Structure-activity relationship studies of protective function of nitroxides in Fenton system

Jolanta Glebska[1], Lukasz Pulaski[1,*], Krzysztof Gwozdzinski[1] & Janusz Skolimowski[2]
[1]*Department of Molecular Biophysics, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland*
[2]*Department of Organic Chemistry, University of Lodz, Narutowicza 68, 90-136 Lodz, Poland*
Author for correspondence (E-mail: lpulaski@biol.uni.lodz.pl)

Abstract

The aim of this study was to evaluate the effect of piperidine nitroxides and their amine precursors on deoxyribose oxidation in the Fenton system. Protecting activity of nitroxides was found to be concentration-dependent and strongly influenced by ring substituents, while secondary amines did not provide any protection. The reported results suggest a mechanism of nitroxide action through iron oxidation rather than through direct scavenging of hydroxyl radicals. Moreover, presented data point to the danger of interference of nitroxides during the TBARS assay procedure.

Abbreviations: MDA – malondialdehyde; SAR – structure-activity relationship; TBA – thiobarbituric acid; TBARS – thiobarbituric acid-reactive substances; SOD – superoxide dismutase; EPR – electron paramagnetic resonance; TCA – trichloroacetic acid.

Introduction

There is increasing evidence that free radicals, predominantly hydroxyl radicals, are involved in a variety of deleterious biological processes. Hydroxyl radicals, extremely reactive species, interact indiscriminately, in a diffusion-controlled manner, with all vitally important cell components and they are highly deleterious to biomolecules (Winterbourn 1991). In biological systems hydroxyl radicals can be produced through irradiation or chemically, via the iron-driven Fenton reaction from low-reactivity species such as superoxide anion or hydrogen peroxide (Franzini *et al.* 1994). They can attack, among others, the sugar deoxyribose forming TBA-reactive products – this reaction is commonly used as a convenient method for quantification of oxidative damage caused by hydroxyl radicals (Winterbourn 1991; Samuni *et al.* 1997). Nevertheless, the chemistry of this process is complex, making the interpretation of experimental results rather diffi-

cult due to unspecificity of the reaction (Knight *et al.* 1988).

While there is no doubt that hydroxyl radicals are formed in the Fenton system (Charloux *et al.* 1995; Lloyd *et al.* 1997), several additional possibilities are also considered, including formation either of the ferryl ions, thought to be iron(IV) species, or of the crypto-hydroxyl radicals, a rather undefined complex of organic molecules with hydrogen peroxide (Koppenol & Liebman 1984; Shen *et al.* 1992; Lloyd *et al.* 1997). These intermediates are proposed to have oxidising properties similar to free hydroxyl radicals, but to exhibit a certain degree of target selectivity (Koppenol & Liebman 1984; Shen *et al.* 1992). Besides, the idea of site-specific damages has been postulated, i.e. the site-localised reaction of hydroxyl radicals in immediate proximity to iron atoms bound to deoxyribose molecules (Gutteridge 1984; Aruoma *et al.* 1989). The relative participation of different reactive species in oxidative damage to deoxyribose molecules also remains controversial (Winterbourn 1991; Samuni *et al.*

1997). The controversies quoted above reveal the need for caution in application of the deoxyribose oxidation method for qualitative or quantitative interpretation of oxidative damage caused by Fenton oxidants. However, bearing these caveats in mind, this method remains a valid option as a sensitive, easy to perform oxidative damage assay (Aruoma 1994).

The accumulating knowledge on the participation of free radicals in many clinical and pathological conditions has consequently led to the search for new versatile antioxidants. In some pathophysiological situations, including among others radiation-induced damage to normal tissue, antracycline-induced cardiomyopathy or iron overload, supplementation with exogenous antioxidants may be necessary (Zhang *et al.* 1998). Efficient antioxidants are needed to augment the normal antioxidant defense in a variety of chronic or acute pathological conditions. A perfect exogenous antioxidant should, apart from its ability to effectively scavenge various reactive species, be efficiently regenerated to its active form. Therefore, small antioxidant molecules possessing these properties, among them nitroxides, are gaining increased interest (Krishna & Samuni 1994; Zhang *et al.* 1998; Offer *et al.* 2000).

Nitroxides are membrane-permeable, low molecular weight compounds that can protect many biological systems from diverse kinds of oxidative insults. Recently, the protective effects of nitroxides have been reported for isolated biomolecules such as enzymes, proteins, lipids or DNA (Antosiewicz *et al.* 1995; Damiani *et al.* 2000), cell cultures (Samuni *et al.* 1991; Mohsen *et al.* 1995; Udassin *et al.* 1998; Zhang *et al.* 1998), and laboratory animals (Metodiewa *et al.* 1998). The unique feature of the antioxidative capacity of nitroxides is their catalytic mode of action (Zhang *et al.* 1999). By undergoing one-electron transfer reactions nitroxides switch among three redox states, being easily reduced to hydroxylamines or oxidised to oxoammonium cations. Several mechanisms underlying the protective activity of nitroxides have been proposed including: (i) oxidation of redox-active reduced transition metals (Krishna & Samuni 1994; Zhang *et al.* 1998; Bar-On *et al.* 1999), (ii) catalytic removal of superoxide anion radicals (Krishna & Samuni 1994; Krishna *et al.* 1996; Zhang *et al.* 1998, 1999), (iii) detoxification of hypervalent metals (De Bono *et al.* 1994) or (iv) termination of radical chain reactions (Damiani *et al.* 2000). However, the implementation of antioxidants in therapeutic settings requires both a better understanding of the molec-

ular mechanisms by which free radicals exert their damaging action in disease as well as the identification of potential side effects of antioxidant molecules. Compounds behaving as antioxidants *in vitro*, but reacting unspecifically, may deleteriously influence cellular metabolism and homeostasis. Therefore, a careful evaluation of each novel antioxidant in several model systems is required before jumping to conclusions on the mode of its activity in physiological settings. Having in mind the wide range of possible nitroxide actions, we studied their protective potency against oxidising species generated in Fenton system, using the deoxyribose oxidation assay with subsequent TBARS measurement. Nitroxides have been shown to be able to efficiently react with both superoxide and hydroxyl radicals and furthermore they are capable of maintaining iron ions in their oxidised form (Krishna *et al.* 1996; Zhang *et al.* 1998; Metodiewa *et al.* 1999). Therefore, questions about the relative importance of these different modes of action for efficiency of nitroxides at protecting molecular targets against oxidative damage need to be answered.
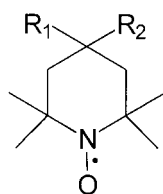
Apart from researching the mechanism of nitroxide activity, structural requirements for nitroxides with regard to providing effective protection should be studied. Some structure-activity relationship (SAR) studies with nitroxide derivatives have recently been published (Morris *et al.* 1991; Glebska & Gwozdzinski 1998; Krishna *et al.* 1998) and increasing interest towards these compounds in pharmacology makes such studies very useful on the practical level. More detailed research in isolated model systems is needed to further clarify the role of structural effects in nitroxide action. Design and optimisation of novel antioxidants is one of the objectives of SAR studies, which aim at drawing theoretical conclusions from experiments on sets of compounds differing in specific molecular structural features.
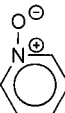
The results we report here provide explanations for possible mechanisms of nitroxide action towards inhibition of oxidative damage, as well as some information about the relationship between structure and function for nitroxide-type protectors.
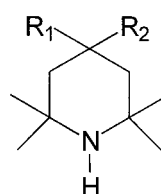
Materials and methods

Reagents

Formulas of investigated nitroxide compounds are shown in Figure 1. Tempo (2,2,6,6-tetramethylpiperi-

Nitroxide

Amine

Nitroxide	R_1	R_2
Tempo	- H	- H
Tempol	- H	- OH
CFoxyl	- H	- NHCOCF$_3$
Tempace	- H	- NHCOCH$_3$
Propioxyl	- H	- NHCOCH$_2$CH$_3$
Tempamine	- H	- NH$_2$
Tempicol-2	- OH	$-CH_2-$ (2-pyridylmethyl)
Tempicol-2-NO	- OH	$-CH_2-$ (N-oxide-2-pyridylmethyl)

Amine	R_1	R_2
compound T	- H	- NHCOCH$_3$
compound P	- H	- NHCOCH$_2$CH$_3$

Fig. 1. Structural formulas of investigated nitroxide radicals and their amine precursors.

dine-1-oxyl), Tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl) and Tempamine (4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl) were purchased from Sigma. Tempicol-2 (4-hydroxy-4-(2-pyridylmethyl)-2,2,6,6-tetramethylpiperidine-1-oxyl), Tempicol-2-NO (4-hydroxy-4-(N-oxide-2-pyridylmethyl)-2,2,6,6-tetramethyl-piperidine-1-oxyl), Tempace (4-acetamido-2,2,6,6-tetramethylpiperidine-1-oxyl), CFoxyl (4-tri-fluoroacetamido-2,2,6,6-tetramethyl-piperidine-1-ox-yl), Propioxyl (4-propionamido-2,2,6,6-tetramethyl-piperidine-1-oxyl), compound T (4-acetamido-2,2,6,6-tetramethylpiperidinium acetate) and compound P (4-propionamido-2,2,6,6-tetramethylpiperidinium propi-onate) were synthesised as described (Rozantsev 1970; Skolimowski 1999). The purity of synthe-sised compounds was assessed by NMR spectroscopy and chromatography methods. Flash column chro-matography of non-radical compounds was performed using Kieselgel 60 (230–400 mesh, Merck). Thin-layer chromatography of nitroxides was performed on silica gel plates (250 μm thickness, Merck) with fluorescent indicator. The ^{1}H NMR spectra were ob-tained at 300 MHz, ^{13}C NMR – at 75 MHz and TMS or the solvent peak(s) were applied as a ref-erence (nitroxides were studied as oxoammonium salt and N-hydroxyammonium mixture, 1: 1). EPR spectra were recorded in a X-band Bruker 200D-SCR spectrometer. Melting points (uncorrected) were determined on a Thomas-Hoover capillary melting point apparatus. Microanalyses (N) were performed at the Microanalytical Laboratory (Center for Mole-cular and Macromolecular Studies, Polish Academy of Science, Lodz). Data from analysis were ob-tained as follows: Tempicol-2, m.p. 101–102 °C, $C_{15}H_{23}N_2O_2$ (263.33); Tempicol-2-NO, m.p. 200–201 °C, $C_{15}H_{23}N_2O_3$ (279.36); Tempace, m.p. 145–

146 °C, $C_{11}H_{21}N_2O_2$ (213.30); CFoxyl, m.p. 152–154 °C, $C_{11}H_{17}F_3N_2O_2$ (266.26); Propioxyl, m.p. 85-86 °C, $C_{12}H_{23}N_2O_2$ (227.33); compound T, m.p. 202-204 °C, (the free base) $C_{11}H_{22}N_2O$ (198.31); and compound P (the free base), m.p. 86–88 °C, $C_{12}H_{24}N_2O$ (212.33).

All other chemicals were of analytical grade commercially available. Millipore Q quality water was used throughout the experiments.

Experimental procedures

Experiments were performed in 50 mM potassium phosphate buffer (pH 7.4). The Fenton-type reactive oxygen species-generating system consisted of 50 μM hydrogen peroxide and 50 μM ferrous sulfate as a source of bivalent iron cations. Ferrous sulfate stock solutions (5 mM) were freshly prepared in 10 mM HCl. Deoxyribose degradation was monitored by reaction with TBA essentially according to Halliwell et al. (Halliwell *et al.* 1987). Routinely, solutions containing deoxyribose alone (5 mM) or deoxyribose and the protecting nitroxide were exposed to the Fenton reagents (added in the following order: hydrogen peroxide, ferrous sulfate) for 30 min at room temperature. Varying the exposition time from 20 min to 40 min did not change the results. TBARS formation was measured by heating the reaction mixtures for 8 min at 100 °C with 2.8% (w/v) trichloroacetic acid and 1% (w/v) TBA. Absorbances at 532 nm were read against appropriate blanks.

In certain experiments nitroxide was added after 30 min of deoxyribose incubation with Fenton reagents.

To exclude the possibility of nitroxides interfering with the colour development stage of the TBARS assay, experiments were repeated using genuine MDA (2 μM) free from other TBA-reactive products generated from deoxyribose. MDA was obtained from tetraethoxypropane according to the method of Yeo et al. (Yeo *et al.* 1994) and quantified spectrophotometrically ($\epsilon_{245} = 13\,700\ M^{-1}\ cm^{-1}$).

Since deoxyribose oxidation studies were carried out under air, the possible involvement of superoxide radicals in the deoxyribose degradation processes was considered and tested by repeating the typical experiment in the presence of 30 U/ml of SOD (using Tempol as the model compound).

Data presentation

Raw absorbance values measured were recalculated for the adequate control and subsequently expressed in percent as relative protection:

relative protection =

100% * ([TBARS]$_c$ - [TBARS]$_n$)/[TBARS]$_c$,

where [TBARS]$_c$ denotes TBARS concentration after the TBARS assay without nitroxide (control) and [TBARS]$_n$ – in the presence of nitroxide. Results are given as means $\pm$ standard deviation (SD) of at least three separate experiments.

Differences between means were determined by Student's *t*-test. A *P* value lower than 0.05 was considered significant.

Results

The extent and the concentration dependence of protective activity towards deoxyribose oxidation in Fenton system were tested for eight nitroxide stable free radicals – three commonly used model compounds (Tempo, Tempol and Tempamine) and five recently synthesised and characterised ones, as well as for two secondary amines, which are nitroxide precursors in some types of chemical reactions, i.a. with the hydroxyl radical (Mathew *et al.* 1980; Floyd & Nagy 1984). All investigated compounds were analogues of Tempo, i.e. piperidine derivatives differing in substituents at the 4-position of the piperidine ring.

Each nitroxide radical was found to inhibit deoxyribose oxidation by Fenton reaction-derived reactive species in a concentration-dependent manner, whereas amines did not provide any inhibition even at the highest concentration used (Figure 2). Experiments were performed in the physiologically relevant concentration range from 50 μM to 1 mM, except for Tempol, where concentrations up to 10 mM were studied to determine the effect of nitroxide excess on its protective ability. Data on their potential adverse effects was considered necessary, since depending on their concentration nitroxides may also exert a prooxidative activity (Offer *et al.* 2000). As shown in Figure 2, for higher nitroxide concentrations a saturation effect can be observed. For Tempol concentrations above 3 mM no further enhancement or decrease of protection was noticed (data not shown) and the maximal protection yield was about 86 $\pm$ 2.9%. The range
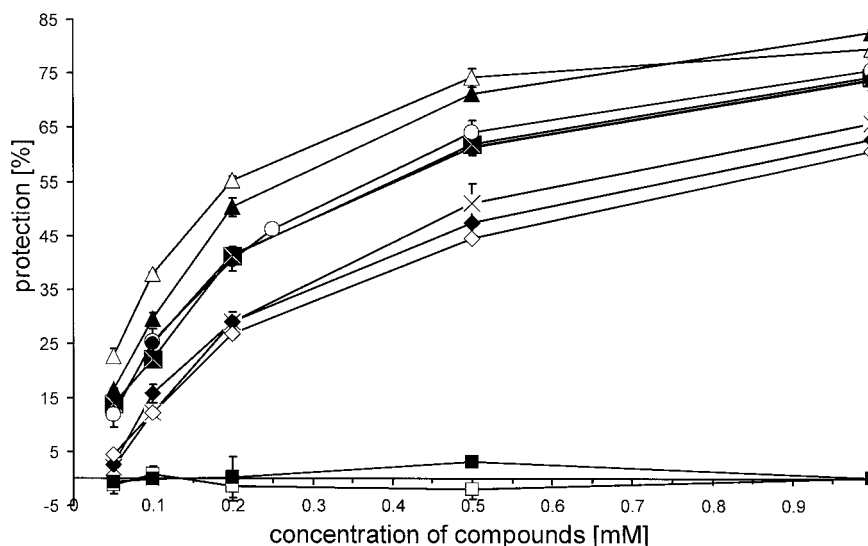
Fig. 2. Relative protection of deoxyribose against oxidation by Fenton reaction products as a function of concentration of investigated nitroxides and amines. Tempo – black crosses, Tempol – empty circles, Tempicol-2 – empty diamonds, Tempicol-2-NO – filled diamonds, Tempace – filled circles, Propioxyl – white crosses, CFoxyl – filled triangles, Tempamine – empty triangles, Compound T – empty squares, Compound P – filled squares. For detailed conditions see Experimental Section.

of concentrations at which an efficient inhibition of deoxyribose oxidation could be obtained (coming down to the micromolar level) is very important and promising in view of potential therapeutic applications of nitroxides, since concentrations in the range of tens of micromoles per litre are possible to attain *in vivo*.

A comparison of the protective capability of nitroxides shows that there is a significant dependence between the observed protection and the nitroxide structure. Among nitroxide radicals examined, Tempamine and CFoxyl were found to be the most effective in preventing oxidative damage to deoxyribose. They afforded a relatively high degree of protection – in the range of 20% at the lowest concentration used, i.e. 50 μM. Slightly less potent were Tempace, Propioxyl and Tempol. The unsubstituted Tempo as well as Tempicol-2-NO and Tempicol-2, possessing a bulky, aromatic group, were the weakest inhibitors among all nitroxides. A strong influence of the substituents at the 4-position of the piperidine ring on the extent of protective effect of nitroxides is evident – thus, the structural factor plays an important role in nitroxide action. Presented data may suggest also a lack of correlation between the effectiveness of nitroxides and the size of substituted groups – therefore electron rather than steric effects are of major importance. Nitroxides with $-NH_2$, $-NH(CO)CF_3$, $-OH$, $-NH(CO)CH_3$, $-NH(CO)CH_2CH_3$ groups as substituents, showing a marked electron-attracting tendency, were consis-

tently more efficient in protecting deoxyribose from degradation then others. Therefore we can conclude that the inductive effect of the substituent is the crucial factor determining the antioxidative potency of nitroxide radicals in this system.

Nitroxides might also be conjectured to interfere with the colour development stage of the TBARS assay. This effect can be excluded by two lines of experimentation: (i) by adding Tempol to the solution containing a known concentration of malondialdehyde and performing the routine reaction with TBA; it is known that deoxyribose oxidation by hydroxyl radicals gives at least eight products, some of which are thought to break down to MDA either on heating with TBA or in an iron-catalysed reaction prior to the heating-step; nevertheless, MDA appears to be the major TBARS product (Winterbourn 1991); (ii) by adding nitroxide after 30 min of incubation of deoxyribose with the Fenton reaction mixture; hydroxyl radicals are known to be produced in a relatively fast reaction and to react extremely quickly (Koppenol & Butler 1985), so any interference of nitroxide in reactions with their participation may be ruled out at this time-scale. Also any unstable intermediates that might be formed in the course of reaction, should be transformed into end-products after this time, since upon varying the incubation time from 20 min to 40 min we found no difference in TBARS yield (data not shown).

The results obtained indicate that Tempol does not interact directly with MDA or modify TBA reactivity towards MDA, precluding the formation of the TBA-MDA adduct and colour development upon heating. No changes in the Tempol EPR signal intensity were detectable after incubation and after heating with MDA alone or in the presence of TBA under standard experimental conditions. Also MDA absorbance (without TBA) as well as the terminal pink chromogen yield were unchanged in studies of interaction between MDA and Tempol (data not shown).

However, as can be seen in Figure 3, Tempol added 30 min after incubation of deoxyribose with the Fenton reagents still provided a certain, albeit decreased, degree of putative 'protection'. This 'late protection' effect may point to possible involvement of residual ferrous ions in catalysing degradation of intermediate deoxyribose oxidation products to MDA and other TBARS during the final heating stage of the TBARS assay. The exact chemistry of the nitroxide 'protective' action at this stage is, however, not known. The observed effect appears rather more complex, probably due not solely to a 'simple' antioxidative activity. Besides, heating a solution containing deoxyribose, nitroxide and Fenton reagents with TBA-TCA mixture caused a complete loss of nitroxide EPR signal, whereas only a non-significant decrease of signal intensity was observed after incubation before heating both with and without TBA and TCA (data not shown). It has been reported that in a trifluoroacetic acid solution, some nitroxides can be converted into their respective oxoammonium cations and hydroxylamine compounds as a 1:1 mixture (Malatesta & Ingold 1973). On the other hand, under physiological-like conditions the comproportionation between oxoammonium cation and hydroxylamine (yielding two nitroxide radicals) is thought to be a fast and spontaneous process (Zhang et al. 1998).

Hence, in view of the complexity of this stage of TBARS assay, the unambiguous interpretation of presented results is impossible and the observed inhibition of TBARS production may be attributed either to additional protection afforded by nitroxides upon heating (e.g. deoxyribose oxidation pathway could involve the iron-dependent breakdown of intermediates to MDA) or to an additional effect of other factors leading to a small decrease in measured absorbance values and not caused by actual nitroxide activity.

Our results underline the need of caution to be taken when interpreting data on inhibition of TBARS production as protection from the action of hydroxyl radicals.

In the presence of superoxide, ferric ions are reduced back to ferrous ions and iron becomes capable of catalysing the production of hydroxyl radicals. Although the reaction of molecular oxygen with ferrous ions is initially slow, if conditions favour the establishment of a radical chain reaction, then the eventual results are similar to the effects of adding hydrogen peroxide (De Bono et al. 1994). Nitroxides might exert an additional protective effect by removing superoxide from the reaction environment, since the redox couple nitroxide/oxoammonium cation has been reported to act as a mimic of SOD (Krishna et al. 1996; Zhang et al. 1998). This alternative mode of protection by nitroxides was investigated using authentic SOD, which is capable of competing with nitroxides for superoxide. The addition of 30 U/ml SOD to the reaction mixture caused a surprising increase in TBARS formation (Figure 4). Similar results were previously described in literature (Winterbourn 1991). In the case of this somewhat unexpected SOD effect, it is not clear whether the reaction has not been affected in a complex way by the superoxide radical. Nevertheless, the most likely explanation of aberrant behaviour of the system upon SOD addition is that in some cases SOD itself may exert a prooxidative effect as it was also shown for high nitroxide and very low superoxide concentrations (Offer et al. 2000).

The presence of SOD did not lead to any significant changes in the extent of protection provided by Tempol (Figure 4) – the total increase of TBARS production was equal to the alteration seen after addition of SOD alone. Thus, the nitroxide protection yield is the same as without SOD, since only the additive effect is observed. The ultimate result of nitroxide action would be expected to change significantly upon competition with SOD due to elimination of some possible pathways of action, if nitroxide activity towards superoxide had an important role in its protective mechanism in the investigated system. Thus, the activity of nitroxides based on their SOD-mimic potency may be excluded in this system.

Discussion

Although the deoxyribose assay is sensitive and simple to perform, it is not easy to interpret mechanistically or to identify the species responsible for deoxyribose oxidation. This reaction is a chain process
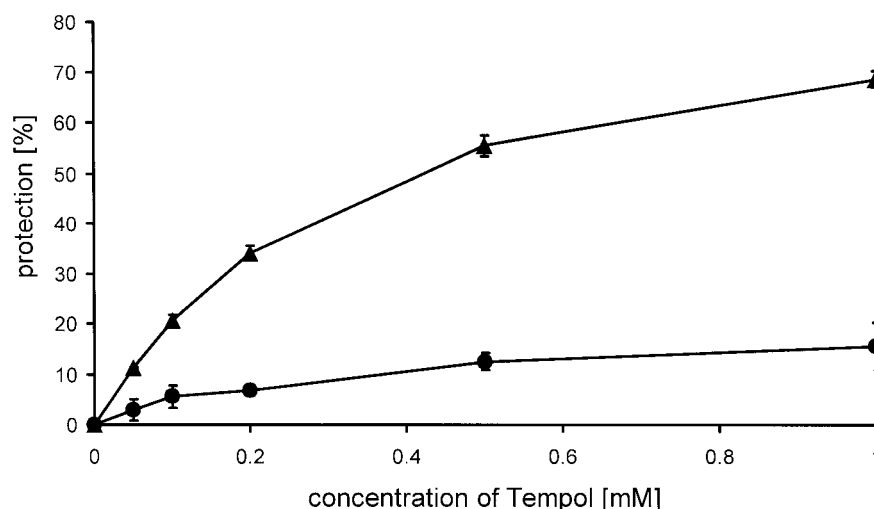
Fig. 3. Effect of time point of addition of Tempol upon its protective efficiency towards deoxyribose in Fenton system. Triangles – Tempol added to deoxyribose solution before Fenton reagents, circles – Tempol added after incubation with the Fenton system, before the TBARS assay. For detailed conditions see Experimental Section.

(Winterbourn 1991), therefore protective agents may act not only on primary oxidants, but also on other intermediates at any chain step. The effect of nitroxides can generally be attributed either to (i) scavenging the Fenton oxidants before they can interact with the susceptible target (deoxyribose) or to (ii) reaction with iron(II) precluding a potential chain reaction sequence in the deoxyribose oxidation process.

The possible chemical mechanisms involved in the protective effect of nitroxides are suggested below. The Fenton reaction is generally thought to yield strong oxidants capable of oxidising targets and the differences in reactivity of target molecules towards these oxidants are of great importance for the reaction course, since any compound present in the reaction mixture will compete for hydroxyl radicals and/or ferryl ions. The extent at which oxidants react with a specific target is a function of the rate constants for reactions involved and of concentrations of molecules presented as targets. It is therefore worth noting that the rate constants for reactions between hydroxyl radical and nitroxide derivatives (e.g. $2.6 \pm 0.5 \times 10^{10}$ M^{-1} s^{-1} for Tempace) (Metodiewa *et al.* 1999) are approximately ten times higher than for the reaction between hydroxyl radical and deoxyribose (2.5×10^9 M^{-1} s^{-1}) (Winterbourn 1991). This implies that nitroxides may act as powerful protecting agents operating in a concentration-dependent manner. One-electron oxidation of nitroxide by hydroxyl radical leads to the formation of oxoammonium cation, presumably via a radical-radical recombination

process and/or via hydroxyl radical addition to the nitrogen atom, with subsequent decomposition into the oxoammonium cation and the hydroxyl anion (reaction 1) (Rozantsev 1970; Deffner & Schimmack 1976; Metodiewa *et al.* 1999).

Nitroxides have also been found to reduce ferryl ions (depicted below as iron(IV) complexes) and this reaction also leads to the formation of oxoammonium cation (reaction 2) (De Bono *et al.* 1994).

However, the mechanism of the reaction between nitroxide derivatives and the hydroxyl radical is not quite clear yet. Since abstraction of a hydrogen atom from an organic molecule is a well established mode of action for the hydroxyl radical, it cannot be excluded that also some nitroxide derivatives undergo this process by losing a hydrogen atom from their side-chain substituent (Deffner & Schimmack 1976). The oxoammonium cation, resulting from the oxidation of nitroxide, may form a sink for removal of nitroxides from redox cycling, but under certain conditions its reduction back to nitroxide cannot be finally excluded (reaction 3). On the other hand, the results obtained from experiments with addition of SOD show that the involvement of superoxide radicals (according to reaction 3 and 4) in the studied model is probably of limited relevance and should not be considered significant.

The hydroxyl radicals that escape scavenging by nitroxide become available to attack deoxyribose molecules. In that case, two scenarios are feasible: (i) deoxyribose degradation resulting from attack of
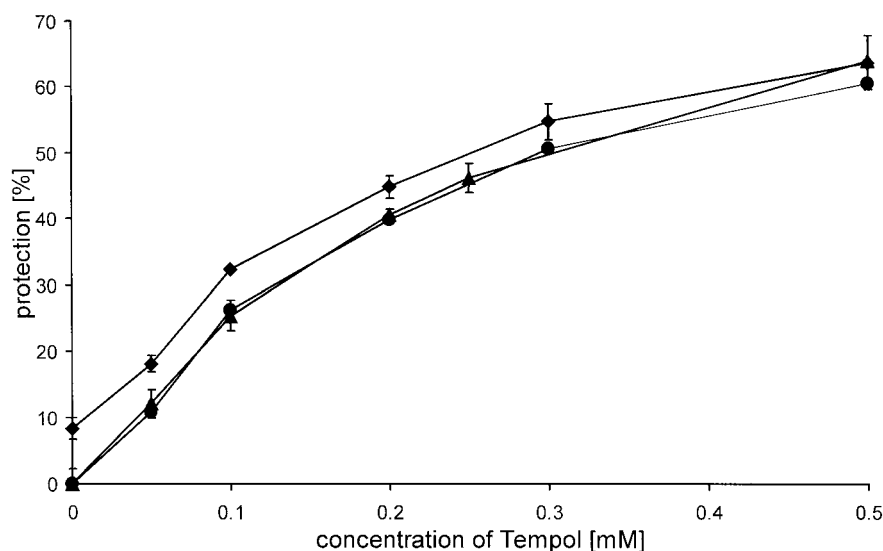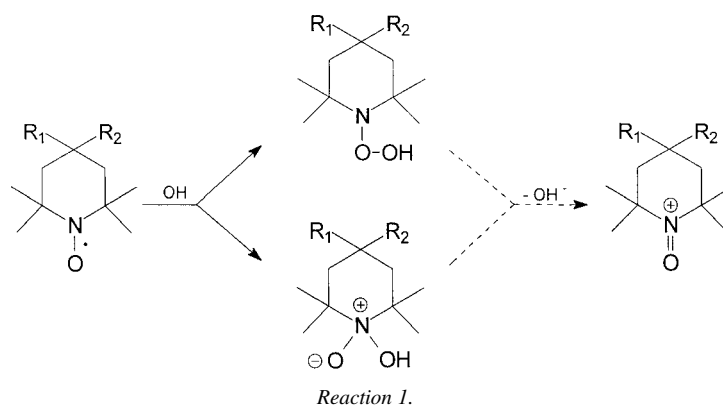
Fig. 4. Effect of SOD upon relative protection of deoxyribose from oxidation, provided by Tempol. Triangles – Tempol alone, diamonds – Tempol in the presence of 30 U/ml SOD, circles – Tempol in the presence of 30 U/ml SOD corrected for the effect of SOD alone. For detailed conditions see Experimental section.
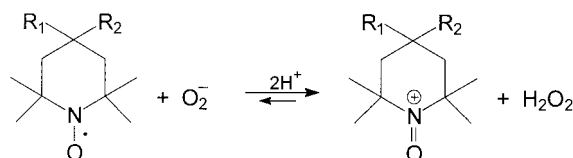

Reaction 1.

free hydroxyl radicals or (ii) deoxyribose degradation resulting from site-specific hydroxyl radicals generation close to the deoxyribose molecule. Data presented here suggest that hydroxyl radicals and/or ferryl ions might not be the only damaging species against which nitroxides are able to provide protection. The relatively high protection yield of nitroxides as well as the total lack of protective effect of amines (Figure 2) might indicate that the investigated compounds could act not only as efficient, recyclable hydroxyl radical scavengers, but also as effective inhibitors of oxidation process in a different manner. Indeed, nitroxides can compete with hydrogen peroxide and oxidise reduced metal ions (here ferrous ions), thus preventing their involvement in Fenton reactions (reaction 5).

Generally, reagents capable of oxidising reduced transition metal ions operate in a stoichiometric manner. Consequently, in that case the inhibitory effect of nitroxides should also be concentration-dependent just as for direct scavenging of hydroxyl radicals and this feature cannot be used for distinguishing between these two processes. Hydroxylamine, when produced, has a propensity to undergo spontaneous oxidation back to nitroxide under the influence of a wide variety of oxidants, including molecular oxygen. This effect might be responsible for an enhancement of the protective effect and the nitroxide/hydroxylamine redox couple may retain its antioxidant capacity in the course of the reaction.
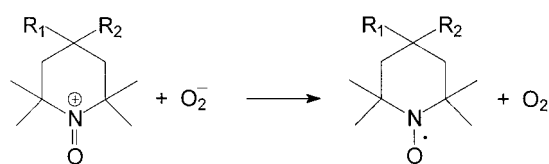
Thus, when considering an explanation for the protective effect of nitroxides against deoxyribose ox-
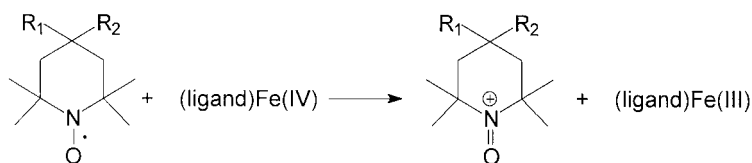
Reaction 2.

Reaction 3.

Reaction 4.

idation in an iron-driven Fenton reaction, at least two modes of nitroxide action should definitely be taken into account: (i) scavenging of Fenton oxidants, thereby terminating the chain reaction sequence, and (ii) blocking the initiation of Fenton reaction by oxidation of ferrous ions.

Accordingly, nitroxides can operate through two different catalytic pathways including reductive (reaction 5) and oxidative (reaction 1 and 2) conversions. If the possibility of experimental separation of these two pathways should arise, kinetic effects would be of great importance for their relative importance and the domination of one mechanism would depend on the ratio of respective reaction rate constants.

The rate-determining step in deoxyribose oxidation by hydroxyl radicals is their generation in the Fenton reaction, since the rate constant for the oxidation of ferrous ions by hydrogen peroxide is dependent on pH and values in the range of 10^2 M^{-1} s^{-1} or greater may be found in literature (Koppenol & Butler 1985; Miller *et al.* 1990). At the same time, rate constants for oxidation of ferrous ions by investigated nitroxides vary from 10^3 M^{-1} s^{-1} to 6.5×10^3 M^{-1} s^{-1} under conditions similar to used in this study (50 mM PPB, pH 7.0) (Glebska *et al.*, in preparation). Comparison of quoted rate constants suggests that the oxidation of iron(II) by nitroxides may in some conditions have a significant or even dominant impact. Therefore, we consider that the major part of protective efficiency

against Fenton-type reactions afforded by nitroxides can be caused by their ability to oxidise reduced metal ions, thus pre-empting metal participation in Fenton chemistry.

These conclusions are in accordance with some recently published data stemming from investigations of inhibition of lipid peroxidation by other nitroxides (Nilsson *et al.* 1989; Samuni *et al.* 1997), whereas another recent report contains discordant data and inferences (Damiani *et al.* 2000).

More detailed result analysis as well as other bodies of data provide further support for our conclusion. Notably, sequence of reactivity of nitroxides similar to the one reported here was found previously for reactions including reduction with Fe(II) (Bar-On *et al.* 1999; Glebska *et al.* in preparation) and with ascorbic acid (Morris *et al.* 1991; Glebska & Gwozdzinski 1998). Nitroxides with high resistance to reduction exhibit a considerably lower degree of protection against deoxyribose oxidation, suggesting that the same reaction pattern may be assumed for this two processes. It would be hardly expected for nitroxides to show the same reactivity order while taking part in two processes as different as oxidation and reduction of the nitroxyl group. Since a decrease of electron density on the reactive group should facilitate its reduction, but not oxidation, the observed dependence between the protection yield and the electron inductive effect of the substituent suggests that reductive mode of nitroxide action is here preferred.

Besides, the proposed mechanism – oxidation of Fe(II) – is attractive in view of nitroxide redox chemistry. The resulting reduced nitroxide derivative, the hydroxylamine, can be oxidised back to nitroxide and is capable of modifying the protected molecule (damaged intermediate of deoxyribose) in the process in such a way that it cannot turn into TBARS anymore, thus providing also secondary scavenging-type protection at this stage. It should be stressed that no unequivocal and final conclusion is possible based on data presented here. Nevertheless, observed effects appear to rule out the possibility of a simple antioxidative mechanism based on a scavenging reaction only.
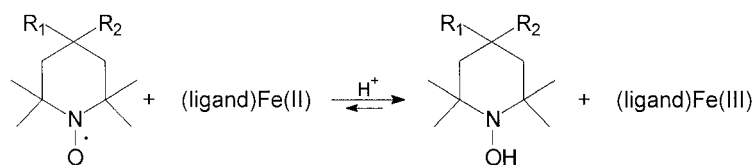
Reaction 5.

Results reported here provide further support for the above conclusion. Interesting assumptions about the mechanism of deoxyribose oxidation and its prevention may come from the fact that secondary amines were ineffective in providing protection against Fenton system-derived oxidants. Compounds T and P did not exhibit any protective activity under our experimental conditions even at the highest concentrations tested. Amines do not react with iron ions, therefore a somewhat lower protective efficiency then their respective nitroxide derivatives was expected for them, but the complete lack of protection was surprising in the light of our knowledge about their chemistry. Our results are, however, in relatively good accordance with recently reported data on investigations of some nitroxides derivatives (Samuni *et al.* 1997; Krishna *et al.* 1998).

Amine compounds of this type were previously reported to scavenge hydroxyl radicals. Moreover, a certain fraction of the initial amine substrate can be converted into the nitroxide free radical. This process might take place either by (i) hydroxyl radical addition to the amine nitrogen followed by the loss of a water molecule and subsequent hydroxylamine oxidation or by (ii) abstraction of the amine hydrogen resulting in a nitrogen-centered radical which subsequently undergoes oxidation (Mathew *et al.* 1980; Floyd & Nagy 1984). The extent of these reactions will be dependent on experimental conditions and other reactions involving the participation of substituents are of importance (Mathew *et al.* 1980). Under experimental conditions similar to the ones used in this study (50 mM PPB, pH 7.0), compound T was found to scavenge radiolytically produced hydroxyl radicals with a rate constant of $5.0 \pm 0.3 \times 10^9$ M^{-1} s^{-1}, which is about twice higher than for the reaction of hydroxyl radicals with deoxyribose (Metodiewa *et al.* 1999).

This could be an argument against free hydroxyl radicals as the major species removed by nitroxides, especially since under conditions of high hydroxyl radical flux amines should be oxidised and provide at least some degree of protection. Hence deoxyribose degradation by reactive species produced site-specifically in the vicinity of the damaged molecule seems a likely assumption. Indeed, the lack of inhibition by hydroxyl radical scavengers may be characteristic for site-specific Fenton reaction. According to the site-specific model, the rate of degradation of deoxyribose molecules is a function of both the rate constant of reaction with hydroxyl radicals and the iron chelating activity of the carbohydrate.

Thus, the ability of a scavenger to inhibit deoxyribose damage inflicted in a site-specific manner depends additionally on its ability to chelate iron, whereas to this time there is no evidence of any stable amine-metal and/or nitroxide-metal complex.

On the other hand, our speculations are not in good agreement with those presented by Winterbourn (Winterbourn 1991) including the assumption that only a very small amount of iron ions (less than 3%) would be bound to deoxyribose under our experimental conditions, while the rest would remain complexed with phosphate ions. In this case, deoxyribose would be degraded mainly by hydroxyl radical produced in the reaction of iron-phosphate complexes with hydrogen peroxide. However, our conclusion may be supported by the previously reported data obtained from gamma radiolysis investigations (Franzini *et al.* 1994).

Taken together, the results reported here indicate that the requirements for the nitroxide derivative to be effective in providing protection in Fenton system are predominantly influenced by ring substituents and the oxidation state. Finally, the most likely assumption is that nitroxides protect deoxyribose molecules against oxidative damage caused by Fenton system through oxidation of reduced metal ions (reductive conversions of nitroxides; reaction 4) rather than by direct scavenging of Fenton oxidants (oxidative conversions of nitroxides; reactions 1, 2). Additional experimental support for our conclusions is needed, especially comparative studies of other models (e.g. mannitol) as well as other techniques including radiolysis.

The relationship between structure and function has been preliminarily explored in this study and its significance underlined in view of proposed reaction mechanisms. The important role which nitroxides

might play in practical prevention of damage involving the participation of reduced metal ions increases the weight of such studies, both in chemical synthesis and *in vivo* experimental design. This type of studies on these promising antioxidants may provide a basis for further investigations and pharmacological applications. We have proven the importance of structural effects of the substituent for the reactivity of the nitroxide moiety. Novel nitroxides, even more effective in their clinically relevant protective activity, may be designed keeping this paradigm in mind. Such custom-designed nitroxides might be especially good candidates for future development of new therapeutic techniques.

Summary conclusions

The results presented in this work show that the major features of inhibition by nitroxide derivatives of deoxyribose oxidation by Fenton system products were: (i) a major impact of the oxidation state of the derivative (nitroxide vs. amine), (ii) a significant influence of the ring substituents and (iii) an increase in protection with increasing nitroxide concentration. Some lines of evidence, including the lack of protection afforded by amines, support the hypothesis that the observed protective effect may be attributed rather to oxidation of Fe(II) and subsequent blocking of iron-dependent processes crucial for production of damage-inducing oxidants than to any processes connected to putative scavenging of hydroxyl radicals and/or ferryl ions. The main conclusion from structure-activity relationship analysis was the significant dependence between protection provided by a nitroxide and the electron inductive effect of its substituents.

Results presented here enhance our knowledge of nitroxides action in providing protection against Fenton system derived oxidation damage. As a consequence, nitroxides may be considered as complementary and/or alternative compounds for combating oxidative damage by acting rather as potent preventive antioxidants than as chain-breaking antioxidants.

Acknowledgement

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