROS, $^{\bullet}OH$, $O_2^{\bullet -}$, H_2O_2, leads to neural damage and cell death. This finding has led to much interest in the development of novel drugs, antioxidants, which might be of benefit in PD treatment as potential complementary or alternative compounds.

We have developed a number of novel substances, JSAKs, nitroxide- and propargylamine-related stable free radicals. The studies presented here investigated their antioxidative and protective abilities and compared their efficiency either with parent nitroxyl (Nx-640) and DEP, the drug developed for the treatment of PD and the most investigated member of adjunct therapies in clinical neurology. The chosen N-propargylamine derivatives of nitroxyl, JSAKs, 641 or 648 (Sch. 1), are members of the newly created class of antioxidant compounds with potential pharmacological application in PD. JSAK-641 and JSAK-648 show excellent *in vitro* reactivity with $^{\bullet}OH$. It is worth noting that the estimated rate constant of the reaction of JSAK-641 with $^{\bullet}OH$ was approximately one order of magnitude higher than that of the common model target (DR) and significantly higher as compared with DEP.

The results presented in this paper also demonstrated high activity of JSAK-641 and its parent nitroxide (Nx-640) devoid of propargyl group, against superoxide ($O_2^{\bullet -}$), which was almost two orders of magnitude higher than those of DEP. The comparison of the reactivity of the novel nitroxide derivatives towards $^{\bullet}OH$ and $O_2^{\bullet -}$ shows that it depends on the structure of substituents at the position 4 of the piperidine moiety of the molecules. Therefore, we can conclude that the inductive structural effect could be the crucial factor determining the reactivity of JSAKs towards oxygen radicals, as has been observed before for other nitroxide derivatives [17, 31, 33, 34, 38, 51]. Thus, design and optimization of the structure of novel antioxidants are one of the objectives of our experimental work aiming at drawing theoretical conclusions from screenings of sets of nitroxide derivatives differing in specific substituents [17, 33]. The results we report here can provide some explanations for a possible comparative mechanism(s) involved in the antioxidant potency of JSAKs which are nitroxides by its chemical nature (Sch. 2). We have proposed this oxidation, which can proceed one-electron oxidation of the nitroxide moiety of JSAK molecule by $^{\bullet}OH$, to be through (i) radical-



Scheme 2. Proposed mechanisms of antioxidative action of novel nitroxide derivatives* against hydroxyl radical (•OH) and superoxide($O_2^{\bullet-}$) actions. *A, B and C, as described in Schema 1

radical recombination the driving force in the pathways in which these compounds participated and (ii) •OH addition to the nitrogen atom of piperidine ring, followed by decomposition of the transient(s) and formation of oxoammonium cation [17, 31, 33]. It could be formed as well in direct reaction of the nitroxide moiety with $O_2^{\bullet-}$ as a part of SOD-mimic dismutation reaction [20, 33, 34, 38]. Once formed, oxoammonium cation can react with other molecule $O_2^{\bullet-}$ to reconstitute nitroxide moiety and JSAK molecule. Thus, nitroxide/oxoammonium redox couple may retain the complete antioxidative activity of JSAKs molecules by switching back and forth between themselves. Therefore, JSAKs could act as efficient, self-replenishing recyclable nitroxyl antioxidants, which may retain their capacity in the course of the oxidative damages *in situ* caused by the reactive oxygen radicals: •OH and $O_2^{\bullet-}$. Generally, a single molecular mechanism cannot account for the observed reactivity of JSAKs towards ROS and the pathways presented in Scheme 2 may not exhaust the list of all the possible antioxidant and antiradical actions of JSAKs either *in vitro* or *in vivo*, which remain to be investigated in the next future.

In this study, we have also found that •OH seems to degrade DEP irreversibly, in oxidative concentration-dependent manner, giving rise through the amphetaminergic metabolites to molecules with an aldehyde function able to react with TBA. Although to date other direct proofs for this pathway are lacking, it is consistent with the observations [46] showing a strong "TBARS-positive false effect of DEP" *in vitro* and *in vivo* experiments involving •OH. Regarding our results presented here, we may assume that JSAKs can protect DEP against •OH-caused degradative oxidation *in situ*, which implies that they may act as complementary and/or alternative protective agents. This offers a considerable advantage since JSAKs could prevent the damage

"downstream" of the generation of undesired metabolites of DEP and their side effects, which may help to find an optimal way to administer DEP in PD.

Having in mind this finding, we have focused attention on the available evidence indicating that JSAKs are able to protect targets against chain reactions of oxidation and degradation initiated by $^{\bullet}OH$, in model $^{\bullet}OH$-generating Fenton-type reaction systems [17, 26, 32, 47, 49]. Notably, although a number of different processes eventually lead to the formation of $^{\bullet}OH$ capable of oxidizing targets indiscriminately, there is some evidence that the Fenton reaction may be particularly important in the degeneration of dopaminergic neurons in PD [4, 18, 23, 26, 27, 32, 36, 37]. As has been indicated by our results, JSAKs and parent nitroxyl (Nx-640) can protect biological target (DR) against its chain oxidation by $^{\bullet}OH$ in concentration-dependent manner. Moreover, the protection is influenced by the piperidine ring substituents in their molecules. In addition, the observed protective effects appear rather to be more complex, probably due not solely to the "simple" antioxidative competitive scavenging of $^{\bullet}OH$ formed in the system. Hence, in view of the complexity of protection afforded by JSAKs and nitroxide, devoid of propargyl group (Nx-640), the unambigous interpretation of the obtained results is impossible. Thus, it is well known and widely accepted that antioxidants and protectors may act not only on primary oxidant ($^{\bullet}OH$) but also on other intermediates, at any step of the chain [18, 19, 32]. Nevertheless, it has been suggested before that nitroxides effectively and rapidly oxidize Fe(II) thereby blocking the initiation of Fenton reaction [2, 17, 38, 51]. Thus, such oxidation of Fe(II) by the nitroxide moiety of JSAKs could be attractive in view of the resulting reduced transient, bioactive hydroxylamine. The later compound can act by itself as a very good general antioxidant, detoxifying lipid radicals and breaking the radical chain reaction [20, 51] or can undergo spontaneous oxidation back to nitroxide, which in turn, may retain the complete protective capacity of JSAK molecule in the course of Fenton reacion. Therefore, we believe that the major part of the protective efficiency of JSAKs may depend on the oxidation of the Fenton reaction participant, Fe(II), thus preempting partially the formation of $^{\bullet}OH$ and its deleterious action. However, the line of evidence considered above needs an additional experimental support, but nevertheless, it has led us to assume that JSAKs should be applied and examined as protectors in *in vivo* models, to establish their effects on very early events in degeneration of dopaminergic cells of SN, where the total concentrations of iron and ROS are significantly increased and the endogenous neuroprotective processes are lost [3, 9, 41]. These model investigations are in progress in our laboratories.

It has been suggested that iron by itself is an endotoxin that mediates SN cell death in PD in reactions that generate H_2O_2 and toxic DA-o-semiquinones [9, 26, 27, 32, 36, 38, 44]. The option that drug-mediated stimulation of the mechanisms responsible for the detoxification of DA oxidation products can be a novel method of neuroprotection in PD [10], prompted us to investigate the JSAKs action in Fenton-type reaction model system [26]. Firstly, in separate experiments, we have found that none of the investigated substances, neither nitroxides nor DEP, affected the peroxidative oxidation of DA to dopaminochrome *via o*-semiquinone radical [43]. This absence of an important effect of JSAKs was taken as evidence for the lack of a direct radical-radical reaction between nitroxide moiety and the formed DA-*o*-semiquinones. Thus, it was excluded that JSAKs might inhibit the first step of the oxidative cascade of DA and *o*-semiquinone reactions yielding DA-*o*-quinone. Secondly, we have found the structure-dependent inhibitory effect of JSAKs, Nx-640 and DEP on the aminochrome-driven melanization of DA in Fenton reaction. However, at this stage of our investigations, the ultimate mechanism(s) that triggered this observed protective effects cannot be offered. We suppose that at least three modes of JSAKs action in DA oxidation could be taken into account: (i) oxidation of Fe(II)ions by nitroxide moiety [2, 51] thus blocking the $^{\bullet}OH$ formation; (ii) blocking the aberrant iron chelation by DA and its transient quinones [36] and (iii) direct reaction of nitroxide moiety with $^{\bullet}OH$ (Sch. 2) thereby terminating the DA oxidative cascade, where the first obligatory step is formation of DA-*o*-semiquinone radicals. Thus, should the possibility of experimental separation arise, the kinetic effects would be of great importance to distinguish the different pathways of the inhibition of dopaminechrome melanization, induced by JSAKs, nitroxyl (Nx-640) and DEP. Notably, a recent study [27] has also revealed a feasibility of DA hydroxylation by $^{\bullet}OH$ generated in Fenton reaction, leading to the formation of neurotoxic

6-OHDA. This potential neurotoxic sequence could be prevented by JSAKs as well, whatever their exact mechanisms of antioxidative protection are.

Accordingly, we conclude that the discovered antioxidant and protective potency of the newly synthesized N-propargylamine nitroxyls, JSAKs, alone or in "combination" with DEP, may provide a proof for the recent "concept of neuroprotective treatment strategies". Further comprehensive studies of their activity *in vitro* and *in vivo* may yield multifunctional antiparkinsonian agents for therapeutic applications in PD, where a site-specific formation of cytotoxic ROS in the basal ganglia is one of the most important neurotoxic mechanisms underlying nigrostriatal degeneration and apoptotic death of neurons.

Acknowledgment. This work was supported in part by a grant no. 6 PO4A 086 19 from the State Committee for Scientific Research, Warszawa, Poland.

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Received: November 12, 2002; in revised form: April 25, 2003.