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SUPEROXIDE REACTION WITH NITROXIDES

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Stable, free radical nitroxides are commonly used ESR spectroscopy tools. However, it has recently been found that ESR observable signal from 5-membered ring spin-adducts or stable label nitroxides is lost or diminished by reaction with superoxide. A similar radical-radical annihilation was not found for six membered ring nitroxide radicals. To discern why six-membered ring nitroxides are not reduced under superoxide flux generated by hypoxanthine/xanthine oxidase, spectrophoptmetric (Cyt C^{III}) and chemiluminescence (lucigenin) and ESR assays were used to follow the reactions. Spectrophotometry and chemiluminescence clearly demonstrated that the six-membered piperidine-1-oxyl compounds (TEMPO, TEMPOL, and TEMPAMIN) rapidly react with superoxide: rate constants at pH 7.8 ranging from 7×10^4 to $1.2 \times 10^5 M^{-1}s^{-1}$. The absence of detectable ESR signal loss results from facile re-oxidation of the corresponding hydroxylamine by superoxide. To fully corroborate the efficiency of the 6-membered nitroxide superoxide dismutase activity, they were shown to protect fully mammalian cells from oxidative damage resulting from exposure to the superoxide and hydrogen peroxide generating system hypoxanthine/xanthine oxidase. Since six-membered cyclic nitroxides react with superoxide about 2 orders of magnitude faster than the corresponding 5-membered ring nitroxides, they may ultimately be more useful as superoxide oxide dismutase mimetic agents.

KEY WORDS: Superoxide, superoxide dismutase mimic, ESR, spin-labels, nitroxides.

ABBREVIATIONS: ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide;TEMPO,2,2,6,6-tetramethyl-piperidinoxyl; CHDO, 2-spirocyclohexane doxyl (2-spirocyclohexane-5,5-dimethyl-3-oxazolidinoxyl); DTPA, diethylenetriaminopenta-acetic acid; DFO, desferrioxamine; XO, xanthine oxidase; HX, hypoxanthine; Cyt-C^{III}, ferricytochrome c; TEMPOL, 4-hydroxy-2,2,6,6,-tetramethylpiperidine-1-oxyl; TEMPAMIN, 4-amino-2,2,6,6-tetramethyl-piperidine-1-oxyl; OXANO, 2-ethyl-2,5,5-trimethyl-3-oxaolidinoxyl; 3-CN-TMP, 3-cyano-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy.

INTRODUCTION

Spin label nitroxides are stable free radicals commonly used in electron spin resonance (ESR) spectroscopy to probe molecular motion in membranes and whole cells, transmembrane potential, and intracellular oxygen and pH.[1-5] Cellular metabolic reduction of the nitroxides,[6,7] taken originally as a drawback for their use, now has been exploited to investigate cellular metabolism.[5,8] In addition, because of their paramagnetism, nitroxides can shorten the relaxation times of protons and have found use as metabolic response contrast agents for *in vivo* NMR studies.[9,10]

Although free radicals are prone to one-electron redox cycling, only a few radical-radical reactions of nitroxides have been reported.[6,11-15] Persistent spin-adducts and stable nitroxides are known to be readily reduced in biological systems.[2,5-8,16,17] Recently, nitroxides were found to be reduced to the corresponding hydroxylamine by superoxide radical anion ($O_2^{\cdot-}$).[18] This finding originally stemmed from the observa-

tion of $O_2^{\cdot-}$ induced spin-loss of OH spin-adduct of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). The ESR signals of persistent nitroxide spin-adducts such as DMPO-OH or DMPO-CH_3 and of several stable nitroxide radicals, such as OXANO (2-ethyl-2,5,5-trimethyl-3-oxazolidinoxyl) rapidly decayed to a lower steady state when exposed to a continuous $O_2^{\cdot-}$ flux.[19,20] The spin loss resulting from reaction of nitroxides with $O_2^{\cdot-}$ demonstrated a so far unrecognized drawback to their use as spin probes. Unfortunately the assessment, by spin trapping technique,[21] of OH yields becomes invalid under high $O_2^{\cdot-}$ flux.[19,22] Analogously, the persistence of stable spin labels appears more complex because in addition to bioreduction, nitroxides are removed (as well as restored) through reaction with $O_2^{\cdot-}$. Since $O_2^{\cdot-}$ can also re-oxidize hydroxylamines to nitroxides,[12] the apparent initial drawback of nitroxides reaction with $O_2^{\cdot-}$ proved to have a potential use; as $O_2^{\cdot-}$ induced depletion and regeneration of the nitroxide appeared to be coupled through reactions 1 and 2:

$$H^+ + O_2^{\cdot-} + \frac{R'}{R} > N^{\cdot}O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} O_2 + R'R > N-OH \qquad (1)$$

$$H^+ + O_2^{\cdot-} + \frac{R'}{R} > N\text{-}OH \xrightarrow{k_2} H_2O_2 + R'R > N_2^{\cdot}O \qquad (2)$$

thus identifying an SOD-mimetic activity of the nitroxides.[20]

These findings stimulated us to extend this study to other nitroxide derivatives. Initially, only 5-membered ring nitroxides seemed to react with $O_2^{\cdot-}$ because the ESR signal of neither phenyl butyl nitrone (PBN) spin-adducts nor of 6-membered ring nitroxides were affected by hypoxanthine/xanthine oxidase (HX/XO) $O_2^{\cdot-}$ generating reaction system. [23] Yet, it appeared very unlikely that piperidine nitroxides which are chemically more facilely reduced than pyrrolidine derivatives[6] would not react with $O_2^{\cdot-}$. To determine if different nitroxides indeed respond differently to $O_2^{\cdot-}$ we have studied using ESR, absorbance, and chemiluminescence spectroscopies the reaction of several 6-membered ring nitroxides with $O_2^{\cdot-}$. In the present study the reaction of 6-membered ring nitroxides such as TEMPAMIN or TEMPOL with $O_2^{\cdot-}$ has been identified using the ferricytochrome c reduction and chemiluminescence assays. No corresponding ESR observable nitroxide spin-loss was observed because of rapid re-oxidation of the corresponding hydroxylamines. Through the combined reduction and oxidation of $O_2^{\cdot-}$, each nitroxide/hydroxylamine couple exhibits a superoxide dismutase-like activity. Similar to their 5-membered ring counterparts, these low molecular weight, cell permeable, non-immunogenic, non-cytotoxic, metal-independent, 6-membered cyclic nitroxides are shown to possess SOD-like activity and to protect mammalian cells against oxidative damage, as was found for 5-membered ring derivatives.[24]

MATERIALS AND METHODS

Chemicals

Desferroxamine (DFO) was a gift from Ciba Geigy. Hypoxanthine (HX) from Calbiochem—Boehring Co.; 4-hydroxypyrazolo[3,4,-d]-pyrimidine (allopurinol); 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), 4-amino-2,2,6,6-tetramethyl-piperidine-1-oxyl

(TEMPAMIN), p-toluene sulfonic acid, 3-cyano-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy, 3-CN-TMP, 3-CN-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy, and 2-amino-2-methyl-1-propanol, 2-butanone, and cyclohexanone were purchased from Aldrich Chemical Co., xanthine oxidase (EC 1.2.3.2 xanthine: oxygen oxidoreductase) (XO) grade III from Buttermilk, superoxide dismutase (SOD), bis-N-methylacridinium nitrate (lucigenin), and ferricytochrome C (Cyt-C^{III}) were obtained from Sigma. All chemicals were prepared and used without further purificiation. Distilled-deionized water was used throughout all experiments. Unless otherwise stated the experiments were conducted at room temperature.

Synthesis of oxazolidine derivatives:

CHDO, 2-spirocyclohenxane doxyl (2-spirocyclohenxane-5,5-dimethyl-3-oxazolidinoxyl) and OXANO, 2-ethyl-2,5,5-trimethyl-3-oxazolidine-1-oxyl were synthesized as previously described.[7] To produce the amine, a ketone was reacted with 2-amino-2-methyl-1-propanol in benzene in the presence of p-toluene sulfonic acid catalyst. The formation of the cyclic structure resulted in the elimination of water. The volume of water collected in a Dean Stark apparatus was monitored and used to gauge the reaction progress. The amines thus produced were purified through fractional distillation under reduced pressure, characterized through NMR, IR, UV, mass spectroscopy, and oxidized to the corresponding nitroxides using m-chloroperbenzoic acid. The nitroxides were purified by silica flash chromatography.

Electron Spin Resonance

Samples (0.05–0.1 ml) for ESR experiments were drawn by a syringe into a gas-permeable teflon capillary (Zeus Industries, Raritran, NJ) of 0.032 inch inner diameter, 0.015 inch wall thickness, and 15 cm long. Each capillary was folded twice,inserted into a narrow quartz tube which was open at both ends (2.5 mm ID), and then placed into the ESR cavity. During the experiments, gases of desired compositions were blown around the sample without having to disturb the alignment of the tube within the ESR cavity. ESR spectra were recorded on a Varian E4 (or E9) X-band spectrometer, with field set at 3357 G, modulation frequency of 100 KHz, modulation amplitude of 1 G (unless otherwise stated) and non-saturating microwave power.

Cell Survival Analysis

Survival of Chinese hamster V79 cells in tissue culture was assessed by clonogenic assay. Inoculated cells were incubated 12–16 hours prior to experimental procedures, exposed to varying nitroxide concentrations in complete medium at 37 °C in the absence and the presence of the HX/XO $O_2^{\cdot-}$ generating system.[23] Following treatment, cells were trypsinized, rinsed, counted, and plated in triplicates for macroscopic colony formation. Following appropriate incubation periods, colonies were fixed, stained, and lastly counted with the aid of a dissecting microscope. Survival curves in the presence and absence of various concentrations of the SOD-mimic were compared.

Chemiluminescence (CL) assay

Superoxide radicals were generated using 4 mM HX + 0.03 U/ml XO at 25 °C in 50 mM phosphate buffer pH 7.8 supplemented with 50 μM DTPA and 250 μM lucigenin,[25] under a continuous air supply, with or without gentle stirring. Following temperature equilibration, the experiments were started by adding XO followed by additions of varying nitroxide concentrations. The rate of CL emission was measured by photon counting using an SLM 8000 spectrofluorimeter.

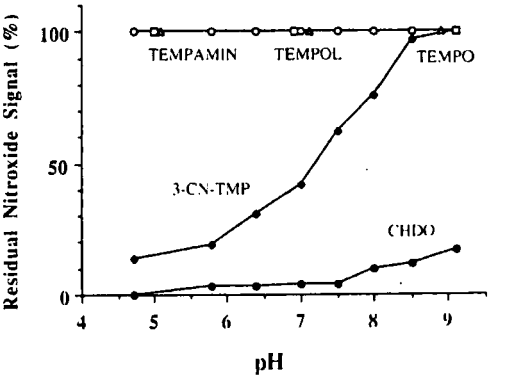
RESULTS

Superoxide effect on the nitroxide ESR signal

Superoxide has been previously shown to reduce OXANO to OXANOH.[20] To examine if other stable nitroxides react with $O_2^{\cdot}$, we exposed 100 μM of the nitroxide under air at pH 7.4 to $O_2^{\cdot}$ generating system (HX/XO) within the ESR cavity and followed the nitroxide spectrum. In the presence of continuous $O_2^{\cdot}$ flux, the ESR signal of 5-membered nitroxide spin-labels partially decayed achieving a pH-dependent steady state level. The residual signal intensity decreased at lower pH (Figure 1) indicating a corresponding decrease in the ratio k_1/k_2, as previously found for other 5-membered ring spin labels.[17] Upon addition of SOD or allopurinol followed by 0.5 mM ferricyanide the original signal intensity was restored, most likely through the one-electron oxidation of the corresponding hydroxyl amine. Contrary, however, to the 5-membered ring derivatives, the ESR spectra of the 6-membered nitroxides studied were not affected by HX/XO, apparently suggesting that these nitroxides do not react with $O_2^{\cdot}$ (Figure 1).

The reaction of $O_2^{\cdot}$ with nitroxides

Failure of $O_2^{\cdot}$ to affect the ESR signal of 6-membered ring nitroxides would be anticipated if reaction 1 does not proceed to any appreciable extent. Alternatively, it



FIGUREI. The pH-dependence of the residual steady-state concentration of 5- and 6-membered nitroxides under superoxide flux. The steady-state residual ESR signal persisting following exposure of 100 μM nitroxide in 50 mM phosphate containing 50 μM DTPA, 4 mM HX, and 0.03 U/ml XO in air at room temperature under various pH values. Residual ESR signal intensity is displayed vs. pH as percentage of the initial value. TEMPOL (□); TEMPO (○); TEMPAMIN (△); 3-CN-TMP (◆); CHDO (●).

could result if re-oxidation of the hydroxylamine to the parent spin-label is instantaneous. To examine this possiblity, the lucigenin-amplified $O_2^{\cdot-}$ – induced chemiluminescence (CL) assay was adopted. The HX/XO reaction system was used to generate a continuous steady state CL.[25] A typical CL trace obtained for TEMPAMIN is seen in Figure 2a. Addition of the nitroxide caused the steady state CL to decrease in dose-dependent (time-independent) manner (Figure 2a.). To analyze the results, the light emitted in the absence (CL) and in the presence (cl) of various nitroxide concentrations (from Figure 2a) was plotted as a function of [nitroxide]. $k_{nitroxide}$ was calculated from the equation $(CL/cl) - 1 = k_{nitroxide} \times [nitroxide]/ k_{lucigenin} \times [lucigenin]$ (see Figure 2b.). With $k_{lucigenin+superoxide}$ equals 3.6×10^3 we calculate $k_{nitroxide+superoxide} = 9 \times 10^4 M^{-1} s^{-1}$ for both TEMPOL and TEMPAMIN.

To verify that 6-membered ring nitroxide reacts with $O_2^{\cdot-}$ the reaction was studied also using the SOD-inhibitable Cyt-C^{III} reduction assay.[23] The effect of nitroxide concentration on the rate of Cyt-C^{III} reduction was investigated. Formation rates of Cyt-C^{III} were monitored spectrophotometrically at 550 nm, in the absence (V) and in the presence (v) of varying nitroxide concentrations. Data were analyzed by plotting V/v as a function of [nitroxide] and k_1 was calcuated, knowing $k_{CytC+superoxide}$, according to: $V/v = 1 + k_1 \cdot [nitroxide] / k_{CytC+superoxide} \cdot [Cyt\text{-}C^{III}]$. Typical plots obtained for TEMPOL and TEMPAMIN are illustrated in Figure 3. Thus $k_{nitroxide+superoxide}$ values

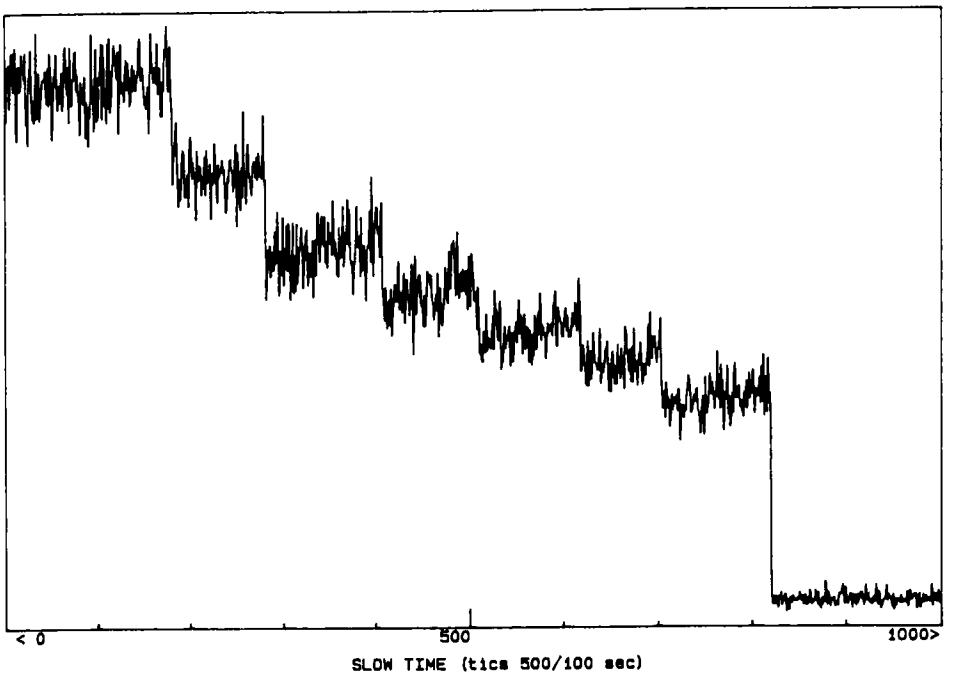


FIGURE 2a. Inhibition of lucigenin-amplified chemiluminescence (CL). Effect of nitroxide concentration on the steady state intensity of the lucigenin-amplified CL induced by $O_2^{\cdot-}$. The arrows indicate the addition of aliquots of TEMPAMIN at various points in time. Superoxide was generated with HX (5 mM) and XO (0.03 U/ml) in aerated phosphate buffer (50 mM, pH = 7.8) containing 50 μM DTPA and 250 μM lucigenin. XO was added last to the solution. The emission of light through the reaction of lucigenin and $O_2^{\cdot-}$ was monitored using the photon counting mode of an SLM 8000 spectrofluorimeter with the solution constantly stirred while in the spectrofluorimeter.

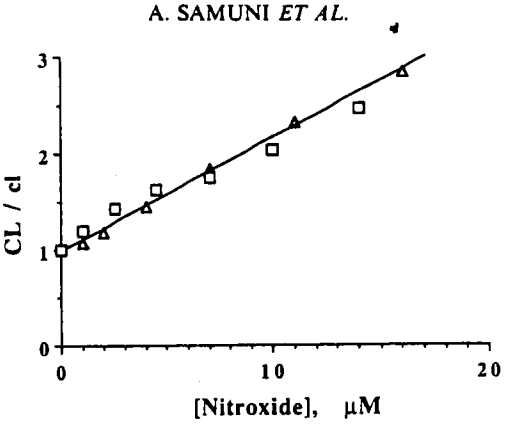


FIGURE 2b. Determination of rate constant of $O_2^{\cdot+}$ reaction with 6-membered ring nitroxides with $O_2^{\cdot+}$ by competition with lucigenin. The emission of light in the absence (CL) and in the presence (cl) of various nitroxide concentrations (from Figure.2A) was plotted as a function of [nitroxide]. $k_{nitroxide}$ was calculated from the equation $(CL/cl) - 1 = k_{nitroxide} \times [nitroxide]/ k_{lucigenin} \times [lucigenin]$. TEMPOL (□); TEMPAMIN (△).

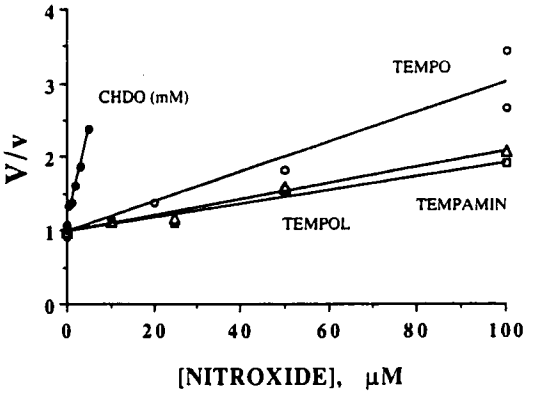


FIGURE 3. Determination of $O_2^{\cdot+}$ reaction with nitroxide by inhibition of superoxide-mediated reduction of Cyt-C^{III}. 10μM ferricytochrome C (Cyt-C^{III}) in 50 mM phosphate buffer pH 7.8 containing 50 μM DTPA, 4 mM hypoxanthine (HX) were incubated at 25 °C with and without nitroxide, and the reaction was started by adding 0.01 U/ml xanthine oxidase XO. Formation rates of Cyt-C^{III} were monitored spectrophotometrically at 550 nm, in the absence (V) and in the presence (v) of varying nitroxide concentrations. Data were analyzed by plotting V/v as a function of [nitroxide] and k_1 was calculated, knowing $k_{CytC+superoxide}$, according to: $V/v = 1 + k_1 \cdot [nitroxide]/k_{CytC+superoxide} \cdot [Cyt-C^{III}]$. TEMPO (○); TEMPOL (○); TEMPAMIN (△); CHDO (value is in millimolar) (●).

determined at pH 7.8 were $6.5 \times 10^4 M^{-1}s^{-1}$, $6.5 \times 10^4 M^{-1}s^{-1}$, $1.2 \times 10^5 M^{-1}s^{-1}$, and $3.5 \times 10^2 M^{-1}s^{-1}$ for TEMPOL, TEMPAMIN, TEMPO, and CHDO respectively.

Nitroxide Protective Effect

To test for protective effects of the 6-membered ring nitroxides against $O_2^{\cdot+}$ induced damage, monolayered Chinese hamster V79 cells were exposed to various fluxes of $O_2^{\cdot+}$

for 1 h in full growth medium, in the absence and in the presence of 5 mM nitroxide. The radicals were generated enzymatically using HX/XO and the cellular damage was assessed by clonogenically monitoring cell viability. In control experiments performed with TEMPO, TEMPOL and TEMPAMIN (up to 5 mM) in the absence of XO, no cytotoxic effect was observed. Experiments performed with several XO concentrations resulted in various degrees of cell killing, not inhibitable by SOD as previously found.[26] Yet, in the presence of the nitroxide no cell killing was observed (Figure 4).

DISCUSSION

Six-membered cyclic nitroxides are more susceptible to reduction than 5-membered cyclic derivatives,[6] although they do not differ much in their redox potentials.[7] The failure of $O_2^{\cdot-}$ to reduce the former would not, therefore, be anticipated. Although $O_2^{\cdot-}$ has been reported to reduce 6-membered cyclic nitroxides, this reaction could be demonstrated only in the presence of thiols.[13] In fact, SOD-inhibitable elimination of TEMPO in the presence of thiol was shown to proceed very rapidly, whereas with 5-membered ring nitroxide the effect was minimal.[13] Oxygen-dependent reduction of nitroxide was attributed also to the effect of transition metal ions.[27] In the present study, however, all nitroxides examined including the 6-membered ring derivatives, inhibited the lucigenin-amplified $O_2^{\cdot-}$ – induced CL, as well as the $O_2^{\cdot-}$ – induced reduction of Cyt-C^{III} (Figure 2 and 3). The reaction system always included DTPA to minimize effects of transition metals. This proves that nitroxides react with superoxide in a metal-independent manner. Moreover, no thiols appear to be required for the reaction to proceed. Previous failures to identify superoxide reaction with the 6-membered cyclic nitroxides is attributable to rapid nitroxide restoration. This assumption is corroborated by the persistence of their ESR signals under continuous $O_2^{\cdot-}$ flux (Figure 1). We conclude, therefore, that this class of spin-labels possess SOD-like activity, as was previously identified in the 5-membered ring derivatives.[20] The present results show that the reaction rate constant (k_1) of the piperidine derivatives are 2 orders of magnitude higher than that of the 5-membered ring class, implying that the

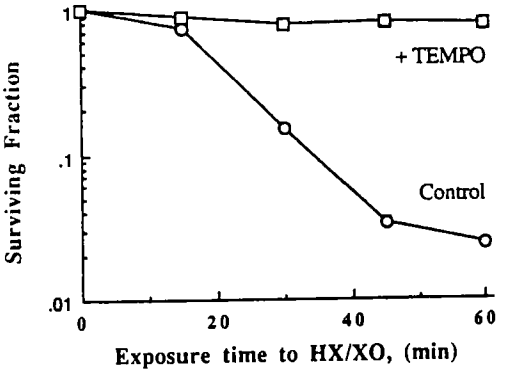


FIGURE 4 The Effects of TEMPO on Chinese Hamster V79 Cells Exposed to HX/XO. Monolayered cells were aerobically incubated for varying duration periods at 37 °C in full medium with 0.5 mM HX in the presence of 0.03 U/ml XO, with and without 5 mM TEMPO, and their surviving fractions were determined.

former may be better SOD-mimics. Moreover, since the steady state distribution of nitroxide/hydroxylamine (Figure 1) reflects the k_2/k_1 ratio, k_2 appears to exceed k_1 about 100-fold.

The 6-membered cyclic nitroxides protected against oxidative damage (Figure 4). Neither endogenous nor exogenously added SOD could prevent HX/XO −induced damage in the cells. Conversely, our cell permeable, non-toxic, metal-independent, stable SOD-mimics manifest extraordinary protective effect. This result might have several implications. Choosing and synthesizing appropriate derivatives that differ by their lipophilicity, charge, and size can serve in directing them toward different cellular compartments. Such synthetically constructed SOD-mimic may help in locating the sites of critical cellular damage, elucidation of damage reaction mechanisms, and may ultimately be applied to the clinical treatment of several acute and chronic maladies.

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