JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 941–949

Received December 12, 2005
Accepted March 6, 2006
DOI 10.1211/jpp.58.7.0009
ISSN 0022-3573

The relationship between structure and antioxidative activity of piperidine nitroxides

Wen-Guang Li, Xiao-Yu Zhang, Yong-Jie Wu, Ming-Tang Gao and Rong-Liang Zheng

Abstract

We have investigated the relationship between structure and antioxidative activity of piperidine nitroxides which were substituted by different groups at the 4-position. All of the tested piperidine nitroxides inhibited malondialdehyde (MDA) generation caused either spontaneously or by a hydroxyl free radical generation system (Fe^{2+}-ascorbic acid) in homogenates of liver, heart and kidney of rats, and antagonized H_2O_2-induced haemolysis from rat erythrocytes in a concentration-dependent manner. The same rank was followed: Bis-(4-amino-2,2,6,6-tetramethyl piperidinooxyl) (4-BIS-Tempo) and 4-azido-2,2,6,6-tetramethyl piperidinooxyl (4-N_3-Tempo) > 4-isothiocyanate-2,2,6,6-tetramethyl piperidinooxyl (4-ISO-Tempo), 4–2′,4′-dinitrophenyl-hydrazone-2,2,6,6-tetramethyl piperidinooxyl (4-D-Tempo), 4-sulfonate-2,2,6,6-tetramethyl piperidinooxyl (4-S-Tempo) and 4-amino-2,2,6,6-tetramethyl piperidinooxyl (4-NH_2-Tempo) > 4-acetate ester-2,2,6,6-tetramethyl piperidinooxyl (4-A-Tempo) and 4-benzoate-2,2,6,6-tetramethyl piperidinooxyl (4-B-Tempo). With the exception of 4-A-Tempo and 4-D-Tempo, the tested piperidine nitroxides inhibited superoxide anion ($O_2^{\bullet -}$) release from neutrophils stimulated by zymosan. The concentration required for inhibiting $O_2^{\bullet -}$ release was higher than that of inhibiting MDA formation and haemolysis. However, 4-amino-2,2,6,6-tetramethyl piperidine (4-NH_2-TempH) and other 4-position substitutes, such as NaN_3 and isothiocyanate, had no effects on MDA formation, haemolysis or $O_2^{\bullet -}$ release. The results indicated that nitroxides have a wide range of scavenging reactive oxygen species (ROS) actions. The nitroxide moiety was the essential group while the 4-position substitutes could influence the activity of nitroxides on scavenging ROS.

School of Life Sciences, Lanzhou University, Lanzhou, Gansu 730 000, P.R. China

W.-G. Li, R.-L. Zheng

Key Laboratory of Preclinical Study for New Drugs of Gansu Province, Lanzhou, Gansu 730 000, P.R. China

W.-G. Li, X.-Y. Zhang, Y.-J. Wu, M.-T. Gao

College of Pharmacy, Lanzhou University, Lanzhou, Gansu 730 000, P.R. China

W.-G. Li, Y.-J. Wu, M.-T. Gao

College of Basic Medicine, Lanzhou University, Lanzhou, Gansu 730 000, P.R. China

X.-Y. Zhang

Correspondence: R.-L. Zheng, School of Life Sciences, Lanzhou University, Lanzhou, Gansu 730 000, P.R. China. E-mail: zhengrl@lzu.edu.cn

Introduction

The nitroxides, a group of metal-free, low molecular weight, water-soluble and stable free radicals, are widely used in electron paramagnetic resonance (EPR) spectroscopy as probes for a number of biophysical and biochemical parameters in biological systems (Swartz 1986; Keana et al 1990). They have been investigated as contrast agents for magnetic resonance imaging (MRI) (Brasch et al 1983; Gallez et al 1992). In addition, it has been shown that nitroxides possess a wide range of biological and pharmacological activity. A number of reports have demonstrated that the nitroxides have antioxidative activity (Wu et al 1997; Hahn et al 2000) and have shown protective effects against oxidative damage in various biological disease models, in-vitro and in-vivo. In-vitro experiments have indicated that the nitroxides protect cells against a variety of oxidative insults, such as the cytotoxicity induced by hydrogen peroxide, superoxide, t-butyl hydroperoxide, and radiation, and against quinone-mediated and other chemotherapeutic agent-mediated cytotoxicity (Hahn et al 1992; Monti et al 1996; Offer & Samuni 2002; Gadjeva et al 2005). Various in-vivo animal models of nitroxides against oxidative damage include closed-head injury (Zhang et al 1998), inflammatory bowel diseases (Karmeli et al 1995; Davies & Jamali 1997), radiation-induced cataractogenesis (Sasaki et al 1998), whole body irradiation (Hahn et al 2000), light-induced retinal degeneration, ischaemia–reperfusion injury (Zeltcer et al 1997, 2002; Udassin et al

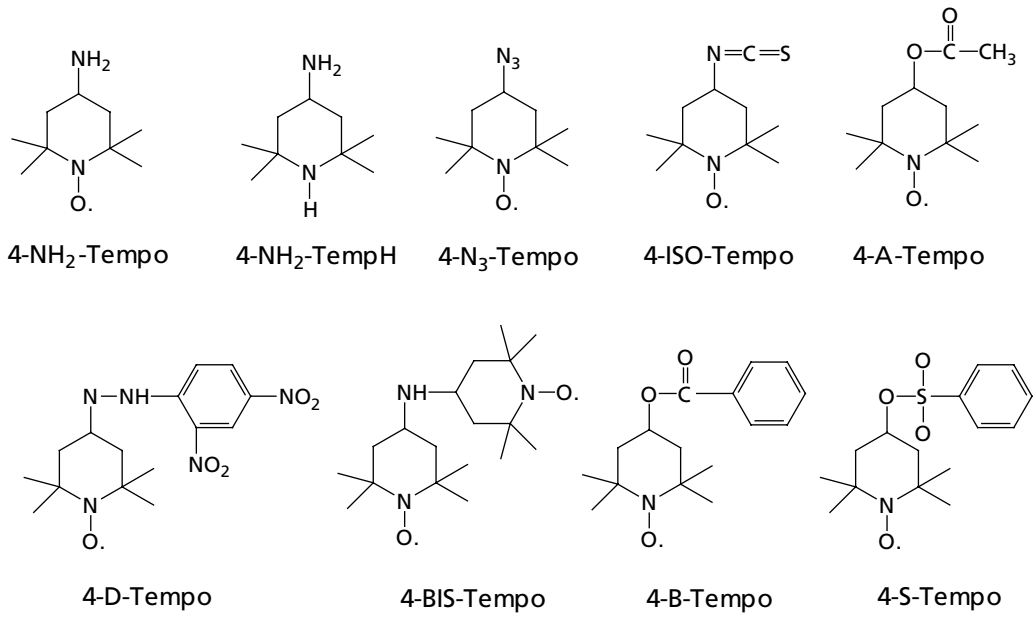

Figure 1 Chemical structure of nitroxides.

1998; Rak et al 2000; Hoffman et al 2003), autoimmune diseases (Zamir et al 1999), and age-related diseases such as tumourigenesis (Gariboldi et al 2003). It is suggested that long-term treatment with nitroxides may provide overall health benefits with decreased obesity and tumour incidence, as shown in mice (Mitchell et al 2003).

However, despite the increasing number of implications for the use of these compounds, the optimal agents for their clinical use and the reports related to their activity–structure relationship are still rare. Apart from the findings of Wu et al (1997) and Krishna et al (1998), few studies have shown that the NO· or NOH group of piperidine nitroxides were essential groups. We have investigated the effects of nitroxides substituted by different groups at the 4-position (structures shown in Figure 1) on antioxidative activity and their structure–activity relationship.

Materials and Methods

Drugs and chemicals

4-Amino-2,2,6,6-tetramethyl piperidine (4-NH_2-TempH), 4-amino-2,2,6,6-tetramethyl piperidinooxyl (4-NH_2-Tempo), 4-azido-2,2,6,6-tetramethyl piperidinooxyl (4-N_3-Tempo), bis-(4-amino-2,2,6,6-tetramethyl piperidinooxyl) (4-BIS-Tempo), 4-isothiocyanate-2,2,6,6-tetramethyl piperidinooxyl (4-ISO-Tempo), 4-acetate ester-2,2,6,6-tetramethyl piperidinooxyl (4-A-Tempo), 4-benzoate-2,2,6,6-tetramethylpiperidinooxyl (4-B-Tempo), 4-2′,4′-dinitrophenylhydrazone-2,2,6,6-tetramethyl piperidinooxyl (4-D-Tempo), 4-sulfonate-2,2,6,6-tetramethyl piperidinooxyl (4-S-Tempo) were synthesized by the State Key Laboratory of Applied Organic Chemistry, Lanzhou University (Lanzhou, China). Purity of the compounds was approximately 98%. The compounds were dissolved in 5% dimethyl sulfoxide (DMSO). Zymosan A (Sigma) was opsonized with rat serum and suspended in 0.15 mol L^{-1} phosphate buffer (pH 7.4). Other agents were of analytical purity.

Animals

Female and male Wistar rats (8 weeks old, 184 ± 19 g) were provided by the Animal Breeding Center of Lanzhou University. The animals were housed two per cage and were maintained in climate controlled and circadian rhythm-adjusted rooms. The mice were provided with standard laboratory chow and water was freely available. All animal experiments were approved by the University Committee on Care and Use of Animals.

Determination of malondialdehyde (MDA)

The heart, liver, and kidney of rats were prepared as 5% tissue homogenates in Tris-KCl buffer (containing trihydroxymethyl aminomethane 0.1 mol L^{-1} and KCl 1 mmol L^{-1}, pH 7.4) as described previously (Wu et al 1997; Li et al 2002). Tissue homogenate (1 mL) was incubated with different concentrations of tested compounds or vehicle (DMSO; 2.5 mL L^{-1}) at 37°C for 10 min, and 50 μL Fe^{2+}-ascorbic acid (AA) solution (50/50 μmol L^{-1}) was added. Following 30-min incubation, MDA was determined using the thiobarbituric acid (TBA) method (Tanizaw et al 1981). The

spontaneous MDA generation from rat liver homogenate was measured after 2-h incubation with the tested compounds and without Fe^{2+}-ascorbic acid solution.

Superoxide anion assay

Leucocytes from the rat abdominal cavity were prepared as described previously (Wu et al 1997; Li et al 2002) and suspended in Hanks' solution ($CaCl_2$ 1.3, $MgSO_4$ 0.8 and glucose 10 mmol L^{-1}, pH 7.4) to a density of 1×10^{10} cells L^{-1}. The leucocyte viability, assessed by trypan blue exclusion, was more than 98%. Neutrophils were 97% of the leucocytes, demonstrated by staining classification. All the procedures were carried out below 4°C. Superoxide anion formation was determined using nitroblue tetrazolium (NBT) reduction assay (Baehner & Nathan 1968). In brief, 0.1 mL potassium cyanide (KCN) 10 mmol L^{-1}, 0.4 mL 0.1% NBT normal saline, 0.4 mL neutrophil suspension, 0.1 mL zymosan A 2.5 g L^{-1} and 50 μL different concentration solution of tested compounds or vehicle were added in plastic tubes. After the mixture was incubated at 37°C for 35 min, the reaction was stopped by adding 5 mL ice-cold HCl 0.5 mol L^{-1}, followed by centrifugation at 4°C for 10 min. The purple pellet was extracted with 3 mL pyridine in boiling water for 20 min. The absorbance of reduced NBT (formazan) was measured at 515 nm with a spectrophotometer.

Haemolysis test

Rat red blood cells (RBC) were washed three times with normal saline and made into 0.5% suspension solution. Following treatment with the tested compounds or vehicle, RBC suspension 1 mL was incubated with H_2O_2 (100 mmol L^{-1}) at 37°C for 1 h. After being diluted ×5 with normal saline, the mixture was centrifuged at 1000 *g* for 10 min. The absorbance (A) of the supernatant was spectrophotometrically measured at 415 nm (Anastassopoulou & Rakintzis 1984). The degree of haemolysis in the control tube was defined as 100%, then the percentage of haemolysis of the other groups was calculated with the following equation: haemolysis $(\%) = A_{415\,\text{Treated}}/A_{415\,\text{Control}} \times 100$ (Wu et al 1997; Li et al 2002).

Statistical analysis

Results were expressed as mean ± s.d. Data analysis was performed using analysis of variance followed by LSD-*t* post-hoc test for multiple comparisons with the computer statistical package SPSS 11.0 (for Windows). Differences were considered statistically significant at $P < 0.05$.

Results

Inhibition of nitroxides on MDA generation

Reactive oxygen species (ROS) can be produced endogenously as a result of oxygen metabolism by tissues. Exogenous chemical agents such as Fe^{2+}-ascorbic acid solution can enhance ROS generation and induce lipid peroxidation (LPO) producing MDA. In this experiment, all the tested nitroxides inhibited spontaneous MDA generation from rat liver homogenate in a concentration-dependent manner (Tables 1 and 2). After stimulation for 30 min by hydroxyl free radical generation system Fe^{2+}-ascorbic acid, MDA generation was 2–8-fold that of the unstimulated liver, heart, and kidney homogenates. All the tested nitroxides concentration-dependently inhibited Fe^{2+}-ascorbic acid-induced MDA formation in diverse tissue homogenates (Tables 2 and 3). In general, the rank of inhibitory effect on MDA formation was as follows: 4-BIS-Tempo and 4-N_3-Tempo > 4-ISO-Tempo, 4-D-Tempo, 4-S-Tempo and 4-NH_2-Tempo > 4-A-Tempo and 4-B-Tempo. 4-NH_2-TempH, a corresponding amine-substituted derivative in the nitroxide moiety of 4-NH_2-Tempo, and other 4-position substitutes, such as NaN_3 or isothiocyanate, had almost no effect on MDA formation in stimulated and unstimulated experiments (data not shown).

Table 1 Effects of nitroxides on the spontaneous malondialdehyde (MDA) generation from liver homogenate of rats using the thiobarbituric acid method

Nitroxides (μmol L^{-1})	MDA (nmol (g weight)$^{-1}$)					
	4-NH_2-Tempo	4-ISO-Tempo	4-D-Tempo	4-N_3-Tempo	4-BIS-Tempo	4-S-Tempo
Control	153.2 ± 11.6	148.2 ± 2.7	134.0 ± 9.3	167.0 ± 13.4	144.4 ± 8.5	142.4 ± 9.0
0.656	150.7 ± 10.2	131.6 ± 16.7	138.5 ± 10.1	143.0 ± 12.3	110.6 ± 7.1^b	144.2 ± 6.4
1.313	134.6 ± 4.9^a	115.0 ± 12.1^b	128.3 ± 4.5	84.0 ± 2.8^b	69.8 ± 4.9^b	140.3 ± 7.6
2.625	116.5 ± 8.5^b	74.2 ± 9.8^b	113.7 ± 8.0^a	46.4 ± 4.8^b	54.2 ± 3.9^b	136.7 ± 8.4
5.25	111.2 ± 6.0^b	60.0 ± 7.3^b	67.4 ± 5.7^b	46.0 ± 6.6^b	51.2 ± 4.9^b	133.1 ± 9.2
10.5	104.8 ± 6.7^b	45.4 ± 4.5^b	29.0 ± 3.1^b	42.6 ± 3.8^b	49.6 ± 5.0^b	99.4 ± 9.3^b
21	94.2 ± 4.4^b	42.0 ± 4.7^b	28.5 ± 2.5^b	37.2 ± 4.8^b	48.0 ± 1.9^b	69.5 ± 6.8^b
42	49.5 ± 7.9^b	41.6 ± 6.9^b	21.0 ± 4.7^b	35.4 ± 4.9^b	33.4 ± 3.7^b	42.6 ± 5.2^b

Results are expressed as mean ± s.d. (n = 5). $^aP < 0.05$, $^bP < 0.01$ compared with control group (analysis of variance, LSD-*t* test).

Table 2 Inhibitory effects of 4-A-Tempo and 4-B-Tempo on malondialdehyde (MDA; nmol (g weight)$^{-1}$) generation from tissue homogenates of rats using the thiobarbituric acid method

Nitroxides (μmol L^{-1})	4-A-Tempo				4-B-Tempo	
	Spontaneously (liver)	Stimulated by Fe^{2+}-ascorbic acid			Spontaneously (liver)	Stimulated by Fe^{2+}-ascorbic acid
		Liver	Heart	Kidney		Liver
Basal	104.6 ± 4.54	31.6 ± 3.7[b]	130.3 ± 8.6[b]	27.1 ± 4.4[b]	168.4 ± 7.9	77.7 ± 4.1[b]
Control		207.0 ± 4.5	379.6 ± 7.1	99.3 ± 5.5		260.7 ± 6.9
5.25	77.7 ± 7.3[b]	180.9 ± 7.7[b]		75.1 ± 7.5[b]		
10.5	64.8 ± 5.3[b]	170.2 ± 6.5[b]	364.6 ± 8.2	54.2 ± 3.7[b]		
21	44.4 ± 9.6[b]	160.1 ± 8.2[b]	350.1 ± 3.3[b]	40.6 ± 8.4[b]	166.5 ± 6.7	212.7 ± 8.1[b]
42	33.4 ± 4.9[b]	136.3 ± 7.0[b]	302.7 ± 8.4[b]	39.4 ± 3.8[b]	154.3 ± 7.4	207.0 ± 6.7[b]
84	23.1 ± 3.0[b]	76.5 ± 6.2[b]	35.7 ± 4.9[b]	35.9 ± 5.5[b]	148.1 ± 5.9[b]	178.5 ± 9.5[b]
168	19.0 ± 2.3[b]	73.7 ± 3.2[b]	29.1 ± 5.9[b]	34.6 ± 7.9[b]	37.0 ± 7.5[b]	100.6 ± 8.8[b]
336					28.9 ± 4.1[b]	39.3 ± 6.1[b]

Results are expressed as mean ± s.d. (n = 5). [a]$P < 0.05$, [b]$P < 0.01$ compared with control or basal group (analysis of variance, LSD-t test).

Table 3 Inhibitory effects of nitroxides on malondialdehyde (MDA) generation from homogenates of rat liver, heart and kidney stimulated by Fe^{2+}-ascorbic acid

Nitroxides (μmol L^{-1})	MDA (nmol (g weight)$^{-1}$)						
	$4NH_2$-Tempo	4-ISO-Tempo	4-D-Tempo	$4-N_3$-Tempo	4-BIS-Tempo	4-S-Tempo	4-B-Tempo
In liver							
Basal	19.2 ± 1.1[b]	20.0 ± 3.0[b]	17.8 ± 2.3[b]	15.8 ± 3.9[b]	21.6 ± 3.4[b]	21.6 ± 4.2[b]	
Control	108.8 ± 13.1	106.8 ± 9.3	134.9 ± 8.4	117.8 ± 7.7	110.3 ± 7.6	98.7 ± 7.3	
0.656	107.7 ± 3.8	111.9 ± 8.6	134.2 ± 10.5	106.8 ± 3.2	100.4 ± 11.4	89.8 ± 9.4	
1.313	95.4 ± 7.9	97.7 ± 7.0	115.9 ± 8.5[a]	101.8 ± 5.6[a]	65.6 ± 11.0[b]	73.6 ± 7.2[b]	
2.625	83.3 ± 8.6[a]	67.5 ± 10.9[b]	101.3 ± 7.6[b]	69.2 ± 5.8[b]	42.6 ± 5.4[b]	71.0 ± 6.1[b]	
5.25	53.8 ± 3.7[b]	34.8 ± 6.0[b]	31.0 ± 4.6[b]	23.6 ± 3.1[b]	20.4 ± 3.0[b]	38.1 ± 4.7[b]	
10.5	22.9 ± 5.3[b]	19.5 ± 4.3[b]	19.0 ± 3.9[b]	21.8 ± 2.5[b]	16.6 ± 1.1[b]	17.6 ± 3.4[b]	
21	21.8 ± 3.1[b]	18.9 ± 4.6[b]	14.6 ± 2.1[b]	20.6 ± 3.1[b]	15.1 ± 3.5[b]	13.8 ± 2.6[b]	
In heart							
Basal	93.9 ± 3.2[b]	102.0 ± 2.8[b]	93.3 ± 6.3[b]	96.4 ± 6.8[b]	81.9 ± 4.7[b]	108.4 ± 4.4[b]	121.8 ± 7.4[b]
Control	304.6 ± 7.2	381.4 ± 14.1	358.3 ± 4.0	321.1 ± 4.6	323.4 ± 14.7	344.4 ± 6.7	357.4 ± 6.2
0.656				240.6 ± 8.8[b]			271.2 ± 8.2[b]
1.313	310.8 ± 7.9	387.3 ± 6.4	357.9 ± 11.5	176.4 ± 8.8[b]	328.2 ± 10.2	328.8 ± 14.1	265.7 ± 4.1[b]
2.625	289.6 ± 6.8	380.5 ± 5.6	350.3 ± 9.8	140.4 ± 7.9[b]	322.1 ± 7.1	324.4 ± 10.6[a]	128.8 ± 5.8[b]
5.25	265.0 ± 11.5[b]	376.5 ± 7.0	343.5 ± 6.5[a]	95.3 ± 7.4[b]	238.3 ± 12.8[b]	188.6 ± 9.1[b]	62.6 ± 4.2[b]
10.5	229.6 ± 22.4[b]	323.6 ± 15.6[b]	81.7 ± 7.5[b]	52.6 ± 6.5[b]	83.1 ± 8.1[b]	62.3 ± 9.8[b]	38.5 ± 4.9[b]
21	115.9 ± 16.0[b]	47.2 ± 1.2[b]	45.7 ± 4.2[b]	33.2 ± 4.5[b]	69.2 ± 6.2[b]	35.4 ± 4.3[b]	30.0 ± 3.1[b]
In kidney							
Basal	45.0 ± 2.5[b]	47.7 ± 4.3[b]	60.5 ± 2.9[b]	46.7 ± 5.9[b]	62.7 ± 7.8[b]	63.7 ± 4.1[b]	61.9 ± 4.6[b]
Control	122.9 ± 10.1	120.2 ± 3.9	116.5 ± 8.8	116.6 ± 7.85	125.2 ± 10.7	136.5 ± 6.4	147.7 ± 5.2
0.656				99.5 ± 4.8[a]	95.8 ± 4.34[b]		
1.313	120.8 ± 4.0	120.4 ± 9.2	113.3 ± 8.8	69.8 ± 6.8[b]	88.8 ± 6.7[b]	128.3 ± 7.4	149.3 ± 7.3
2.625	117.8 ± 8.8	119.2 ± 7.2	108.3 ± 8.2	48.0 ± 4.8[b]	76.9 ± 4.7[b]	116.5 ± 9.3[b]	143.2 ± 8.9
5.25	109.5 ± 5.4	95.2 ± 7.3[b]	92.0 ± 9.1[b]	45.7 ± 4.1[b]	59.0 ± 5.5[b]	89.2 ± 9.6[b]	137.9 ± 8.5
10.5	91.3 ± 6.3[b]	66.2 ± 6.5[b]	26.1 ± 2.7[b]	26.7 ± 5.4[b]	47.8 ± 6.3[b]	31.6 ± 4.6[b]	122.1 ± 4.8[b]
21	48.1 ± 5.8[b]	52.6 ± 3.7[b]	25.4 ± 5.1[b]	25.6 ± 4.9[b]	47.4 ± 7.3[b]	27.5 ± 5.8[b]	32.8 ± 3.8[b]

Results are expressed as mean ± s.d. (n = 5). [a]$P < 0.05$, [b]$P < 0.01$ compared with control group (analysis of variance, LSD-t test).

Table 4 Effects of nitroxides on rat RBC haemolysis stimulated by H_2O_2

Nitroxides (μmol L^{-1})	Haemolysis (%)							
	4-NH_2-Tempo	4-ISO-Tempo	4-A-Tempo	4-D-Tempo	4-N_3-Tempo	4-BIS-Tempo	4-B-Tempo	4-S-Tempo
Basal	2.91 ± 0.90^b	5.72 ± 0.65^b	1.73 ± 0.001^b	4.93 ± 1.64^b	2.60 ± 0.16^b	3.05 ± 2.31^b	7.94 ± 2.11^b	5.60 ± 1.32^b
Control	100	100	100	100	100	100	100	100
5.25						100.82 ± 17.49		
10.5	99.30 ± 3.07				89.50 ± 2.35^b	52.54 ± 11.00^b		
21	90.14 ± 1.41^b	96.00 ± 8.42			39.64 ± 0.76^b	5.56 ± 0.28^b		
42	13.92 ± 6.59^b	70.16 ± 5.41^b	103.32 ± 4.48	95.02 ± 1.36^a	3.71 ± 0.90^b		102.59 ± 3.56	85.3 ± 5.33^b
84	5.94 ± 0.85^b	7.54 ± 2.23^b	80.06 ± 7.90^b	86.83 ± 8.39^b			28.69 ± 1.30^b	78.10 ± 4.84^b
168			37.86 ± 3.90^b	70.05 ± 6.43^b			8.26 ± 1.30^b	66.1 ± 4.50^b
336				52.16 ± 7.51^b				51.50 ± 3.63^b

Results are expressed as mean ± s.d. (n = 5). $^aP < 0.05$, $^bP < 0.01$ compared with control group (analysis of variance, LSD-t test).

Inhibition of nitroxides on haemolysis

H_2O_2 can cause oxidative injury of RBC membrane and induce haemolysis. All the tested nitroxides inhibited RBC haemolysis induced by H_2O_2 (100 μmol L^{-1}) in a concentration-dependent manner (Table 4). Among all the tested nitroxides, the inhibitory effects of 4-BIS-Tempo and 4-N_3-Tempo were the strongest, whereas those of 4-A-Tempo, 4-D-Tempo, and 4-S-Tempo were the weakest. In general, the rank of inhibiting RBC haemolysis was as follows: 4-BIS-Tempo and 4-N_3-Tempo > 4-ISO-Tempo and 4-NH_2-Tempo > 4-B-Tempo > 4-A-Tempo, 4-D-Tempo and 4-S-Tempo. However, as with MDA generation, 4-NH_2-TempH and other 4-position substitutes, such as NaN_3 and isothiocyanate, were ineffective in RBC haemolysis (data not shown).

Inhibitory effects on $O_2^{\bullet -}$ generation

NBT was reduced to formazan, a purple colour, by $O_2^{\bullet -}$ from neutrophils stimulated by zymosan. The formazan indirectly reflected the amount of $O_2^{\bullet -}$ release. 4-ISO-Tempo was the most potent scavenger of $O_2^{\bullet -}$ in all the nitroxides tested (Table 5). The scavenging activity of 4-B-Tempo, 4-BIS-Tempo, and 4-N_3-Tempo exhibited weaker inhibitory effect on formazan formation while 4-A-Tempo and 4-D-Tempo had no effect. Moreover, 4-NH_2-TempH, NaN_3 and isothiocyanate were also ineffective (data not shown).

Structure–activity relationship

4-NH_2-TempH (336 μmol L^{-1}) was ineffective in MDA generation spontaneously or induced by Fe^{2+}-ascorbic acid from rat tissue homogenates, RBC haemolysis induced by H_2O_2, and $O_2^{\bullet -}$ release from activated neutrophils. However, 4-NH_2-Tempo itself inhibited MDA formation, haemolysis and $O_2^{\bullet -}$ release. Therefore, the nitroxide moiety was the essential group in nitroxide activity.

In addition, the substituents of the 4-position also affected piperidine nitroxide activity and may be functional groups. 4-BIS-Tempo with two nitroxide moieties, had at least twofold inhibitory effects on MDA formation, haemolysis and $O_2^{\bullet -}$ release compared with the corresponding single nitroxide moiety 4-NH_2-Tempo, as calculated using IC50 values (Tables 6 and 7). NaN_3 and isothiocyanate had almost no effect (data not shown), while their corresponding nitroxides, 4-N_3-Tempo and 4-ISO-Tempo, had more potent activity than those of 4-NH_2-Tempo. These results indicated that NaN_3 and

Table 5 Effects of nitroxides on the release of superoxide anion ($O_2^{\bullet -}$) from rat neutrophils stimulated by zymosan

Nitroxides (μmol L^{-1})	4-NH_2-Tempo	4-ISO-Tempo	4-A-Tempo	4-D-Tempo	4-N_3-Tempo	4-BIS-Tempo	4-B-Tempo	4-S-Tempo
Basal	0.106 ± 0.018^b	0.049 ± 0.006^b	0.216 ± 0.021^b	0.214 ± 0.023^b	0.049 ± 0.006^b	0.049 ± 0.006^b	0.214 ± 0.023^b	0.214 ± 0.023^b
Control	0.242 ± 0.009	0.131 ± 0.004	0.449 ± 0.016	0.449 ± 0.014	0.131 ± 0.004	0.131 ± 0.004	0.449 ± 0.014	0.449 ± 0.014
21		0.103 ± 0.003^b						
42		0.084 ± 0.002^b				0.125 ± 0.007		
84	0.236 ± 0.009	0.070 ± 0.001^b	0.446 ± 0.012	0.451 ± 0.020	0.132 ± 0.040	0.120 ± 0.004^a	0.420 ± 0.012^a	0.413 ± 0.014^a
168	0.230 ± 0.003^a	0.051 ± 0.002^b	0.432 ± 0.006	0.443 ± 0.025	0.123 ± 0.011	0.112 ± 0.001^b	0.333 ± 0.005^b	0.403 ± 0.008^b
336	0.213 ± 0.004^b	0.050 ± 0.003^b	0.435 ± 0.004	0.454 ± 0.017	0.083 ± 0.008^b	0.082 ± 0.002^b	0.304 ± 0.012^b	0.401 ± 0.008^b

Results are expressed as mean ± s.d. (n = 4). $^aP < 0.05$, $^bP < 0.01$ compared with control group (analysis of variance, LSD-t test).

Table 6 The IC50 values (μmol L^{-1}) of nitroxides on MDA formation in rat tissue homogenates

Nitroxides	Liver (spontaneously)	Liver (Fe^{2+}-ascorbic acid)	Heart (Fe^{2+}-ascorbic acid)	Kidney (Fe^{2+}-ascorbic acid)
4-NH_2-Tempo	25.47 ± 1.02	3.73 ± 0.38	10.16 ± 0.42	9.07 ± 0.46
4-ISO-Tempo	4.97 ± 0.33	3.02 ± 0.20	9.33 ± 0.64	6.73 ± 0.35
4-A-Tempo	18.31 ± 0.94	44.04 ± 1.46	32.05 ± 0.85	6.27 ± 0.40
4-D-Tempo	6.64 ± 0.36	3.11 ± 0.30	5.67 ± 0.44	3.46 ± 0.35
4-N_3-Tempo	1.95 ± 0.16	2.68 ± 0.24	0.89 ± 0.15	0.91 ± 0.12
4-BIS-Tempo	2.18 ± 0.12	1.48 ± 0.16	5.49 ± 0.28	0.78 ± 0.20
4-B-Tempo	125.59 ± 4.35	60.42 ± 1.76	1.07 ± 0.15	8.08 ± 0.45
4-S-Tempo	24.48 ± 0.74	2.50 ± 0.18	3.83 ± 0.22	3.12 ± 0.62

IC50, 50% inhibition concentration, was calculated by linear regression analysis.

Table 7 The IC50 values (μmol L^{-1}) of nitroxides on haemolysis and formazan reduced by $O_2^{\bullet -}$

Nitroxides	H_2O_2-induced haemolysis	Formazan reduced by $O_2^{\bullet -}$
4-NH_2-Tempo	30.20 ± 2.48	> 336
4-ISO-Tempo	45.45 ± 1.56	34.96 ± 1.45
4-A-Tempo	136.55 ± 3.05	ND
4-D-Tempo	365.18 ± 4.52	ND
4-N_3-Tempo	18.75 ± 0.85	320.00 ± 12.65
4-BIS-Tempo	10.68 ± 0.22	326.34 ± 17.50
4-B-Tempo	74.08 ± 3.42	215.5 ± 14.38
4-S-Tempo	345.12 ± 4.28	> 336

IC50 (50% inhibition concentration) was calculated by linear regression analysis. ND, not detected.

isothiocyanate substituted for NH_2 at the 4-position enhanced the activity of the nitroxide moiety.

Moreover, the 4-position substitutes influenced the selectivity of nitroxides on ROS. Based on the Fenton reaction, Fe^{2+} and ascorbic acid produced ·OH, induced lipid peroxidation and formed MDA in tissue homogenates. MDA generation indirectly reflected the amount of ·OH formation. Formazan, the NBT-reduced product by $O_2^{\bullet -}$, represented the amount of $O_2^{\bullet -}$ generation from activated neutrophils. H_2O_2-induced RBC haemolysis mainly reflected the extent of lipoperoxidation of RBC membrane. In this study, all the tested nitroxides had an inhibitory effect on MDA formation and haemolysis. Although the rank of inhibitory effect of nitroxides on MDA formation was the same as that of haemolysis (4-BIS-Tempo and 4-N_3-Tempo > 4-ISO-Tempo, 4-D-Tempo, 4-S-Tempo and 4-NH_2-Tempo > 4-A-Tempo and 4-B-Tempo) the IC50 value of inhibiting MDA formation was lower than that of haemolysis (Tables 6 and 7). With the exception of 4-A-Tempo and 4-D-Tempo, the other tested nitroxides were effective in inhibiting $O_2^{\bullet -}$ release. Meanwhile, the concentration of inhibiting $O_2^{\bullet -}$ release was higher than that of MDA formation and haemolysis. As shown in Tables 6 and 7, the rank of IC50 value of nitroxides in scavenging ROS was as follows: $O_2^{\bullet -}$ > H_2O_2 > ·OH. Therefore, the substitutes of the 4-position could influence piperidine nitroxide activity on scavenging ROS.

Discussion

Many diseases are linked directly or indirectly to free radical processes including stroke, allergies, ischaemia/reperfusion injury, ageing, Parkinson's disease, and carcinogenesis (Halliwell & Gutteridge 1999). Free radicals are produced exogenously by ionizing radiation or specific chemical agents and endogenously as a result of oxygen metabolism. Oxygen is an abundant electron sink that sustains life, yet oxygen metabolites such as superoxide anion, hydroxyl radical, and hydrogen peroxide are potentially toxic ROS. ROS are major promoters of lipid peroxidation (LPO). LPO is a major damaging process in membranes and liposomal dispersions containing polyunsaturated acyl chains. Prevention of LPO may become an important therapy in many related diseases that involve LPO, and is an important aspect in the preparation and preservation of liposomes and similar lipid assemblies.

The common antioxidative defence strategies include enzymatic and especially nonenzymatic mechanisms. Nitroxides are synthetic, stable radicals that vary in size, structure, charge, and lipophilicity, and have been identified as novel antioxidants protecting isolated macromolecules, cells, organs, and whole animals from diverse insults (Hahn et al 1992; Monti et al 1996; Offer & Samuni 2002; Gadjeva et al 2005). Based on the prominent antioxidative activity of nitroxides, these compounds may be potential therapeutic agents in disease and injury involving oxidative stress. It has been confirmed that as a novel nonenzymatic antioxidant, the antioxidative mechanisms of nitroxides include: oxidation of transition metal ions to pre-empt the Fenton reaction (Risso-de Faverneya et al 2000; Zeltcer et al 2002); superoxide dismutase (SOD)-like

activity, cyclic nitroxide stable free radicals oxidize $O_2^{\bullet -}$ radicals, yielding O_2 and H_2O_2, while the nitroxide itself is recycled. Unlike the exogenously added SOD, which does not enter cells, nitroxides are small, nonimmunogenic and cell-permeate molecules, readily crossing biological membranes and barriers (Samuni et al 1988; Samuni & Krishna 1997; Hoffman et al 2003) and scavenging other toxic free radicals (Offer & Samuni 2002). Our results showed that all the tested nitroxides inhibited MDA formation and haemolysis in a concentration-dependent manner. Except for 4-A-Tempo and 4-D-Tempo, the tested nitroxides inhibited $O_2^{\bullet -}$ release from activated neutrophils, whereas the corresponding amine nitroxide 4-NH_2-TempH had no effect. Nitroxide stable radicals may function as antioxidants via the above mechanisms and operate through a cyclic mechanism of electron transfer among three oxidation states: the oxoammonium cation, the nitroxide and the hydroxylamine. It has been confirmed that the nitroxides are rapidly reduced to the corresponding intracellular hydroxylamine, which has antioxidative activity (Wu et al 1997; Krishna et al 1998; Hahn et al 2000). Therefore, through a rapid exchange between them, the two forms can be recycled and can act catalytically while the corresponding amine nitroxides have no antioxidative activity. It was evident that the nitroxide moiety was the essential group in the activity of the nitroxides.

Fe^{2+} induces hydroxyl radical (·OH) generation under existence of ascorbic acid according to the Fenton reaction. ·OH is the most toxic ROS that brings about lipoperoxidation accompanying MDA generation. In this study, MDA generation was used as an indicator of ·OH-caused LPO and indirectly reflected the amount of ·OH formation in tissue homogenates spontaneously or stimulated by Fe^{2+}-ascorbic acid. H_2O_2, as a sort of ROS, could produce ·OH according to the Fenton reaction, causing haemolysis that mainly reflected the extent of lipoperoxide of RBC membrane. The formazan was a NBT-reduced product by superoxide anion during neutrophil activation, representing the amount of $O_2^{\bullet -}$ generation from activated neutrophils. Although in general the inhibition effect of nitroxides on MDA formation followed the same order as haemolysis (4-BIS-tempo and 4-N_3-Tempo > 4-ISO-tempo, 4-D-Tempo, 4-S-Tempo and 4-NH_2-Tempo > 4-A-Tempo and 4-B-Tempo), inhibition for MDA formation needed lower concentrations of corresponding nitroxides than haemolysis. Apart from 4-A-Tempo and 4-D-Tempo, all tested nitroxides were effective in inhibiting $O_2^{\bullet -}$ release at the highest concentration. It was demonstrated that nitroxides scavenged ROS with the order ·OH > H_2O_2 > $O_2^{\bullet -}$. Nitroxides may oxidize (transition metal ions) Fe^{2+} to pre-empt the Fenton reaction, stop initiation of Fenton reaction and terminate the oxidative chain reaction, and then prevent the formation of secondary ·OH (Zeltcer et al 2002). Thus, nitroxides have a stronger and more rapid scavenging effect on ·OH than other ROS.

Our data also showed that NaN_3 and isothiocyanate had almost no effect, while all the tested nitroxides had the same rank in inhibiting MDA formation and haemolysis: 4-BIS-Tempo and 4-N_3-Tempo > 4-ISO-Tempo, 4-D-Tempo, 4-S-Tempo and 4-NH_2-Tempo > 4-A-Tempo and 4-B-Tempo. 4-ISO-Tempo was the most potent scavenger of $O_2^{\bullet -}$ of all the tested nitroxides, and 4-B-Tempo, 4-BIS-Tempo, and 4-N_3-Tempo exhibited lower inhibitory effects on formazan formation, while 4-A-Tempo and 4-D-Tempo had no effect. It was concluded that the substituents of the 4-position affected the functions of piperidine nitroxides, thus they may be functional groups. Krishna et al (1998) reported that nitroxides had better radioprotective and cytoprotective actions on exposure to H_2O_2 when the substitutes contained an amino group in the side chain. Consistent with this notion, the substitutes of 4-BIS-Tempo, 4-N_3-Tempo, 4-ISO-Tempo, 4-D-Tempo, and 4-NH_2-Tempo contained an amino group or nitrogen atom and possessed more potent activity in scavenging ROS. In general, 4-NH_2-Tempo had higher activity than 4-oxy-2,2,6,6-tetramethyl piperidinooxyl (4-O-Tempo) and 4-hydroxy-2,2,6,6-tetramethyl piperidinooxyl (4-OH-Tempo), without the amino group in the 4-position in the same experiment system as reported by Wu et al (1997).

Recently, many efforts have been made in the search for efficient antioxidants to augment the commonly used antioxidant defence. An ideal and effective antioxidant must have the ability to: present and act in both lipophilic and hydrophilic compartments; react with and detoxify a wide range of reactive species, stopping initiation and propagation, and terminating the oxidative chain reaction; and act in a recycling manner that enables replenishment of the antioxidant (Samuni & Barenholz 2003). In summary, as a novel nonenzymatic antioxidant, cyclic nitroxides may well fulfil these requirements and have a challenging prospect for clinical use. Previous studies have demonstrated that spin-labelled derivatives of anti-tumour agents such as triazenes or podophyllic acid hydrazine showed low toxicity and potent anti-tumour activity. These results could account for a possible correlation among tissue distribution and the selective anti-tumour activity of the spin-labelled derivatives and their antioxidative activity (Gadjeva 2002; Li et al 2002; Tian et al 2002). This may be one aspect of the uses of nitroxides. A number of reports have demonstrated that antioxidants may be useful as a functional food or drug against reactive species-mediated diseases such as inflammation (Cho et al 2006), hypotension, hyperglycaemia, cytokine immunoreactivity (Shen et al 2005), cancers, osteoporosis and cardiovascular diseases (Paulo & Mota-Filipe 2006), and radiation-induced damage (Goel et al 2005). Nitroxides as a kind of novel antioxidant may also have these functions.

Conclusions

All of the tested piperidine nitroxides inhibited MDA generation from homogenates, and antagonized H_2O_2-induced haemolysis from rat erythrocytes. With the exception of 4-A-Tempo and 4-D-Tempo, the tested piperidine nitroxides inhibited $O_2^{\bullet -}$ release from neutrophils stimulated by zymosan. It was concluded that the nitroxide moiety was the essential group of nitroxides; the substitutents of the 4-position could influence piperidine nitroxide activity on scavenging ROS. The substitutes containing an amino group or nitrogen atom could significantly potentiate the activity of the nitroxide moiety in scavenging ROS.

References

Anastassopoulou, J. D., Rakintzis, N. T. (1984) Reaction of OH radicals with 2,2,6,6-tetramethyl-4-piperidinol-oxyl (TEMPOL) in a aqueous solution. *Z. Phys. Chem. Neu. Folge*. **141**: S53–58

Baehner, R. L., Nathan, D. G. (1968) Quantitative nitroblue tetrazolium test in chronic granulomatous disease. *N. Engl. J. Med.* **278**: 971–976

Brasch, R. C., London, D. A., Wesbey, G. E., Tozer, T. N., Nitecki, D. E., Williams, R. D., Doemeny, J., Tuck, L. D., Lallemand, D. P. (1983) Work in progress: Nuclear magnetic resonance study of a paramagnetic nitroxide contrast agent for enhancement of renal structures in experimental animals. *Radiology* **147**: 773–779

Cho, J. Y., Park, S. C., Kim, T. W., Kim, K. S., Song, J. C., Kim, S. K., Lee, H. M., Sung, H. J., Park, H. J., Song, Y. B., Yoo, E. S., Lee, C. H., Rhee, M. H. (2006) Radical scavenging and anti-inflammatory activity of extracts from *Opuntia humifusa* Raf. *J. Pharm. Pharmacol.* **58**: 113–119

Davies, N. M., Jamali, F. (1997) Pharmacological protection of NSAID induced intestinal permeability in the rat: effect of tempo and metronidazole as potential free radical scavengers. *Hum. Exp. Toxicol.* **16**: 345–349

Gadjeva, V. (2002) Two spin labeled triazenes: relationship between biochemical and biological activities. *Int. J. Pharmaceutics* **247**: 39–45

Gadjeva, V., Kuchukova, D., Tolekova, A., Tanchev, S. (2005) Beneficial effects of spin-labelled nitrosourea on CCNU-induced oxidative stress in rat blood compared with vitamin E. *Pharmazie* **60**: 530–532

Gallez, G., Demeure, R., Debuyst, R., Leonard, D., Dejehet, F., Dumont, P. (1992) Evaluation of nonionic nitroxyl lipids as potential organ-specific contrast agents for magnetic resonance imaging. *Magn. Reson. Med.* **10**: 445–455

Gariboldi, M. B., Ravizza, R., Petterino, C., Castagnaro, M., Finocchiaro, G., Monti, E. (2003) Study of in vitro and in vivo effects of the piperidine nitroxide Tempol – a potential new therapeutic agent for gliomas. *Eur. J. Cancer* **39**: 829–837

Goel, H. C., Gupta, D., Gupta, S., Garg, A. P., Bala, M. (2005) Protection of mitochondrial system by *Hippophae rhamnoides* L. against radiation-induced oxidative damage in mice. *J. Pharm. Pharmacol.* **57**: 135–143

Hahn, S. M., Wilson, L., Krishna, C. M., Liebmann, J., DeGraff, W., Gamson, J., Samuni, A., Venzon, D., Mitchell, J. B. (1992) Identification of nitroxide radioprotectors. *Radiat. Res.* **132**: 87–93

Hahn, S. M., Krishna, C. M., Deluca, A. M., Coffin, D., Mitchell, J. B. (2000) Evaluation of the hydroxylamine tempol-h as an in vivo radioprotector. *Free Radic. Biol. Med.* **28**: 953–958

Halliwell, B., Gutteridge, J. M. C. (1999) Free radicals, other reactive species, and disease. In: Halliwell, B., Gutteridge, J. M. C. (eds) *Free Radicals in biology and medicine*. Oxford University Press, Oxford, UK, pp 617–783

Hoffman, A., Goldstein, S., Samuni, A., Borman, J. B., Schwal, H. (2003) Effect of nitric oxide and nitroxide SOD-mimic on the recovery of isolated rat heart following ischemia and reperfusion. *Biochem. Pharmacol.* **66**: 1279–1286

Karmeli, F., Eliakim, R., Okon, E., Rachmilewitz, D. (1995) Ketotifen and nitroxides decrease capsaicin-augmented ethanol-induced gastric damage in rats. *Dig. Dis. Sci.* **40**: 1140–1146

Keana, J. F. W., Lex, L., Mann, J. S., May, J. M., Park, J. H., Pou, S., Prabhu, V. S., Rosen, G. M., Sweetman, B. J., Wu, Y. (1990) Novel nitroxides for spin-labelling, -trapping, and magnetic resonance imaging applications. *Pure Appl. Chem.* **62**: 201–205

Krishna, M. C., DeGraff, W., Hankovszky, O. H., Sar, C. P., Kalai, T., Jeko, J., Russo, A., Mitchell, J. B., Hideg, K. (1998) Studies of structure–activity relationship of nitroxide free radicals and their precursors as modifiers against oxidative damage. *J. Med. Chem.* **41**: 3477–3492

Li, W. G., Zhang, X. Y., Wu, Y. J., Tian, X. (2002) Antioxidative activity of spin labelled derivatives of podophyllilic acid hydrazide. *Acta Pharmacol. Sin.* **23**: 727–732

Mitchell, J. B., Xavier, S., Deluca, A., Sowers, A. L., Cook, J. A., Krishna, M. C., Hahn, S. M., Russo, A. (2003) A low molecular weight antioxidant decreases weight and lowers tumor incidence. *Free Radic. Biol. Med.* **34**: 93–102

Monti, E., Cova, D., Guido, E., Morelli, R., Oliva, C. (1996) Protective effect of the nitroxide TEMPOL against the cardiotoxicity of adriamycin. *Free Radic. Biol. Med.* **21**: 463–470

Offer, T., Samuni, A. (2002) Nitroxides inhibit peroxyl radical-mediated DNA scission and enzyme inactivation. *Free Radic. Biol. Med.* **32**: 872–881

Paulo, A., Mota-Filipe, H. (2006) Effects of some natural 5-hydroxy-isoflavones on cultured human endothelial cells in presence and absence of hydrogen peroxide. *J. Pharm. Pharmacol.* **58**: 101–105

Rak, R., Chao, D. L., Pluta, R. M., Mitchell, J. B., Oldfield, E. H., Watson, J. C. (2000) Neuroprotection by the stable nitroxide Tempol during reperfusion in a rat model of transient focal ischemia. *J. Neurosurg.* **92**: 646–651

Risso-de Faverneya, C., Lafaurie, M., Girard, J. P., Rahmani, R. (2000) The nitroxide stable radical tempo prevents metal-induced inhibition of CYP1A1 expression and induction. *Toxicol. Lett.* **111**: 219–227

Samuni, A., Krishna, C. M. (1997) Antioxidant properties of nitroxides and nitroxide SOD-mimics. In: Packer, L., Cadenas, E. (eds) *Handbook of synthetic antioxidants*. Marcel Dekker, New York, pp 351–373

Samuni, A. M., Barenholz, Y. (2003) Site-activity relationship of nitroxide radical's antioxidative effect. *Free Radic. Biol. Med.* **34**: 177–185

Samuni, A., Krishna, C. M., Riesz, P., Finkelstein, E., Russo, A. (1988) A novel metal-free low molecular weight superoxide dismutase mimic. *J. Biol. Chem.* **263**: 17921–17924

Sasaki, H., Lin, L. R., Yokoyama, T., Sevilla, M. D., Reddy, V. N., Giblin, F. J. (1998) TEMPOL protects against lens DNA strand breaks and cataract in the x-rayed rabbit. *Invest. Ophthalmol. Vis. Sci.* **39**: 544–552

Shen, K. P., Lo, Y. C., Yang, R. C., Liu, H. W., Chen, I. J., Wu, B. N. (2005) Antioxidant eugenosedin-A protects against lipopolysaccharide-induced hypotension, hyperglycaemia and cytokine immunoreactivity in rats and mice. *J. Pharm. Pharmacol.* **57**: 117–125

Swartz, H. M. (1986) Interaction between cells and nitroxides and their implications for their uses as biophysical probes and as metabolically responsive contrast agents for in vivo NMR. *Bull. Mag. Res.* **8**: 172–175

Tanizaw, H., Sazuka, Y., Takino, Y. (1981) Micro-determination of lipoperoxide in the mouse myocardium by thiobarbituric acid fluorophotometry. *Chem. Pharm. Bull.* **29**: 2910–2914

Tian, X., Zhang, F. M., Li, W. G. (2002) Antitumor and antioxidant activity of spin labeled derivatives of podophyllotoxin (GP-1) and congeners. *Life Sci.* **70**: 2433–2443

Udassin, R., Haskel, Y., Samuni, A. (1998) Nitroxide radical attenuates ischemia/reperfusion injury to the rat small intestine. *Gut* **42**: 623–627

Wu, Y. J., Li, W. G., Zhang, Z. M., Tian, X. (1997) Antioxidative activity of 4-oxy and 4-hydroxy nitroxides in tissues and erythrocytes from rats. *Acta Pharmacol. Sin.* **18**: 150–154

Zamir, E., Zhang, R., Samuni, A., Kogan, M., Pe'er, J. (1999) Nitroxide stable radical suppresses autoimmune uveitis in rats. *Free Radic. Biol. Med.* **27**: 7–15

Zeltcer, G., Berenshtein, E., Samuni, A., Chevion, M. (1997) Nitroxide radicals prevent metal-aggravated reperfusion injury in isolated rat heart. *Free Radic. Res.* **27**: 627–635

Zeltcer, G., Berenshtein, E., Kitrossky, N., Chevion, M., Samuni, A. (2002) Time window of nitroxide effect on myocardial ischemic-reperfusion injury potentiated by iron. *Free Radic. Biol. Med.* **32**: 912–919

Zhang, R., Shohami, E., Beit-Yannai, E., Bass, R., Trembovler, V., Samuni, A. (1998) Mechanism of brain protection by nitroxide radicals in experimental model of closed-head injury. *Free Radic. Biol. Med.* **24**: 332–340