ELSEVIER

European Journal of Pharmacology 303 (1996) 129–139

ejp

N-Allylsecoboldine as a novel antioxidant against peroxidative damage

Che-Ming Teng [a,*], George Hsiao [a], Feng-Nein Ko [a], Dong-Tsamn Lin [b], Shoei-Sheng Lee [c]

[a] *Pharmacological Institute, College of Medicine, National Taiwan University, Taipei, Taiwan*
[b] *Department of Clinical Pathology, College of Medicine, National Taiwan University, Taipei, Taiwan*
[c] *School of Pharmacy, College of Medicine, National Taiwan University, Taipei, Taiwan*

Received 3 January 1996; revised 31 January 1996; accepted 2 February 1996

Abstract

N-Allylsecoboldine was evaluated for antioxidant properties by studying its ability to react with relevant reactive oxygen species, and its protective effect on human erythrocytes under oxidative stress. Using brain homogenates, we found that *N*-allylsecoboldine dose dependently inhibited lipid peroxidation ($IC_{50} = 4.80 \pm 0.16$ μM) and markedly scavenged stable nitrogen-centered radicals. *N*-Allylsecoboldine was a very efficient scavenger for inhibiting peroxyl radical-mediated destruction of B-phycoerythrin, with a stoichiometry factor of 4.40 ± 0.59. It also trapped the hydroxyl radicals with a second-order rate constant of $6.92 \pm 0.86 \times 10^9$ M^{-1} S^{-1}. Additionally, human erythrocyte oxidative hemolysis induced by aqueous peroxyl radical or hydrogen peroxide was suppressed by *N*-allylsecoboldine. It not only attenuated the extent of lipid peroxidation but also decreased the formation of the high-molecular weight proteins and degradation of the band 6 protein in radical-treated erythrocytes. It also inhibited the shortening of Russell's viper venom-clotting time mediated by prelytic radical-treated erythrocytes. In the presence of exogenous oxidative stress, hemolysis and lipid peroxidation were significantly enhanced in β-thalassemic erythrocytes, as compared to the normal control. These elevated detrimental effects could be prevented by *N*-allylsecoboldine. It is concluded that *N*-allylsecoboldine may act as an effective antioxidant and protect cells against oxidative damage.

Keywords: *N*-Allylsecoboldine; Antioxidant; Lipid peroxidation; Peroxyl radical; Hydroxyl radical; Hypercoagulability; β-Thalassemia; Oxidative hemolysis

1. Introduction

There is increasing evidence that oxidative stress is a major contributor to the etiology of degenerative diseases, including cardiovascular diseases (Powell and Tortolani, 1992) and some hematopathies (Saltman, 1989). Under oxidative stress, reactive oxygen species will emerge and overwhelm the native cellular defense system. Subsequently, these substances will overflow and attack the cellular macromolecules directly or indirectly. Especially their oxidative damage to phospholipids could lead to lipid peroxidation and then cause alterations in the structure of the cellular membrane, giving rise to reactive lipid derivatives which are capable of modifying the membrane proteins (Dean et al., 1986). The peroxidative attack on erythrocyte membranes could disturb the cell deformability, leading to circulatory impairment, and may lead to hemolysis (Chiu et al., 1989). Such oxidative damage also plays an important role in the denaturation of β-thalassemic erythrocytes (Shinar and Rachmilewitz, 1990); α-tocopherol as an antioxidant could diminish these deleterious effects (Giardini et al., 1981). Therefore, lipid peroxidation may be critically involved in some pathophysiological events.

It has been suggested that reactive oxygen-derived free radicals, such as superoxide anions, hydroxyl radicals and peroxyl radicals, formed during the early moment of reperfusion, and lipid peroxidation proarrhythmic-products (4-hydroxynonenal, etc.), have been implicated in myocardial ischemia/reperfusion injury (Downey, 1990; Bhatnagar, 1995). Therefore, several scavengers of oxygen-derivated radicals have been found to be effective to reduce reperfusion-induced damage (Abadie et al., 1993; Gelvan et al., 1991). Recently, it has been reported that a secoaporphine derivative called *N*-allylsecoboldine (Fig. 1) is effective to reduce reperfusion-induced arrythmia (Wu et al., 1994). In

* Corresponding author. Pharmacological Institute, College of Medicine, National Taiwan University, No. 1, Jen-Ai Road, 1 st Section, Taipei 10018, Taiwan. Fax: 886-2-322-1742.

PII S0014-2999(96)00102-1

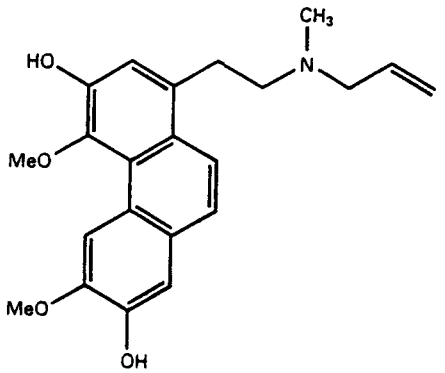

Fig. 1. Chemical structure of *N*-allylsecoboldine.

the present study, we further investigated the antioxidant properties of *N*-allylsecoboldine and evaluated its ability to protect human erythrocytes, especially those from β-thalassemia patients, from peroxidative attack. The results suggest strongly that *N*-allylsecoboldine, in addition to its ionic channel blocking activity (Wu et al., 1994), is a novel antioxidant which could protect cells from exogenous oxidative damage.

2. Materials and methods

2.1. Chemicals

N-Allylsecoboldine was prepared from boldine by exhaustive *N*-alkylation (Lee et al., 1992). Desferrioxamine mesylate, α-tocopherol, probucol, butylated hydroxytoluene, 2-thiobarbituric acid, tetramethoxypropane, diphenyl-*p*-picrylhydrazyl, B-phycoerythrin, 2-deoxy-D-ribose, xanthine oxidase (Grade IV, from buffermilk), cytochrome c (Type III, from horse heart), catalase, superoxide dismutase (Type I, from bovine liver), D-mannitol, hydrogen peroxide (30% solution) and Russell's viper venom were purchased from Sigma Chemical Co. (USA). Trolox was purchased from Aldrich Chemical Co. (USA). 2,2′-Azo-bis(2-amidinopropane)dihydrochloride (AAPH) was obtained from Wako Pure Chemical (Japan). All the other chemicals used in this study were of the highest purity grade for research.

2.2. Antioxidant activity in the supernatant of rat brain homogenate

The supernatant of Wistar rat brain homogenate was freshly prepared according to the methods of Braughler et al. (1988) with some modifications. In brief whole brain tissue, excluding the cerebellum, was washed and homogenized in 10 volumes of ice-cold Krebs buffer [10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), 10 mM glucose, 140 mM NaCl, 3.6 mM KCl, 1.5 mM $CaCl_2$, 1.4 mM KH_2PO_4, 0.7 mM $MgSO_4$, pH 7.4] using a glass Dounce homogenizer. The homogenate was centrifuged at low speed ($1000 \times g$) for 10 min, and the resulting supernatant (adjusted to 2 mg/ml) was used immediately in lipid peroxidation assays. The reaction mixture with test compounds or vehicle was incubated for 10 min, then stimulated by addition of ferrous ion (200 μM, freshly prepared), and maintained at 37°C for 30 min. The reactions were terminated by adding 10 μl of ice-cold trichloroacetic acid solution [4% (w/v) in 0.3 N HCl] and 200 μl of thiobarbituric acid-reactive substance reagent [0.5% (w/v) thiobarbituric acid in 50% (v/v) acetic acid]. After boiling for 15 min, the samples were cooled and extracted with *n*-1-butanol. The extent of lipid peroxidation was estimated as thiobarbituric acid-reactive substances and was read at 532 nm in a spectrophotometer (Hitachi, Model U 3200). Tetramethoxypropane was used as a standard, and the results were expressed as nanomoles of malondialdehyde equivalents per milligram protein of the supernatant of rat brain homogenates. The protein contents of the brain homogenates and other preparations were determined with the Bio-Rad method (Marion, 1976), employing bovine serum albumin as a standard.

2.3. Stable free radical scavenging action

Diphenyl-*p*-picrylhydrazyl, a stable nitrogen-centered free radical, was dissolved in ethanol to give a 100 μM solution. To 1.0 ml of ethanolic diphenyl-*p*-picrylhydrazyl in a cuvette was added the test compound or vehicle. The decrease in absorption at 517 nm was correlated with the scavenging action of the test compound (Mellors and Tappel, 1966), and the concentration of the antioxidant that induced a change of 0.20 in absorbance during 30 min observation time was taken as evaluation of antioxidant activity.

2.4. Peroxyl radical scavenging action

The scavenging effect of compounds on the peroxyl radical was determined with the method described by Tsuchiya et al. (1992). The final reaction mixtures contained 5 nM B-phycoerythrin, 25 mM AAPH, and any other additives in 2.0 ml of 75 mM phosphate buffer, pH 7.0, at 40°C. The decrease in relative fluorescence emission of B-phycoerythrin by AAPH-generated peroxyl radical was monitored at 575 nm with excitation at 540 nm in a Hitachi F4000 fluorescence spectrophotometer. Dimethyl sulfoxide (DMSO), the solvent for *N*-allylsecoboldine, had little effect on this reaction. Ascorbic acid or Trolox was used as a positive control. The stoichiometry factor of *N*-allylsecoboldine with the peroxyl radical was calculated with the equation mentioned (Tsuchiya et al., 1992). At 40°C, the rate of peroxyl radical formation from AAPH is 1.6×10^{-6} [AAPH] per second (Teras and Niki, 1986).

2.5. Superoxide anion scavenging action

The superoxide scavenging activity of the compounds was determined by monitoring their competition with cy-

tochrome c for superoxide anion generated by the xanthine/xanthine oxidase system (Fridovich, 1970). Different concentrations of compounds, 0.5 mg/ml cytochrome c, 0.3 mM K_2H_2-EDTA and 100 μM xanthine in 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.4) were incubated for 1 min, and 0.02 U/ml xanthine oxidase was then added. Reduction of cytochrome c was measured spectrophotometrically at 550 nm at 25°C, using the extinction coefficient for the reduction of ferricytochrome c as given by Massey (1959), $\Delta\epsilon$ 550 = 21 000 M^{-1} cm^{-1}. The first minute rate of superoxide-induced cytochrome c reduction was determined by subtraction of that of cytochrome c reduction in the presence of 100 U/ml superoxide dismutase. Superoxide dismutase was used as a positive control.

The effect of compounds on the activity of xanthine oxidase was determined as described by Fridovich (1970) with minor modifications. The formation of urate from the enzyme system was monitored spectrophotometrically at 295 nm. A molar extinction coefficent of 11 000 M^{-1} cm^{-1} for uric acid was used for calculation.

2.6. Scavenging of hydrogen peroxide

The content of hydrogen peroxide (H_2O_2) was measured indirectly using a catalase-based method, with a Clark-type electrode (YSI model 5331, Yellow Spring Instruments Co., OH, USA). The reaction mixtures contained 0.5 mM or 1.0 mM H_2O_2 and test compound (100 μM) in 50 mM potassium phosphate buffer with 0.2 mM NaCl, pH 7.4. After the mixture was incubated for 40 min at 25°C, catalase (5.96 U/ml) was added and O_2 release was monitored polarographically for 0.8 min. After the experiments the amount of H_2O_2 remaining was calculated using the standard curve made for O_2 production vs. H_2O_2 concentration (0.1–2.0 mM).

2.7. Scavenging of the hydroxyl radical generated by the ascorbate / iron / H_2O_2 systems

The reaction mixture contained 2.8 mM 2-deoxyribose, 1.42 mM H_2O_2, 20 μM $FeCl_3$ or with 100 μM EDTA and test compound in 10 mM KH_2PO_4-KOH buffer, pH 7.4. The reaction was triggered by the addition of 50 μM ascorbate and the mixture was incubated for 60 min at 37°C. *N*-Allylsecoboldine solution was prepared by dissolving the material in acidic solution and then readjusting the pH to 7.4 immediately before use. Solutions of $FeCl_3$, H_2O_2 and ascorbate were made up fresh just before use. The extent of deoxyribose degradation by $HO^{\cdot}$ was measured with the thiobarbituric acid method (Halliwell and Gutteridge, 1981). After heating at 100°C for 15 min, and cooling, the absorbance at 532 nm was measured. The second-order rate constants for scavengers were calculated from the slope of competition plots as described by Halliwell et al. (1987) assuming that deoxyribose reacts with $HO^{\cdot}$ with a constant of 3.1×10^9 M^{-1} S^{-1}.

2.8. Preparation and oxidative treatment of human erythrocytes

Blood samples were obtained from healthy or from β-thalassemic individuals by venipuncture after informed consent had been given. The citrated blood was centrifuged and washed three times with phosphate-buffered saline (PBS), the plasma and buffy coat were carefully removed by aspiration after each wash. Washed erythrocytes were finally resuspened in buffered saline [125 mM NaCl and 10 mM sodium phosphate buffer (pH 7.4)] or PBS, and the concentration of erythrocytes was measured in a cell counter (Hemalaser, Sebia) and adjusted to 10^9 erythrocytes/ml with the same buffer.

Erythrocyte ghosts were prepared according to the method of Dodge et al. (1963) with some modifications. One volume of washed erythrocytes was lysed in 30 volumes of hypotonic phosphate buffer (5 mM NaH_2PO_4, 0.5 mM EDTA, pH 8.0, 4°C), and the erythrocyte ghosts were washed three times with the buffer. The final concentrated pellet of ghosts were adjusted to the concentrations indicated with PBS and used within 2 days.

The erythrocyte suspensions (10^9 erythrocytes/ml, in the buffered saline) were added to the same volume of water-soluble radical initiator, AAPH (in 10 mM PBS). The erythrocytes were preincubated with *N*-allylsecoboldine or vehicle for 10 min before addition to the oxidative solution. Unless specified otherwise, incubations with H_2O_2 (20 mM) were carried out in the presence of 2 mM sodium azide to inhibit catalase. The reaction mixture was shaken gently while being incubated at 37°C for the time indicated. The oxidation parameters determined are specified below.

2.9. Assay system for hemolysis

AAPH- or H_2O_2-induced hemolysis was measured by the method of Miki et al. (1987). At specific intervals two samples were taken out from the same reaction mixture, one sample (A) was diluted with 20 volumes of PBS as control and the other (B) with cold distilled water to induce hemolysis. Both samples were centrifuged at 1000 $\times g$ for 10 min. The absorbance of both supernatants at 540 nm was read. Percentage hemolysis was calculated from the ratio of the readings, (A/B) $\times$ 100.

2.10. Assessment of lipid peroxidation of erythrocytes

Oxidative (AAPH)-induced thiobarbituric acid-reactive substance formation was measured by the method of Stocks and Dormandy (1971). An aliquot of the reaction mixture was combined with half parts of trichloroacetic acid-arsenite solution. The precipitate was removed by centrifugation, and an aliquot (4 ml) of the supernatant was heated with 1 ml of thiobarbituric acid reagent for 15 min. Since Gilbert et al. (1984) reported that intracellular com-

ponents of erythrocytes produce thiobarbituric acid-reactive substances other than malondialdehyde, the correct malondialdehyde values were obtained by subtracting 20% of the absorbance at 453 nm from the absorbance at 532 nm. The results were expressed as nanomoles of malondialdehyde equivalents per 2×10^9 erythrocytes of the suspension.

Oxygen consumption was measured polarographically in a Clark-type oxygen electrode (YSI model 5331), providing kinetic monitoring of the rate of membrane oxidation. The ghosts (1 mg/ml final concentration) were added to the PBS (saturated with oxygen) and incubated in a rapidly stirred, thermostatically controlled (37°C) chamber and the reagents were pretreated for 6 min before the addition of azo initiator AAPH (10 mM final concentration). Oxygen consumption was monitored with vehicle, *N*-allylsecoboldine or butylated hydroxytoluene (as positive control, at 100 μM) and was compared to the resting only presence of ghosts. According to the oxygen solubility assay (Robinson and Cooper, 1970), the solubility of oxygen was taken as approximately 602 μM in the initial incubation mixture at 37°C. The results were expressed as O_2 consumption as a percentage of total oxygen content at the beginning.

2.11. Russell's viper venom clotting time

Russell's viper venom clotting time was determined as described by Zwaal et al. (1977) with minor modifications: 0.1 ml of citrated human plasma (centrifuged for 15 min, $5000 \times g$) was incubated in a fibrometer at 37°C for 40 s with 14 ng of Russell's viper venom, followed by the addition of 0.1 ml of various radical-treated, washed unhemolyzed packed erythrocytes (71.6 ± 0.4% hematocrit) and 0.1 ml of 25 mM calcium chloride, and the clotting time was determined (a coagulation time of 82.9 ± 3.2 s was given for intact packed erythrocytes on recalcification).

2.12. Analysis of erythrocyte membrane proteins

After ghosts had been prepared from radical-treated erythrocytes, three parts of ghost suspension were mixed with seven parts of sodium dodecyl sulfate sample buffer [2% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, 10% (v/v) glycerol, 0.03% bromophenol blue, 63 mM Tris-HCl, pH 6.8] and incubated at 95°C for 5 min. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on a 1.5-mm-thick slab gel with 4% and 10% gels for condensation and separation, respectively, according to the method of Laemmli (1970). The amount of protein layered was 25 μg. The gel system was calibrated for molecular weight determination by measuring the migration of standard proteins (ranging from 42.7 kDa, ovalbumin, to 200 kD, myosin). Staining and destaining of protein bands with Coomassie brilliant blue was performed as described by Fairbanks et al. (1971).

2.13. Statistical analysis

The data are presented as the means ± S.E.M. for the number of experiments indicated. Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ was regarded as significantly different. The IC_{50} value was obtained by regression analysis.

3. Results

3.1. Effects of N-allylsecoboldine on lipid peroxidation

N-Allylsecoboldine was tested for its ability to inhibit non-enzymatic lipid peroxidation in rat brain homogenates stimulated by ferrous ion. At 2, 5, 10, 20 and 50 μM, *N*-allylsecoboldine inhibited the ferrous-induced lipid peroxidation by 15.7 ± 1.1, 59.1 ± 3.5, 72.2 ± 1.5, 78.2 ± 0.9 and 80.0 ± 1.4%, respectively. At the highest concentration tested (50 μM), *N*-allylsecoboldine also inhibited spontaneous lipid peroxidation by even more than 80% (data not shown). *N*-Allylsecoboldine did not interfere with the thiobarbituric acid test, since the color formation was not changed if it was added after the incubation with thiobarbituric acid reagents. Trolox, α-tocopherol or desferrioxamine all inhibited iron-dependent lipid peroxidation in brain homogenates in a dose-dependent manner. Table 1 shows the IC_{50} values for these compounds to inhibit lipid peroxidation, and *N*-allylsecoboldine was nearly as potent as α-tocopherol. It also displayed a greater antiperoxidative effect compared to either Trolox (i.e., the antioxidant with the ring structure of α-tocopherol) or desferrioxamine (i.e., the iron sequestor).

3.2. The interaction with stable free radical, diphenyl-p-picrylhydrazyl

The diphenyl-*p*-picrylhydrazyl decolorization was increased by *N*-allylsecoboldine in a concentration-dependent manner; the concentration resulting in a decrease of 0.20 in the absorbance of diphenyl-*p*-picrylhydrazyl was approximately 7.91 ± 0.51 μM (Table 2, $n = 5$). This showed that *N*-allylsecoboldine was a good scavenger to

Table 1
Inhibitory effects of *N*-allylsecoboldine and various antioxidants on iron-induced lipid peroxidation in rat brain homogenates

Compound	IC_{50} (μM)
N-Allylsecoboldine	4.80 ± 0.16
Trolox	14.86 ± 0.99
α-Tocopherol	3.78 ± 0.26
Desferrioxamine	95.07 ± 7.83

The antioxidant activity of test compounds was calculated as percent inhibition of iron (200 μM)-induced lipid peroxidation. Concentrations causing 50% inhibition (IC_{50} values) are presented as means ± S.E.M. of five independent experiments.

interact with the nitrogen-centered stable free radical, diphenyl-*p*-picrylhydrazyl. In this assay, *N*-allylsecoboldine was a more potent scavenger than classical antioxidants, including probucol, α-tocopherol, Trolox or butylated hydroxytoluene. The biphasic pattern of the decay trace of diphenyl-*p*-picrylhydrazyl decolorization by *N*-allylsecoboldine was similar to that by α-tocopherol, but not that by butylated hydroxytoluene (data not shown).

3.3. The interaction with peroxyl radical

The interaction of *N*-allylsecoboldine with aqueous peroxyl radical, as monitored by the decrease in fluorescence intensity of B-phycoerythrin, is shown in Fig. 2. The addition of a peroxyl radical initiator (AAPH) to the reaction mixture induced a transient decrease of B-phycoerythrin fluorescence intensity. Under these assay conditions, the rate of B-phycoerythrin fluorescence decay parallels the rate of oxidative destruction of fluorescence properties. *N*-Allylsecoboldine effectively protected against the oxidative degradation of B-phycoerythrin by the peroxyl radical, to prolong the lag time in a concentration-dependent manner. It was clearly shown that 20 μM *N*-allylsecoboldine almost completely prevented the oxidative degradation of B-phycoerythrin within 10 min. The stoichiometry factor of *N*-allylsecoboldine with the peroxyl radical was calculated to be 44 ± 0.6, by comparing it with that of Trolox or ascorbate, whose value was 2.5 ± 0.1 or 0.9 ± 0.1, respectively ($n = 5$). This suggests that *N*-allylsecoboldine is significantly more efficient as a peroxyl radical scavenger than Trolox or ascorbate.

3.4. The interaction with superoxide anion and hydrogen peroxide

The xanthine-xanthine oxidase system generated superoxide anions as measured by the reduction of cytochrome c, the initial rate of reduction had been inhibited by superoxide dismutase in a concentration-dependent manner with an IC_{50} value (the concentration required for 50% inhibition of initial rate of O_2^- fomation) of approximatly 1.4 U/ml. *N*-Allylsecoboldine inhibited the reaction with a higher IC_{50} (> 200 μM), and the percent inhibition of *N*-allylsecoboldine (100 μM) on this system was 21.4 ± 3.3% ($n = 4$). In addition, *N*-allylsecoboldine (100 μM) also inhibited xanthine oxidase [In % = 7.8 ± 2.6 ($n = 4$)] and the same concentration of a positive control, allopurinol [In % = 88.2 ± 0.8 ($n = 4$)]. These data indicate that *N*-allylsecoboldine is not an effective O_2^- scavenger and has a very weak inhibitory action on xanthine oxidase at 100 μM.

N-Allylsecoboldine was not capable of reacting directly with hydrogen peroxide in our assay method. At 100 μM, it caused no significant loss of H_2O_2 (0.5–1.0 mM), because the rate of O_2 production was not changed, as measured in a catalase-based assay (results not shown).

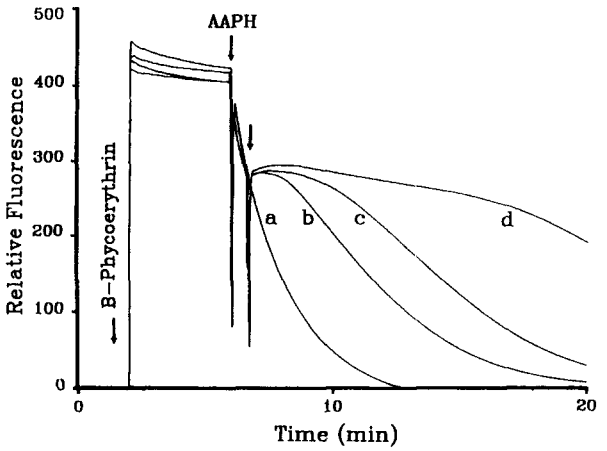

Fig. 2. Effect of *N*-allylsecoboldine on the peroxyl radical-dependent loss of B-phycoerythrin fluorescence. The incubation system contained B-phycoerythrin (5 nM) and AAPH (25 mM) in sodium phosphate buffer (75 mM, pH 7.0) at 40°C. The fluorescence intensity was measured with 540 nm excitation and 575 nm emission. After the addition of AAPH, there were fluorescence spectra of B-phycoerythrin treated with (a) DMSO; or *N*-allylsecoboldine (b) 1 μM, (c) 2 μM, (d) 5 μM (as indicated by the arrow).

Table 2
Antioxidant effects of *N*-allylsecoboldine and various antioxidants in the diphenyl-*p*-picrylhydrazyl test

Compound	Antioxidant effect $IC_{0.20}$ (μM)
N-Allylsecoboldine	7.91 ± 0.51
Probucol	9.29 ± 0.23
α-Tocopherol	12.77 ± 0.81
Trolox	9.82 ± 0.20
Butylated hydroxytoluene	11.35 ± 0.79

The stable free radical scavenging action was evaluated as the concentration of the compounds that decreased to 0.20 the absorbance of the stable free radical diphenyl-*p*-picrylhydrazyl within 30 min, as described under Materials and methods. Results show means ± S.E.M. of $IC_{0.20}$ from five determinations.

3.5. The interaction with hydroxyl radical generated by ascorbate / iron ion / H_2O_2 system

The effect of *N*-allylsecoboldine on deoxyribose degradation by hydroxyl radicals which were generated by a mixture of ascorbic acid, H_2O_2 and iron ion was tested with or without EDTA. Either *N*-allylsecoboldine or mannitol (i.e., as a well known HO· scavenger) inhibited deoxyribose degradation concentration dependently in the presence of EDTA (data not shown). According to the slope of the competition plot obtained, the second-order rate constants for *N*-allylsecoboldine and mannitol were calculated to be $6.9 \pm 0.9 \times 10^9$ ($n = 4$), 2.1×10^9 M^{-1} S^{-1} ($n = 7$), respectively. It was clear that the scavenging effect of *N*-allylsecoboldine on hydroxyl radicals was approximately three times more potent than that of manni-

tol under these conditions. Additionally, *N*-allylsecoboldine or mannitol concentration dependently inhibited deoxyribose degradation in the reaction mixture without EDTA (data not shown). At the highest concentration (1 mM), the two compounds, respectively, produced approximately 65% and 15% inhibition in the assay.

3.6. Effects of N-allylsecoboldine on oxidation-induced erythrocyte hemolysis and lipid peroxidation

As shown in Fig. 3, human erythrocyte hemolysis was time dependently increased following addition of the aqueous radical initiator (AAPH). In this setup, the start of oxidative hemolysis was within 120–150 min. The start of hemolysis was delayed as the concentrations of *N*-allylsecoboldine were added. In the presence of *N*-allylsecoboldine (20 μM), oxidative hemolysis did not occur before approximately 210 min. The time-response curves were shifted to the right in a concentration-dependent manner. Additionally, the times for 50% inhibition of oxidative hemolysis (IT_{50}) of *N*-allylsecoboldine at 5, 10 and 20 μM were 178.4 ± 4.5, 214.3 ± 5.3 and 249.0 ± 5.2 min, respectively, as compared to vehicle, 182.6 ± 2.4 min. In the other experiments, the hemolysis induced by H_2O_2 (20 mM) was also prevented by *N*-allylsecoboldine (20 μM); the percent inhibition was 45.7 ± 8.6% ($n = 4$) (after 4 h of incubation, H_2O_2-induced hemolysis was 11.4 ± 0.6%). When human erythrocyte suspensions were incubated in air at 37°C, they were relatively stable and little hemolysis (< 5%) was observed within 5 h. Furthermore, oxidative hemolysis was significantly attenuated by butylated hydroxytoluene as a positive control of oxidative stress (data not shown).

The addition of AAPH to the human erythrocyte suspensions caused the time-dependent generation of thiobarbituric acid-reactive substances which are a product of lipid peroxidation. In this setup, thiobarbituric acid-reactive substance formation started within 90–100 min, and these oxidative products (equivalent to malondialdehyde) had increased from 0.3 ± 0.1 to 6.8 ± 0.6 nmol/2 × 10^9 erythrocytes ($n = 4$) after 180 min. The increase of thiobarbituric acid-reactive substance formation was completely inhibited even after 3 h more by the treatment of erythrocytes with *N*-allylsecoboldine or butylated hydroxytoluene at a concentration of 20 μM (data not shown).

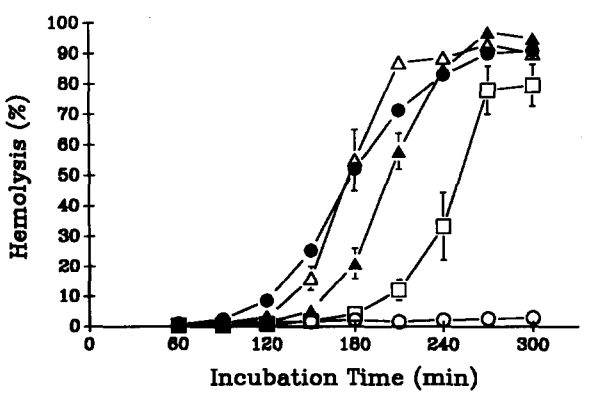

Fig. 3. Effect of *N*-allylsecoboldine on peroxidative hemolysis of human erythrocytes. In erythrocyte suspensions, oxidative hemolysis was triggered by AAPH (50 mM) at 37°C in air. Under this oxidative stress, the mixture was preincubated with vehicle (●) or *N*-allylsecoboldine 5 μM (△), 10 μM (▲), 20 μM (□), respectively. No challenge under resting conditions (○). The values shown were means ± S.E.M. from six experiments.

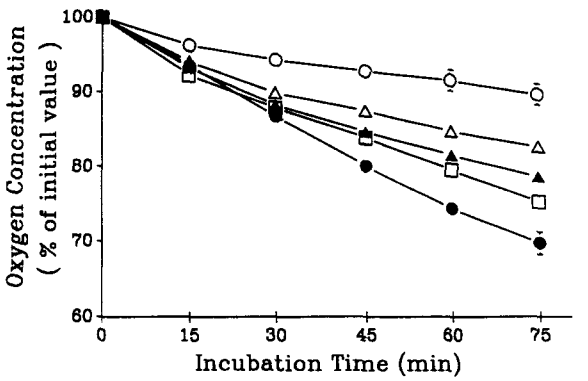

Fig. 4. *N*-Allylsecoboldine diminished oxygen consumption during human ghost lipid peroxidation. The oxidation of ghosts (1 mg/ml) was carried out by addition of AAPH (10 mM) in the absence of (●) or presence of *N*-allylsecoboldine 5 μM (□), 10 μM (▲), 20 μM (△) under 37°C in PBS (with saturated oxygen). Open circle (○) represents conditions without any oxidative challenge. Data are the means ± S.E.M. of five independent experiments.

Fig. 4 shows the increased rates of oxygen consumption during the oxidation of human erythrocyte ghosts as compared with values from a blank experiment at 37°C initiated with AAPH (10 mM). Under these conditions, the lag time for oxygen consumption induced by AAPH was less than 15 min (data not shown). However, it was clearly shown that oxygen consumption was suppressed and the lag time was prolonged by *N*-allylsecoboldine. It also progressively depressed the rates of oxygen consumption in a concentration-dependent manner. However, in the absence of erythrocyte ghosts, *N*-allylsecoboldine (20 μM) did not interfere with the oxygen consumption rate induced by AAPH itself within 60 min. This suggests that *N*-allylsecoboldine can react without primary carbon radicals thermally derived from AAPH.

3.7. Effects of N-allylsecoboldine on hypercoagulability induced by radical-treated erythrocytes

Fig. 5 illustrates the time-dependent effect on the Russell's viper venom-mediated clotting time using AAPH-treated erythrocytes, indicating hypercoagulability induced by prelytic oxidative erythrocytes. It is clearly shown that the extent of clotting time was significantly shortened to the peak value by radical-treated erythrocytes at 90 min of incubation, and then remained constant for up to 120 min. The effect of oxygen radicals on erythrocyte membrane

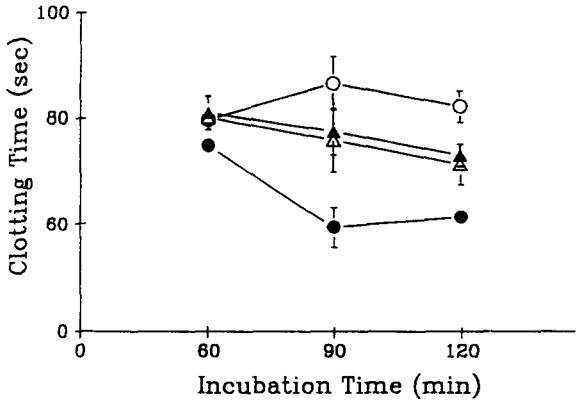

Fig. 5. Effects of *N*-allylsecoboldine on hypercoagulability of AAPH (50 mM)-treated human erythrocytes. Aliquots were removed at the indicated time points and assayed for Russell's viper venom-clotting time as described in Materials and methods. Key: resting (○), vehicle control (●), *N*-allylsecoboldine (20 μM, △), and butylated hydroxytoluene (20 μM, ▲). Values are the means ± S.E.M. of four independent experiments (each in duplicate). All the data points for *N*-allylsecoboldine and butylated hydroxytoluene are significantly different from those for control values ($P < 0.001$).

alterations in coagulability was markedly reversed when erythrocytes were pretreated with *N*-allylsecoboldine or butylated hydroxytoluene (20 μM) within 90–120 min.

3.8. Effects of N-allylsecoboldine on changes in membrane proteins induced by peroxyl radicals

Fig. 6 shows that SDS-PAGE of ghost membrane proteins with oxidation induced by AAPH. The membrane proteins were damaged by oxidative attacks, as shown by the formation of high-molecular-weight protein shown at the top of the gel and upside of band 1 and the decreased intensity of band 6, but not that of bands 1, 2, 4 and 5. This high-molecular-weight protein generation was significantly prevented by *N*-allylsecoboldine in a concentration-dependent fashion. The decrease in intensity of band 6

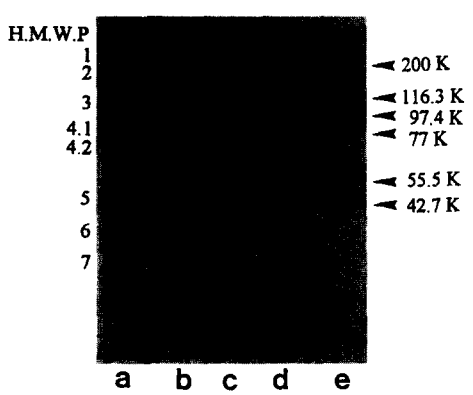

Fig. 6. SDS-polyacrylamide gel electrophoresis of human erythrocyte ghosts. Erythrocytes were incubated with 20 μM (lane a), 10 μM (lane b) of *N*-allylsecoboldine or vehicle (lane c) in the presence of 50 mM AAPH at 37°C for 3.5 h. Lane d: intact erythrocyte membrane proteins. Lane e: high-molecular weight standard proteins. The amount of protein layered was 25 μg in each gel.

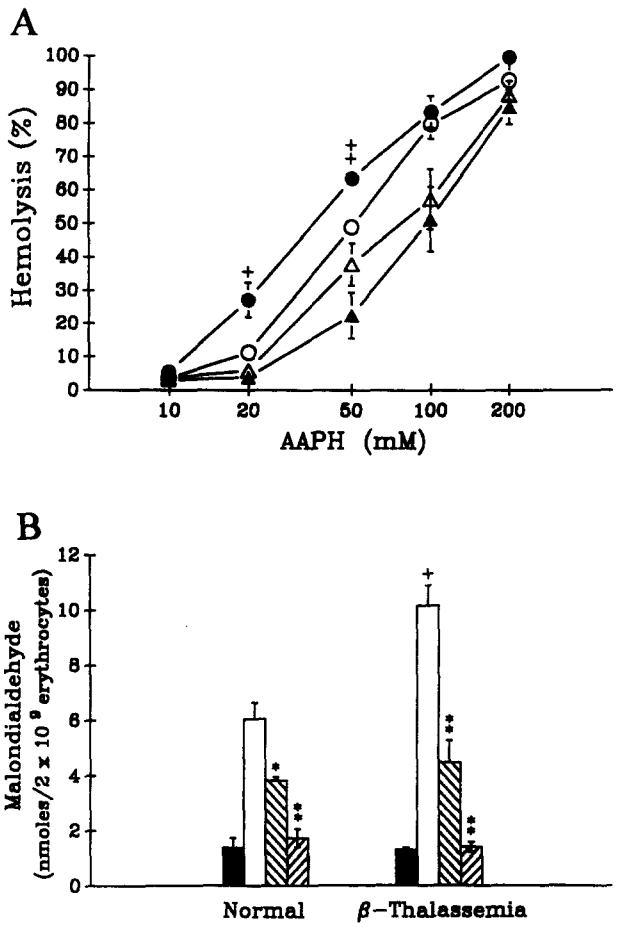

Fig. 7. The effect of *N*-allylsecoboldine on peroxidative damage in human β-thalassemic and normal erythrocytes. (A) Concentration-response curves of AAPH-induced hemolysis in normal or β-thalassemic erythrocytes in the presence of vehicle (○, ●), or *N*-allylsecoboldine 10 μM (△), 20 μM (▲). (B) AAPH (50 mM)-induced lipid peroxidation of normal and β-thalassemic erythrocytes. The reaction mixtures were also pretreated with vehicle (open columns), *N*-allylsecoboldine 10 μM (right obligue hatched columns) or 20 μM (left obligue hatched columns). No challenge under resting conditions (black columns). The values given are the means ± S.E.M. of five independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$, when compared with each respective control; $^{+}P < 0.05$, $^{++}P < 0.001$, normal v.s β-thalassemic control.

was partially reversed by *N*-allylsecoboldine in a concentration-dependent manner.

3.9. Effects of N-allylsecoboldine on oxidative hemolysis and lipid peroxidation in β-thalassemic erythrocytes

When β-thalassemic erythrocytes were challenged with AAPH, the oxidative hemolysis was more deleterious than in normal erythrocytes. As shown in Fig. 7A, the concentration-hemolysis curve for AAPH in normal erythrocytes was shifted to the left by substitution with β-thalassemic erythrocytes. *N*-Allylsecoboldine (10–20 μM) caused concentration-dependent rightward shifts in the concentration-response curve of AAPH. With β-thalassemic erythrocytes under challenge by AAPH (50 mM), *N*-allylsecoboldine at concentrations of 10 μM or 20 μM

could inhibit nearly 61% or 80% of maximal hemolysis, respectively. Additionally, AAPH (50 mM) markedly induced the formation of thiobarbituric acid-reactive substances in normal and β-thalassemic erythrocytes within 3 h. Apparently, the formation of thiobarbituric acid-reactive substances (malondialdehyde equivalent) in β-thalassemic erythrocytes was also greater than that in normal erythrocytes challenged with AAPH (Fig. 7B). *N*-Allylsecoboldine concentration dependently (10–20 μM) inhibited AAPH-induced thiobarbituric acid-reactive substance formation in these two erythrocyte preparations.

4. Discussion

In this study, a series of in vitro tests were used to assess the possible radical scavenging activity of *N*-allylsecoboldine, a new cardioprotective compound, plus its efficacy as a protectant of human erythrocytes against oxidative stress. Among cell-free systems, brain homogenates are usually chosen to evaluate antioxidant agent effects on lipid peroxidation (Braughler et al., 1988). Erythrocytes are a convenient system for the study of the degree of oxidative injury (Alloisio et al., 1982) or of the antiperoxidative properties of an antioxidant on cells (Fernandes et al., 1992).

Undoubtedly, transition metals are involved in both initiation and propagation of lipid peroxidation (Halliwell and Gutteridge, 1984). Rat brain homogenates exposed to ferrous ion exhibit lipid peroxidation in air by a mechanism whose induction step may primarily involve site-bound iron-mediated decomposition of lipid hydroperoxides to yield alkoxy or peroxyl radicals, leading to the chain reaction of lipid peroxidation (Braughler et al., 1987). In this system, *N*-allylsecoboldine effectively inhibits lipid peroxidation. Its antioxidant effect is similar to that of α-tocopherol and greater than those of desferrioxamine and Trolox. The antiperoxidative activity of *N*-allylsecoboldine could be exerted through different mechanisms: desferrioxamine-like metal chelation, free radical scavenging or membrane stabilization.

A potent iron chelator, desferrioxamine, which inhibits lipid peroxidation by 50%, does so at a concentration nearly the half molar equivalent of the total added iron. *N*-Allylsecoboldine, on the other hand, effectively inhibited lipid peroxidation to the same degree at a concentration 40-fold less than the concentration of exogenously added iron in the assay. It is clear that the metal-chelating activity of *N*-allylsecoboldine may not predominantly contribute to the inhibition of lipid peroxidation. If this peroxidative inhibitory effect is attributable to the free radical scavenging activity, such an antioxidant should reach the vicinity where the site-bound iron is located (Vile and Winterbourn, 1987), and therefore reduce the induction and propagation of lipid peroxidation in the membrane phospholipid (Braughler et al., 1988). As our results showed, α-tocopherol and Trolox had different potencies. This is probably due to the phytyl side chain (McCay, 1985) which contributes greater lipophilic solubility to α-tocopherol compared to the more polar Trolox, allowing the former to more readily associate with the homogenized brain tissue.

The diphenyl-*p*-picrylhydrazyl tests provided direct information about the reactivity of *N*-allylsecoboldine with a stable free radical, while the AAPH-induced B-phycoerythrin bleaching test was addressed to quantify the specific reactivity with $ROO^{\cdot}$. In the former test, *N*-allylsecoboldine appeared more potent than other classical antioxidants. The results of the B-phycoerythrin bleaching tests indicated *N*-allylsecoboldine was able to react with peroxyl radicals, the major radicals involved in lipid peroxidation (Dix and Aikens, 1993). Since transition metals were not used for peroxyl radical generation, this effect was not due to the chelation of transition metals by *N*-allylsecoboldine. According to the stoichiometric factor calculated, *N*-allylsecoboldine should possess a quenching efficacy with $ROO^{\cdot}$ on a mole-to-mole stoichiometric ratio of 1 : 4.5, and thus its scavenging activity was more potent than that of Trolox or ascorbate. It seems that the reaction with $ROO^{\cdot}$ leaves two hydroxyl groups to be radicals, which initiate chain reactions for hydroxyl radicals of secoaporphine rings with two more $ROO^{\cdot}$. However, *N*-allylsecoboldine had only weak measurable reactivity with superoxide anions. Under the reaction conditions, it seems that *N*-allylsecoboldine does not react with H_2O_2.

The hydroxyl radical is a very aggressive oxygen radical that reacts with almost all macromolecules found in living cells (Halliwell and Gutteridge, 1984). It is involved in many pathophysiological states, for example, ischemia-reperfusion injury (Powell and Tortolani, 1992). *N*-Allylsecoboldine was proved to be a scavenger of the hydroxyl radical with a second-order rate constant of $6.9 \pm 0.9 \times 10^9$ M^{-1} S^{-1}. It was more effective than mannitol for scavenging of $HO^{\cdot}$. *N*-Allylsecoboldine also inhibited deoxyribose degradation in reaction mixtures without EDTA. This could suggest that it either has some degree of metal pulling and binding capacity (Halliwell et al., 1987) or that it occupies the available coordination sites of iron (Graf et al., 1984). According to aforementioned findings, we proposed that the cardioprotective activity of *N*-allylsecoboldine may be partially contributed to by its radical trapping properties, as with the other hydroxyl radical scavengers (Gelvan et al., 1991).

It is known that erythrocytes are susceptible to oxidative stress and the resulting damage is associated with many hematopathies (Hatherill et al., 1991). Oxidative damage may disturb the erythrocyte membrane, e.g. its asymmetric organization and protein composition (Alloisio et al., 1982), finally leading to a decrease in cell deformability and hemolysis (Carrell et al., 1975). It has been shown that AAPH thermally generates aqueous free radicals which attack erythrocyte membranes from the outside

(Yamamoto et al., 1985). These free radicals induce the chain oxidation of phospholipids and degradation of proteins (Dean et al., 1986) in the erythrocyte membranes, leading to disturbances in membrane organization and eventually hemolysis (Miki et al., 1987). We found in the studies on AAPH-induced erythrocyte oxidation that *N*-allylsecoboldine could interrupt the free radical chain efficiently, diminishing not only thiobarbituric acid-reactive substance formation but also oxygen consumption in erythrocytes or ghosts. We found that when *N*-allylsecoboldine scavenged the derivative radicals, the intermediate products of *N*-allylsecoboldine were stable but did not promote further recycling peroxidation, including elevated oxygen consumption followed lipid hydroperoxide formation (Sugihara et al., 1993). The hemolysis which follows the peroxidation of membrane lipids was also inhibited. Therefore, *N*-allylsecoboldine really was an inhibitor of membrane lipid peroxidation in human erythrocytes. In another experiment, we found that *N*-allylsecoboldine could also prevent the membrane damage from intracellular stress, and protect erythrocytes from H_2O_2-induced hemolysis. Because the permeable oxidant, H_2O_2, could trigger oxidation of hemoglobin in erythrocytes, this may cause peroxidation of membrane lipids (Puppo and Halliwell, 1988) and thus result in membrane destabilization which could lead to hemolysis (Barker and Brin, 1975). Additionally, the HMW proteins observed in the SDS-PAGE may have been formed by direct cross-linking in protein molecules through free radicals and/or interaction of proteins with oxidized membrane lipids (Koster and Slee, 1983; Dean et al., 1986). We thus showed that *N*-allylsecoboldine played a protective role against erythrocyte membrane protein oxidation and degradation. Furthermore, the bands of cytoskeletal proteins of erythrocytes in the SDS-PAGE did not change during peroxidation, as would happen if hydrophilic radicals attack membranes from the outside of human erythrocytes.

Oxidative stress inducers could induce peroxidative damage and cause a hypercoagulable state in erythrocytes (Jain, 1985). The mechanism responsible for hypercoagulability mediated by oxidatively modified erythrocytes has not been well analyzed. However, previous reports suggest that either the accumulated malondialdehyde (Jain, 1984) or the oxidative inhibition of flippase (Connor and Schroit, 1988) leads to disturbance of the asymmetric distribution of phospholipids in the erythrocyte membrane, followed by externalization of the procoagulant, phosphatidylserine, and finally induce hypercoagulability (Zwaal et al., 1989). These suggestions point to a possible role of erythrocytes in coagulation, at least when these cells are exposed to excess oxygen radicals as well as when erythrocyte antioxidant defences are diminished in some hematopathies. AAPH could induce lipid peroxidation in human erythrocytes, and the prelytic erythrocytes could shorten the clotting time on recalcification of 'normal' human platelet-poor plasma in the presence of Russell's viper venom. In our opinion, N-allylsecoboldine works as an antioxidant, diminishing the peroxidative damage from peroxyl radicals. Both the elevated malondialdehyde or the reduction in Russell's viper venom-clotting time were blocked when erythrocytes were treated with *N*-allylsecoboldine. This suggests that hypercoagulability mediated by radical-treated human erythrocytes is indeed associated with the peroxidative lipid damage.

Oxidative damage is thought to be a major factor in β-thalassemia pathology (Shinar and Rachmilewitz, 1990; Grinberg et al., 1995). Therefore, oxidation of cellular components has been implicated as an important factor for the complex pathophysiology of the β-thalassemic erythrocytes. When erythrocytes were challenged with exogenous peroxyl radical initiator (AAPH), both lipid peroxidation and hemolysis were significantly enhanced in the β-thalassemic more than in normal erythrocytes. The effect was similar to that of other exogenous oxidants, showing elevated oxidative damage on β-thalassemic erythrocytes. Both decreased levels of intracellular antioxidants (Zannose-Mariolea et al., 1974) and high concentrations of membrane-associated hemoglobin, heme or iron (Repka et al., 1993) are correlated with a significantly enhanced sensitivity to exogenous oxidative stress (Scott et al., 1993). *N*-Allylsecoboldine, as an antioxidant, protected β-thalassemic erythrocytes against exogenous free radical damage. Although pathological changes occur not only in the membrane lipids, however, we could establish a role for exogenous free radical-induced membrane peroxidative damage in the degenerative process of β-thalassemia. These findings support the hypothesis that oxidative mechanisms contribute to the cellular pathophysiology of β-thalassemic erythrocytes.

In conclusion, our studies showed that *N*-allylsecoboldine displays multiple antioxidant effects. It was found to be: (1) an inhibitor of iron-induced lipid peroxidation; (2) a scavenger of stable free radical, DPPH; (3) a scavenger of peroxyl radical in aqueous systems with a stoichiometry factor of 4.5; and (4) a scavenger of hydroxyl radicals with a second-order rate constant of 7×10^9 M^{-1} S^{-1}. In addition, *N*-allylsecoboldine was effective to inhibit peroxyl radical-induced human erythrocyte oxidative hemolysis, lipid peroxidation and membrane protein degradation. It could protect the asymmetrical organization of the erythrocyte membrane from being disturbed by free radicals. Furthermore, *N*-allylsecoboldine prevented the enhanced damage from exogenous oxidative stress in susceptible β-thalassemic erythrocytes. It is of interest to study further the antioxidant activities of this compound in various radical-mediated pathological events, particularly for in vivo situations, to evaluate its possible use in therapeutics.

Acknowledgements

This work was supported by a research grant (NSC 85-2622-B-002-001) from the National Science Council,

Taiwan and the China Chemical and Pharmaceutical Company, Taipei, Taiwan.

References

Abadie, C., A.B. Baouali, V. Maupoil and L. Rochette, 1993, An α-tocopherol analogue with antioxidant activity improves myocardial function during ischemia reperfusion in isolated working rat hearts, Free Radic. Biol. Med. 15, 209.

Alloisio, N., D. Michelon, E. Bannier, A. Revol, Y. Beuzard and J. Delaunay, 1982, Alterations of red cell membrane proteins and hemoglobin under natural and experimental oxidant stress, Biochim. Biophys. Acta 691, 300.

Barker, M.O. and M. Brin, 1975, Mechanism of lipid peroxidation in erythrocytes of vitamine E-deficient rats and in phospholipid model systems, Arch. Biochem. Biophys. 166, 32.

Bhatnagar, A., 1995, Electrophysiological effects of 4-hydroxynonenal, an aldehydic product of lipid peroxidation, on isolated rat ventricular myocytes, Circ. Res. 76, 293.

Braughler, J.M., R.L. Chase and J.F. Pregenzer, 1987, Oxidation of ferrous iron during peroxidation of various lipid substrates, Biochim. Biophys. Acta 921, 457.

Braughler, J.M., P.S. Burton, R.L. Chase, J.F. Pregenzer, E.J. Jacobsen, F.J. VanDoornik, J.M. Tustin, D.E. Ayer and G.L. Bundy, 1988, Novel membrane localized iron chelators as inhibitiors of iron-dependent lipid peroxidation, Biochem. Pharmacol. 37, 3853.

Carrell, R.W., C.C. Winterbourn and E.A. Rachmilewitz, 1975, Activated oxygen and hemolysis, Br. J. Haematol. 30, 259.

Chiu, D., F. Kuypers and B. Lubin, 1989, Lipid peroxidation in human red cells, Semin. Hematol. 26, 257.

Connor, J. and A.J. Schroit, 1988, Transbilayer movement of phosphatidylserine in erythrocytes; inhibition of transport and preferential labelling of a 31 kD protein by sulfhydryl reactive reagents, Biochemistry 27, 848.

Dean R.T., S.M. Thomas and A. Garner, 1986, Free-radical-mediated fragmentation of monoamine oxidase in the mitochondrial membrane. Role for lipid radicals, Biochem. J. 240, 489.

Dix, T.A. and J. Aikens, 1993, Mechanisms and biological relevance of lipid peroxidation initiation, Chem. Res. Toxicol. 6, 2.

Dodge, J.T., C. Mitchell and D.J. Hanahan, 1963, The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes, Arch. Biochem. Biophys. 100, 119.

Downey, J.M., 1990, Free radicals and other involvement during long-term myocardial ischemia and reperfusion, Annu. Rev. Physiol. 52, 487.

Fairbanks, G., T.L. Steck and D.F.H. Wallach, 1971, Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane, Biochemistry 10, 2606.

Fernandes, A.C., P.M. Filipe and C.F. Manso, 1992, Protective effects of a 21-aminosteroid against copper-induced erythrocyte and plasma lipid peroxidation, Eur. J. Pharmacol. 220, 211.

Fridovich, I., 1970, Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase, J. Biol. Chem. 245, 4053.

Gelvan, D., P. Saltman and S.R. Powell, 1991, Cardiac reperfusion damage prevented by a nitroxide free radical, Proc. Natl. Acad. Sci. USA 88, 4680.

Giardini, O., A. Cantani and A. Donfrancesco, 1981, Vitamin E therapy in homozygous beta-thalassemia, New Engl. J. Med. 350, 644.

Gilbert, H.S., D.D. Stump and E.F. Roth, 1984, A method to correct for errors caused by generation of interfering compounds during erythrocyte lipid peroxidation, Anal. Biochem. 137, 282.

Graf, E., J.R. Mahoney, R.G. Bryant and J.W. Eaton, 1984, iron-catalyzed hydroxyl radical formation, J. Biol. Chem. 259, 3620.

Grinberg, L.N., E.A. Rachmilewitz, N. Kitrossky and M. Chevion, 1995, Hydroxyl radical generation in β-thalassemic red blood cells, Free Radic. Biol. Med. 18, 611.

Halliwell, B. and J.M.C. Gutteridge, 1981, Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts, FEBS Lett. 128, 347.

Halliwell, B. and J.M.C. Gutteridge, 1984, Oxygen toxicity, oxygen radicals, transition metal and diseases, Biochem. J. 219, 1.

Halliwell, B., J.M.C. Gutteridge and O.I. Aruoma, 1987, The deoxyribose method: a simple 'test-tube' assay for determination of rate constants for reactions of hydroxyl radicals, Anal. Biochem. 165, 215.

Hatherill, J.R., G.O. Till and P.A. Ward, 1991, Mechanisms of oxidant-induced changes in erythrocytes, Agents and Actions 32, 351.

Jain, S.K., 1984, The accumulation of malonyldialdehyde, a product of fatty acid peroxidation, can disturb aminophospholipid organization in the membrane bilayer of human erythrocytes, J. Biol. Chem. 259, 3391.

Jain, S.K., 1985, In vivo externalization of phosphatidylserine and phosphatidylethanolamine in the membrane bilayer and hypercoagulability by the lipid peroxidation of erythrocytes in rats, J. Clin. Invest. 76, 281.

Koster, J.F. and R.G. Slee, 1983, Lipid peroxidation of human erythrocyte ghosts induced by organic hydroperoxides, Biochim. Biophys. Acta 752, 233.

Laemmli, U.K., 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature (London) 227, 680.

Lee, S.S., Y.J. Lin, M.Z. Chen, Y.C. Wu and C.M. Chen, 1992, A facile semisynthesis of litebamine, a novel phenanthrene alkaloid, from boldine via a biogenetical approach, Tetrahedron Lett. 33, 6309.

Marion, M.B., 1976, A rapid and sensitive method for quantitation of microgram quantities of protein utiliting the principle of protein-dye binding, Anal. Biochem. 2, 248.

Massey, V., 1959, Microestimation of succinate and the extinction coefficient of cytochrome c, Biochim. Biophys. Acta 34, 255.

McCay, P.B., 1985, Vitamin E: interactions with free radicals and ascorbate, Annu. Rev. Nutr. 5, 323.

Mellors, A. and A.L. Tappel, 1966, The inhibition of mitochondrial peroxidation by ubiquinone and ubiquinol, J. Biol. Chem. 241, 4353.

Miki, M., H. Tamai, M. Mino, Y. Yamamoto and E. Niki, 1987, Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol, Arch. Biochem. Biophys. 258, 373.

Powell, S.R. and A.J. Tortolani, 1992, Recent advances in the role of reactive oxygen intermediates in ischemic injury, J. Surg. Res. 53, 417.

Puppo, A. and B. Halliwell, 1988, Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Is haemoglobin a biological fenton reagent?, Biochem. J. 249, 185.

Repka, T., O. Shalev, R. Reddy, J. Yuan, A. Abramov, E.A. Racmilewitz, P.S. Low and R.P. Hebbel, 1993, Nonrandom association of free iron with membranes of sicke and β-thalassemic erythrocytes, Blood 82, 3204.

Robinson, J. and J.M. Cooper, 1970, Method of determining oxygen concentrations in biological media, suitable for calibration of the oxygen electrode, Anal. Biochem. 33, 390.

Saltman, P., 1989, Oxidative stress: a radical view, Semin. Hematol. 26, 249.

Scott, M.D., J.J.M. Van den Berg, T. Repka, P.H. Rouyer-Fessard, R.P. Hebbel, Y. Beuzard and B.H. Lubin, 1993, Effect of excess α-hemoglobin chains on cellular and membrane oxidation in model β-thalassemic erythrocytes, J. Clin. Invest. 91, 1706.

Shinar, E. and E.A. Rachmilewitz, 1990, Oxidative denaturation of red blood cells in thalassemia, Semin. Hematol. 27, 70.

Stocks, J. and T.L. Dormandy, 1971, The autoxidation of human red cell lipids induced by hydrogen peroxide, Br. J. Haematol. 20, 95.

Sugihara, T., G. Rao and R.P. Hebbel, 1993, Diphenylamine: an unusual antioxidant, Free Radic. Biol. Med. 14, 381.

Teras, K. and D. Niki, 1986, Damage to biological tissues induced by radical initiator 2,2′-azobis(2-aminopropane) dihydrochloride and its

inhibition by chain breaking antioxidants, Free Radic. Biol. Med. 2, 193.

Tsuchiya, M., G. Scita, H.J. Freisleben, V.E. Kagan and L. Packer, 1992, Antioxidant radical-scavenging activity of carotenoids and retinoids as compared to alpha-tocopherol, Meth. Enzyinol. 213, 460.

Vile, G.F. and C.C. Winterbourn, 1987, Iron binding to microsomes and liposomes in relation to lipid peroxidation, FEBS Lett. 215, 151.

Wu, M.H., M.J. Su, S.S. Lee and M.L. Young, 1994, The electrophysiological effects of antiarrhythmic potential of a secoaporphine, *N*-allylsecoboldine, Br. J. Pharmacol. 113,221.

Yamamoto, Y., E. Niki, J. Eguchi, Y. Kamiya and H. Shimasaki, 1985, Oxidation of biological membranes and its inhibition. Free radical chain oxidation of erythrocyte ghost membranes by oxygen, Biochim. Biophys. Acta 819, 29.

Zannose-Mariolea, L., F. Tzortzatou, K. Dendaki-Svolaki, C.H. Katerellos, M. Kavallari and N. Matsanoitis, 1974, Serum vitamin E levels with beta-thalassemia major: preliminary report, Br. J. Haematol. 26, 193.

Zwaal, R.F.A., P. Comfurius and L.L.M. van Deenen, 1977, Membrane asymmetry and blood coagulation, Nature (London) 268, 358.

Zwaal, R.F.A., E.M. Bevers, P. Comfurius, J. Rosing, R.H.J. Tilly and P.F.J. Verhallen, 1989, Loss of membrane phospholipid asymmetry during activation of blood platelet and sickled red cells; mechanisms and physiological significance, Mol. Cell. Biochem. 91, 23.