Current Topics in Biophysics 2000, 24(2), 57–63

NITROXIDES AS PROTECTORS AGAINST OXIDATIVE DAMAGE INDUCED BY THE FENTON SYSTEM

JOLANTA GŁĘBSKA*, KRZYSZTOF GWOŹDZIŃSKI

Department of Molecular Biophysics, University of Łódź, Łódź, Poland

The protective effect of nitroxides, six-membered piperidine derivatives, on deoxyribose oxidation to thiobarbituric acid-reactive substances by the Fenton system consisting of hydrogen peroxide and ferrous ions has been studied. Several newly synthesized nitroxides, analogs of Tempo (2,2,6,6-tetramethylpiperidine-1-oxyl), containing various substituents at the 4-position were tested and compared to Tempo. All nitroxides were found to be highly efficient antioxidants inhibiting the deoxyribose oxidation process in a concentration-dependent manner, while the extent of protection provided by nitroxides depends strongly upon their structure. The data obtained suggest that the oxidation of iron(II) rather than scavenging of hydroxyl radicals is the main mechanism of nitroxide protective action under the assay conditions used. Thus, the proven potency of nitroxides as good hydroxyl radical scavengers in simple systems may have little relevance in biological systems.

INTRODUCTION

Reactive oxygen species (ROS), including superoxide radical anions, hydroxyl radicals, as well as oxidants such as hydrogen peroxide have been implicated in the pathogenesis of various types of diseases. Biologically, three important ways of producing ROS are through enzyme systems, by autooxidation and by the Fenton reaction (Saran & Bors, 1990; Winterbourn, 1991a; Candeias, Folkes & Wardman, 1995). The Fenton reaction, i.e. the conversion of hydrogen peroxide and/or superoxide to hydroxyl radicals catalyzed by metal ions, is the major pathway for generation of highly reactive, hazardous hydroxyl radicals, leading to the oxidative damage of biological macromolecules (Candeias *et al.*, 1995; Winterbourn, 1991a; Shen, Tian, Li, Li & Chen, 1992). The exact mechanism of this process and nature of species involved in this reaction system are not well understood yet and are also strongly dependent on the system investigated, i.e. the target molecules studied (Winterbourn, 1991a; Franzini, Sellak, Hakim & Pasquier, 1994; Winterbourn, 1991b).

If radical processes spread within the cells, endogenous antioxidants may interfere preventing the formation and/or deleterious action of ROS, what would limit the injury of critical cellular components. Under normal physiological conditions, the protection provided by cellular defense systems is sufficient, but in most pathological situations complementation by exogenous antioxidants is necessary to minimize the oxidative disruption by free radicals of cellular metabolism and homeostasis.

Recently, much physiological and pharmacological interest has been focused on nitroxides, low molecular-weight cell-permeable stable radicals, which may be considered as promising therapeutic agents against oxidative injury (Zhang, Pinson & Samuni, 1998; Hahn, Krishna & Mitchell, 1994). In order to suppress or control the oxidation of biological macromolecules it is essential to understand the fundamental aspects of protective actions of a potential antioxidant. Nitroxides easily undergo one-electron redox reactions and they can act in a catalytic fashion as self-replenishing antioxidants (Krishna & Samuni, 1994; Krishna, Russo, Mitchell, Goldstein, Dafni & Samuni, 1996a). Nitroxide radicals were found to exhibit superoxide dismutase activity (Krishna, Samuni, Taira, Goldstein, Mitchell & Russo, 1996a; Zhang, Goldstein & Samuni, 1999) and to scavenge other free radicals without forming any reactive secon-

*Corresponding author; e-mail: jolka@taxus.biol.uni.lodz.pl

Abbreviations: ROS – reactive oxygen species; MDA – malondialdehyde; TCA – trichloroacetic acid; TBA – thiobarbituric acid; TBARS – thiobarbituric acid-reactive substances; DR – deoxyribose; >N˙–O and Nx – nitroxide radical; >N–OH – hydroxylamine; >N+=O – oxoammonium cation

dary species, what prevents free radical-induced damage (Damiani, Kalińska, Canapa, Canestrari, Woźniak, Olmo & Greci, 2000; Metodiewa, Kochman, Skolimowski, Gębicka & Koska, 1999). Moreover, nitroxides may also protect targets from biological injury by detoxifying metals, either reducing hypervalent metals (Krishna *et al.*, 1996b) or oxidizing reduced transition metal ions (Krishna & Samuni, 1994; Bar-On, Mohsen, Zhang, Feigin, Chevion & Samuni, 1999) that could potentiate deleterious effects by producing ROS.

To further our knowledge of the antioxidant behavior of this class of nitroxide compounds, and before extending their use into more complex biological systems such as living cells and whole animals, it is essential to have more information about their operation in simple model systems.

These experiments were performed to determine the ability of a variety of nitroxides to react with oxidizing species generated chemically in Fenton system. Because of the high reactivity of hydroxyl radicals which interact indiscriminately, in a diffusion-controlled manner with all vitally important cell components, we examined the capacity of nitroxides to protect a model target such as deoxyribose. The 'deoxyribose assay' is a sensitive and convenient method for oxidative damage quantification by measurement of the aldehyde end-products formation from deoxyribose (Aruoma, 1994; Winterbourn, 1991b).

The results presented here show a significant dependence between structure and activity of nitroxide compounds ascribed to the effect of substituent. Besides, the possible mechanisms responsible for nitroxide potency against Fenton system-derived oxidative damages are discussed.

MATERIALS AND METHODS

Materials

Chemical structures of investigated nitroxides are shown in Fig. 1.

Tempo (2,2,6,6-tetramethylpiperidine-1-oxyl) and Tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl) were purchased from Sigma (Poznań, Poland) whereas 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) and Tempace (4-acetamido-2,2,6,6-tetramethylpiperidine-1-oxyl) were synthesized in our laboratory by the methods described previously (Metodiewa, Skolimowski, Kochman, Gwoździński & Głębska, 1998; Rozantsev, 1970). All other chemicals used were of the highest quality commercially available.

Fenton reaction

A typical experimental procedure was as follows: the reaction mixture containing potassium phosphate buffer (50 mM, pH 7.4), deoxyribose (1, 5 or 10 mM) and an appropriate concentration of nitroxide was exposed to the Fenton system consisting of 50 μM hydrogen peroxide and 50 μM ferrous sulfate for 30 min at room temperature. Reactions were started by addition of hydrogen peroxide and subsequently the iron salt. Stock ferrous sulfate solutions (5 mM) were prepared in 10 mM HCl immediately before use. Nitroxide concentration ranged from 0 (control) to 1 mM, except for Tempol, where concentrations up to 10 mM were studied to determine the effect of high nitroxide concentrations on its protective ability.

Deoxyribose oxidation assay

Deoxyribose degradation to thiobarbituric acid-reactive substances (TBARS), mainly to malondialdehyde (MDA), after exposure to oxidizing species generated chemically in Fenton system was monitored by the method described by Halliwell and Gutteridge (Halliwell & Gutteridge, 1981). TBA-reactivity was measured as follows: 1 ml of 2.8% (w/v) trichloroacetic acid (TCA) and 0.5 ml of 1% (w/v) TBA in 50 mM NaOH were added to the reaction mixture with subsequent heating at 100°C for 8 min for production of the TBA-MDA adduct. The absorbance of the final pink chromo-

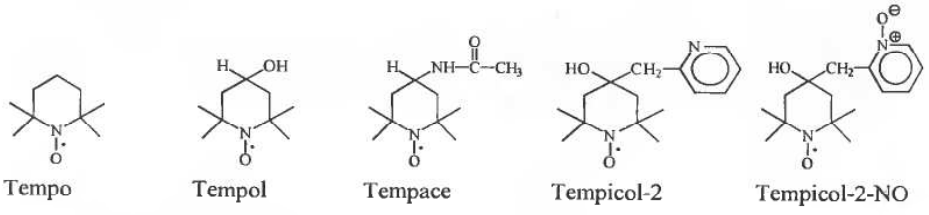

Fig. 1. Chemical structures of investigated nitroxides

gen was assessed against an appropriate blank at 532 nm and deoxyribose oxidation was expressed as TBARS formation.

Data presentation

Inhibition by nitroxide of deoxyribose oxidation was expressed in percent as relative protection and calculated from the following formula:

$$\%P = \frac{[TBARS] - [TBARS]_{Nx}}{[TBARS]} \times 100\%,$$

where [TBARS] and $[TBARS]_{Nx}$ denote TBARS concentration without (control) and in the presence of nitroxide, respectively. Results are given as means ± standard deviation of three or more separate experiments. The differences between means were determined by Student's "t" test and P values lower than 0.05 were considered significant.

RESULTS AND DISCUSSION

In this study we verified the antioxidant efficiency of nitroxides, piperidine derivatives differing in substituents at the 4-position of piperidine ring, against oxidative damage to deoxyribose exposed to the Fenton system. Three newly synthesized nitroxides, analogs of Tempo: Tempicol-2, Tempicol-2-N-oxide and Tempace, as well as others used more commonly, Tempo and Tempol were tested. The relative protection by nitroxides estimated in comparison to the control, nitroxide free sample, is displayed in Fig. 2 and 3. The extent of nitroxide action was investigated in the concentration range which is achievable *in vivo*, i.e. 50 μM –1.0 mM, apart from Tempol, where concentrations up to 10 mM were tested to verify the influence of nitroxide excess on its protective ability, since adverse effect of nitroxides have also been observed at high concentrations (Offer, Russo & Samuni, 2000).

As shown in Fig. 3, under these experimental conditions Tempol did not show any prooxidative effect and above 3 mM of Tempol relative protection achieved a maximal, constant value of ca. 86%. All nitroxides inhibited deoxyribose oxidative degradation in a concentration dependent way suggesting a stoichiometric course of the reaction underlying this process with respect to nitroxide.

The results presented in Fig. 2 clearly demonstrate the influence of the nitroxide structure on its protective efficiency. In general the antioxidant activity of nitroxides followed the order related to the electron inductive effect of piperidine ring substituents at the 4-position. Markedly, the ni-

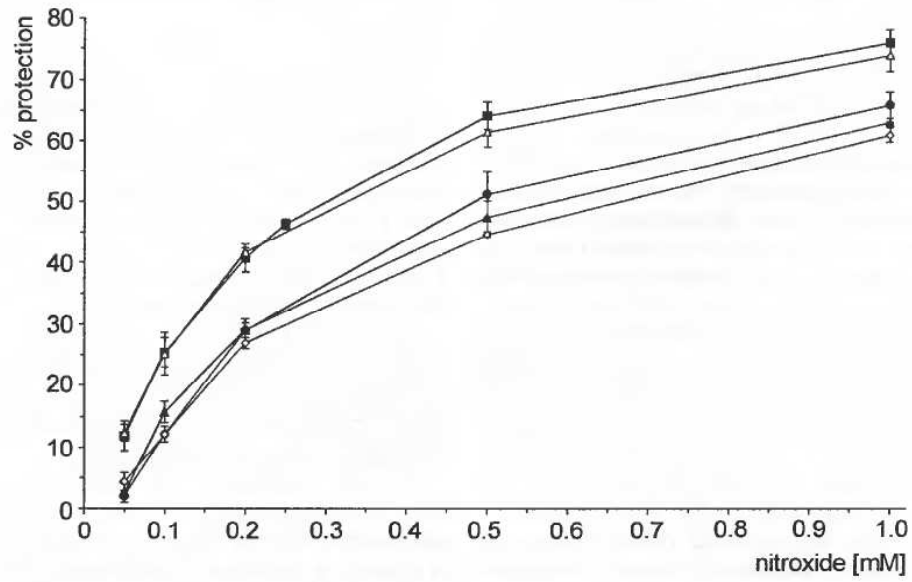

Fig. 2. Relative protection (%) of deoxyribose against oxidative degradation by the Fenton system. The reaction mixture containing deoxyribose (5 mM) and various concentrations of a nitroxide (Tempo, black circles; Tempol, black squares; Tempicol-2, empty diamonds; Tempicol-2-NO, black triangles and Tempace, empty triangles) was exposed to the Fenton system mixture (50 μM H_2O_2, 50 μM $FeSO_4$) for 30 min. Experimental conditions are described in the Materials and Methods section. Results are given as means ± standard deviation from at least three sets of experiments

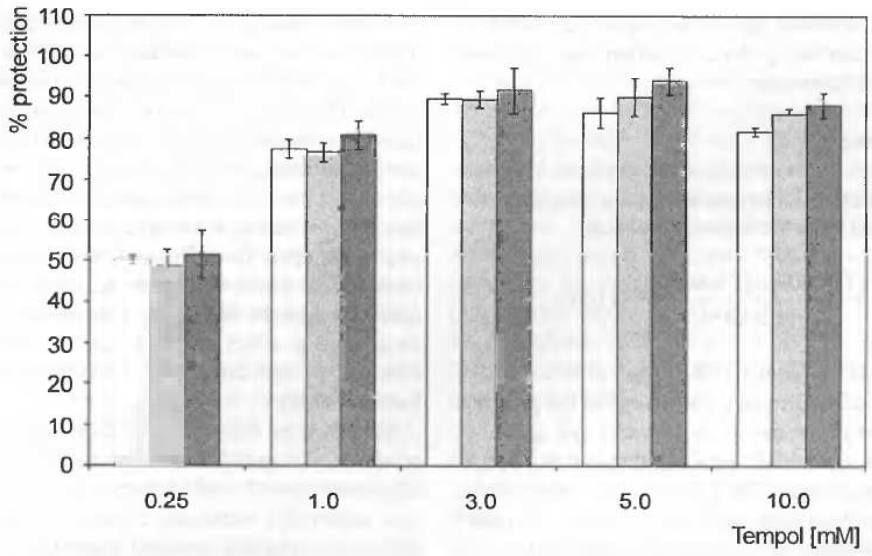

Fig. 3. Effect of varying deoxyribose and Tempol concentrations on the relative protection afforded by Tempol. Black bars, 10 mM deoxyribose; gray bars, 5 mM deoxyribose; white bars, 1 mM deoxyribose. Experimental conditions are described in the Materials and Methods section. Results are given as means ± standard deviation from at least three sets of experiments

troxides with –OH or –$NH(CO)CH_3$ group substituted, where a hydrogen atom is replaced by more electronegative atoms such as oxygen or nitrogen, were better protectors than Tempo. However, in the case of Tempicol-2 and Tempicol-2-NO, theoretical prediction suggests that the total inductive effect of their substituents should be a little greater than it is in the case of Tempo, but the inhibitory potency seen for these compounds was slightly lower than that of Tempo (explanation below). Nevertheless, the more electronegative substituents show a stronger tendency for the electrons withdrawal, which should facilitate the reduction of nitroxide group, but not its oxidation. Indeed, the same compounds which displayed relatively higher protection yields, i.e. Tempol and Tempace, were more quickly and efficiently reduced by ferrous ions (Głębska, unpublished data) or ascorbic acid (Głębska & Gwoździński, 1998; Morris, Sosnovsky, Hui, Huber, Rao & Swartz, 1991). Substituents enhanced protective ability of nitroxides in the same order in which they augmented their reduction rates with iron(II) ions. Therefore, it seems likely that nitroxides protect deoxyribose rather through oxidation of reduced redox-active iron ions accompanied by nitroxide reduction (Bar-On *et al.*, 1999; Krishna & Samuni, 1994; Zhang *et al.*, 1998) than through scavenging of hydroxyl radicals or reduction of ferryl ions accompanied by nitroxide oxidation (Deffner & Schimmack, 1976; Krishna *et al.*, 1996b; Metodiewa *et al.*, 1999). Thus, the antioxidant activity of nitroxide radicals against deoxyribose oxidation by Fenton system may be mainly connected with their ability to maintain iron ions in their oxidized form.

In addition, the crucial role of the reaction between nitroxide and iron(II) in the investigated Fenton system can give an explanation for the disagreement between theoretical assumptions and experimental results observed for Tempicol-2 and Tempicol-2-NO. This discrepancy was not observed in the reaction between nitroxide and ascorbic acid (Głębska & Gwoździński, 1998) and might be ascribed to some additional effects, such as a possible interaction between nitrogen atom from the pyridyl moiety and ferrous ions, perhaps resulting in formation of weak complexes. This can make iron not so easily accessible for the nitroxide group, also of other nitroxide molecules, thus limiting electron transfer reactions.

To study restrictions in protection stemming from nitroxide/deoxyribose concentration ratio, antioxidative effect of Tempol was investigated as a function of deoxyribose concentration. Surprisingly, the relative protection did not significantly vary with deoxyribose concentration in the applied range of 1–10 mM (Fig. 3). A simple explanation based on insufficient amount of Fenton reaction-derived oxidants is unlikely, though, since in-

creasing the deoxyribose concentration leads to a parallel increase in absolute TBARS concentration (data not shown), while only the relative protection remains unchanged.

The observed phenomenon can be discussed based on a simplified scheme, considering independently two mechanisms, which have been proposed to underlay the nitroxide inhibitory effect. The deoxyribose molecule (DR) is degraded to TBARS (reaction 1) by oxidants produced in the Fenton reaction (reaction 2)

$$DR + {}^{\cdot}OH \xrightarrow{k_1} TBARS \qquad \text{(reaction 1)}$$

$$Fe^{2+} + H_2O_2 \xrightarrow{k_2} {}^{\cdot}OH + OH^- + Fe^{3+} \qquad \text{(reaction 2)}$$

and the possible chemical reactions involved in the protective action of nitroxides are (i) scavenging of Fenton oxidants, exemplified by hydroxyl radicals,

$$>N^{\cdot}-O + {}^{\cdot}OH \xrightarrow{k_3} >N^{+}=O + OH^- \qquad \text{(reaction 3)}$$

or an alternative pathway – (ii) oxidation of the reduced iron ions

$$>N^{\cdot}-O + Fe^{2+} \xrightarrow{k_4} >N-OH + Fe^{3+} \qquad \text{(reaction 4).}$$

The relative protection value is given by equation 1 (square brackets always denote the concentration of the respective reagent and values with an 'Nx' index correspond to conditions with nitroxide in the reaction mixture).

$$P = \frac{[TBARS] - [TBARS]_{Nx}}{[TBARS]} \qquad [1]$$

Formula 1 can be converted into

$$P = 1 - \frac{[TBARS]_{Nx}}{[TBARS]} \qquad [2]$$

and since [DR] » [$^{\cdot}$OH], one can assume that TBARS arise stoichiometrically from reaction 1 and the final concentration of TBARS equals the total concentration of hydroxyl radical having participated in the reaction

$$P = 1 - \frac{[{}^{\cdot}OH]_{Nx}}{[{}^{\cdot}OH]} \qquad [3]$$

When it is assumed that nitroxide takes part only in reaction 3, the rate of hydroxyl radical production and disappearance is formulated as follows:

$$\frac{d[{}^{\cdot}OH]_{Nx}}{dt} = k_2[Fe^{2+}]_{Nx}[H_2O_2]_{Nx} - k_1[{}^{\cdot}OH]_{Nx}[DR]_{Nx} - k_3[{}^{\cdot}OH]_{Nx}[Nx] \qquad [4]$$

$$\frac{d[{}^{\cdot}OH]}{dt} = k_2[Fe^{2+}][H_2O_2] - k_1[{}^{\cdot}OH][DR] \qquad [5]$$

In the case of $k_2 \ll k_1$ and/or k_3 (Winterbourn, 1991b) the steady-state approximation leads to

$$\frac{d[{}^{\cdot}OH]_{Nx}}{dt} = 0 \qquad [6]$$

$$\frac{d[{}^{\cdot}OH]}{dt} = 0 \qquad [7]$$

Hence:

$$[{}^{\cdot}OH]_{Nx} = \frac{k_2[Fe^{2+}]_{Nx}[H_2O_2]_{Nx}}{k_1[DR]_{Nx} + k_3[Nx]} \qquad [8]$$

$$[{}^{\cdot}OH] = \frac{k_2[Fe^{2+}][H_2O_2]}{k_1[DR]} \qquad [9]$$

Since the nitroxide presence does not alter the hydrogen peroxide and iron concentrations as well as assuming conditions of pseudo first-order reaction ([DR] » [$^{\cdot}$OH]), substitution of the equations [8] and [9] to [3] yields

$$P = 1 - \frac{k_1[DR]}{k_1[DR] + k_3[Nx]} \qquad [10]$$

and upon rearrangement

$$P = \frac{k_3[Nx]}{k_1[DR] + k_3[Nx]} \qquad [11]$$

In that case, the amount of oxidants available for reaction with the target should be constant and dependent only on the protector concentration. Therefore, the relative protection would decrease with increasing deoxyribose concentration. How-

ever, this result is inconsistent with the obtained experimental data.

If the second mechanism, oxidation of ferrous ions, is presumed to be the only one involved in nitroxide action against deoxyribose damage, the rate of hydroxyl radical concentration change is described by the following equation:

$$\frac{d[^{\cdot}OH]}{dt} = k_2[Fe^{2+}][H_2O_2] - k_1[^{\cdot}OH][DR] \quad [12]$$

Because $k_2 \ll k_1$ (Winterbourn, 1991b), the concentration of hydroxyl radical can be determined in a manner similar to the previous case, assuming steady-state conditions, yielding

$$[^{\cdot}OH] = \frac{k_2[Fe^{2+}][H_2O_2]}{k_1[DR]} \quad [13]$$

Unfortunately, under this mechanism the iron(II) concentration is influenced by the nitroxide presence, thus equation [13] breaks up into two separate expressions depending on nitroxide involvement

$$[^{\cdot}OH]_{Nx} = \frac{k_2[Fe^{2+}]_{Nx}[H_2O_2]_{Nx}}{k_1[DR]_{Nx}} \quad [14]$$

$$[^{\cdot}OH] = \frac{k_2[Fe^{2+}][H_2O_2]}{k_1[DR]} \quad [15]$$

The rate at which iron(II) is oxidized is given by the equations

$$\frac{d[Fe^{2+}]_{Nx}}{dt} = -k_2[Fe^{2+}]_{Nx}[H_2O_2]_{Nx} - k_4[Fe^{2+}]_{Nx}[Nx] \quad [16]$$

or

$$\frac{d[Fe^{2+}]}{dt} = -k_2[Fe^{2+}][H_2O_2] \quad [17]$$

so that

$$[Fe^{2+}]_{Nx} = \frac{-\frac{d[Fe^{2+}]_{Nx}}{dt}}{k_2[H_2O_2]_{Nx} + k_4[Nx]} \quad [18]$$

and

$$[Fe^{2+}] = \frac{-\frac{d[Fe^{2+}]}{dt}}{k_2[H_2O_2]} \quad [19]$$

For time $t_{\to\infty}$ $-\frac{d[Fe^{2+}]}{dt} \to -\Delta[Fe^{2+}]$ and $-\frac{d[Fe^{2+}]_{Nx}}{dt} \to -\Delta[Fe^{2+}]_{Nx}$ giving, after the end of the reaction, $-\Delta[Fe^{2+}]_{Nx} = -\Delta[Fe^{2+}]$, which in turn equals the initial iron ion concentration $[Fe^{2+}]_0$ due to the pseudo first-order reaction conditions. Also, when $[DR] \gg [Fe^{2+}]$, $[DR] = [DR]_{Nx} \approx [DR]_0$.

Hence, the relative protection can be computed by substituting the values from equation [18] and [19] into [14] and [15], respectively and subsequently merging equations [14], [15] and [3], yielding

$$P = 1 - \frac{k_2[H_2O_2]_{Nx}}{k_2[H_2O_2]_{Nx} + k_4[Nx]} \quad [20]$$

and after rearrangement

$$P = \frac{k_4[Nx]}{k_2[H_2O_2]_{Nx} + k_4[Nx]} \quad [21]$$

Thus, the TBARS yield would vary with deoxyribose concentration as for pseudo first-order reaction, but relative protection would remain unchanged exactly in the same way as it is shown in Fig. 3.

The 'iron oxidation' model seems to be quite close to experimental findings in this study suggesting that the reduced metal oxidation may play a dominant role in the protective mechanism of nitroxide action.

In summary, this work provides some information concerning mechanisms of inhibition of oxidative processes and the relationships between structure and function for nitroxide antioxidants. The results presented clearly show that the relative protection of deoxyribose degradation to TBA-reactive products depends strongly upon the nitroxide structure. Even though some general trends can be observed, they do not allow for universal prediction of nitroxide efficiency because of some additional (e.g. affinity-related) aspects which may complicate predictions even in well-defined, sim-

ple chemical systems. However, these results should help to guide the development of nitroxides for specific pharmacological application as therapeutic agents by providing a basis for future prediction of their antioxidant potency.

The main conclusions from mechanistic studies are that (i) the obtained results attest to the potency of antioxidants from the nitroxide family against oxidative injury induced by redox-active transition metal ions and (ii) nitroxides pre-empt the Fenton chemistry rather by oxidizing ferrous ions than by scavenging hydroxyl radicals.

In any case, these findings show the need for further careful evaluation and indicate that nitroxides might have an application as protectors from biological damage caused by redox active transition metal ions, but presented suggestions require further support from more extensive studies planned in the future.

Acknowledgements

The authors are very grateful to Dr Diana Metodiewa and Prof. Grzegorz Bartosz for their useful suggestions and fruitful discussions.

REFERENCES

Aruoma O. I. (1994). Deoxyribose assay for detecting hydroxyl radicals. *Methods Enzymol.*, **233**, 57–66.

Bar-On P., Mohsen M., Zhang R., Feigin E., Chevion M. & Samuni A. (1999). Kinetics of nitroxide reaction with iron(II). *J. Am. Chem. Soc.*, **121**, 8070–8073.

Candeias L. P., Folkes L. K. & Wardman P. (1995). Is Fenton chemistry that important? *Congress Proceedings. Congress Lectures*, 2.

Damiani E., Kalinska B., Canapa A., Canestrari S., Wozniak M., Olmo E. & Greci L. (2000). The effects of nitroxide radicals on oxidative DNA damage. *Free Radic. Biol. Med.*, **28**, 1257–1265.

Deffner U. & Schimmack W. (1976). Radiation effects on aqueous solutions of the nitroxyl free radical TMPN (2,2,6,6-tetramethyl-4-piperidinol-N-oxyl). *Int. J. Radiat. Biol.*, **29**, 71–75.

Franzini E., Sellak H., Hakim J. & Pasquier C. (1994). Comparative sugar degradation by $(OH)^{\cdot}$ produced by the iron-driven Fenton reaction and gamma radiolysis. *Arch. Biochem. Biophys.*, **309**, 261–265.

Głębska J. & Gwoździński K. (1998). Oxygen-dependent reduction of nitroxides by ascorbic acid and glutathione. An EPR study. *Curr. Topics Biophys.*, **22**(suppl. B), 75–82.

Hahn S. M., Krishna M. C. & Mitchell J. B. (1994). New directions for free radical cancer reserch and medical applications, [In:] *Free Radicals in Diagnostic Medicine*. Plenum Press, New York.

Halliwell B. & Gutteridge J. M. C. (1981). Formation of a thiobarbituric acid-reactive substance from deoxyribose in the presence of iron salts – The role of superoxide and hydroxyl radicals. *FEBS Lett.*, **128**, 347–351.

Krishna M. C. & Samuni A. (1994). Nitroxides as antioxidants. *Methods Enzymol.*, **234**, 580–589.

Krishna M. C., Russo A., Mitchell J. B., Goldstein S., Dafni H. & Samuni A. (1996a). Do nitroxide antioxidants act as scavengers of $O_2^{\cdot}$ or as SOD mimics? *J. Biol. Chem.*, **271**, 26026–26031.

Krishna M. C., Samuni A., Taira J., Goldstein S., Mitchell J. B. & Russo A. (1996b). Stimulation by nitroxides of catalase-like activity of hemeproteins. Kinetics and mechanism. *J. Biol. Chem.*, **271**, 26018–26025.

Metodiewa D., Kochman A., Skolimowski J., Gebicka L. & Koska C. (1999). Metexyl (4-methoxy-2,2,6,6-tetramethylpiperidine-1-oxyl) as an oxygen radicals scavenger and apoptosis inducer *in vivo*. *Anticancer Res.*, **19**, 5259–5264.

Metodiewa D., Skolimowski J., Kochman A., Gwoździński K. & Głębska J. (1998). Tempicol-2 (4-hydroxy-4-(2-picolyl)-2,2,6,6-tetramethylpiperidine-1-oxyl), a stable free radical, is a novel member of nitroxides class of antioxidants and anticancer agents. *Anticancer Res.*, **18**, 369–377.

Morris S., Sosnovsky G., Hui B., Huber C. O., Rao N. U. M. & Swartz H. M. (1991). Chemical and electrochemical reduction rates of cyclic nitroxides (nitroxyls). *J. Pharm. Sci.*, **80**, 149–152.

Offer T., Russo A. & Samuni A. (2000). The prooxidative activity of SOD and nitroxide SOD mimics. *FASEB J.*, **14**, 1215–1223.

Rozantsev E. G. (1970). In: *Svobodnyie iminoksilnyie radikaly*. Khimiya. Moscow.

Saran M. & Bors W. (1990). Radical reactions *in vivo* – an overview. *Radiat. Environ. Biophys.*, **29**, 249–262.

Shen X., Tian J., Li J., Li X. & Chen Y. (1992). Formation of the excited ferryl species following Fenton reaction. *Free Radic. Biol. Med.*, **13**, 585–592.

Winterbourn C. C. (1991a). Free radical biology of iron. In: *Trace Elements, Micronutrients, and Free Radicals*. The Humana Press Inc.

Winterbourn C. C. (1991b). Factors that influence the deoxyribose oxidation assay for Fenton reaction products. *Free Radic. Biol. Med.*, **11**, 353–360.

Zhang R., Goldstein S. & Samuni A. (1999). Kinetics of superoxide-induced exchange among nitroxide antioxidants and their oxidized and reduced forms. *Free Radic. Biol. Med.*, **26**, 1245–1252.

Zhang R., Pinson A. & Samuni A. (1998). Both hydroxylamine and nitroxide protect cardiomyocytes from oxidative stress. *Free Radic. Biol. Med.*, **24**, 66–75.