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Antioxidant Activity of Diethyldithiocarbamate

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Diethyldithiocarbamate (DDC), a potent copper chelating agent, has long been used for the treatment of oxygen toxicity to the central nervous system, as an immunomodulator to treat cancer, and in HIV-infected patients. We evaluated the antioxidant properties of DDC, including its scavenging of reactive oxygen species, its reducing properties, its iron-chelating properties, and its protective effects on oxidant-induced damage to brain tissue, protein, human LDL, and DNA. It is found that DDC is a powerful reductant and antioxidant since it scavenges hypochlorous acid, hydroxyl radical and peroxynitrite; it chelates, then oxidizes ferrous ions; it blocks the generation of hydroxyl radicals and inhibits oxidative damage to deoxyribose, protein, DNA, and human LDL. These findings may provide an explanation for the apparent beneficial effects of DDC against oxidative stress-related diseases that have been observed in experimental and clinical studies.

Key words: Diethyldithiocarbamate (DDC), hypochlorous acid, hydroxyl radicals, DNA damage, NO toxicity

INTRODUCTION

Diethyldithiocarbamate (DDC) is the reduced form of disulfiram (tetraethylthiuram disulfide) and a potent copper chelating agent. DDC inhibits superoxide dismutase (SOD),[1,2] and in rodents has long been used *in vivo* for the treatment of oxygen toxicity to the central nervous system (CNS).[3–5] More recently it has been shown that DDC is an inhibitor of cytochrome P450 activity[6–9] and of endothelium-derived relaxing factor (EDRF).[10] DDC has been used to boost the immune system in the treatment of cancer,[11–13] and in human immunodeficiency virus (HIV)- infected patients.[14–16]

Explanations for the protective effects of DDC have focused on its inhibition of SOD and cytochrome oxidase activities.[1,4,5] DDC also appears to be an antioxidant *in vitro* as it has been shown to be radioprotective[17], to inhibit lipid peroxidation,[18,19] and to inactivate hydrogen peroxide and superoxide.[20]

Because of DDC's therapeutic use in the treatment of human disease, we examined the antioxidant activity of DDC with hypochlorous acid, hydroxyl radical and 3-morpholinosydnonimine N-ethylcarbamide (SIN-1)-generated oxygen species. We have also characterized its reducing and iron-chelating properties, and effects on oxidative

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damage to brain tissue, protein, DNA and low-density lipoprotein (LDL). DDC is an antioxidant with powerful scavenging activity towards oxidants, chelates ferrous ions to block hydroxyl radical generation, and protects tissues and other macromolecules such as proteins and DNA from oxidative damage.

MATERIALS AND METHODS

Materials

DDC, double-strand calf thymus DNA, cytochrome c, bathophenanthroline sulphonate, and nitro blue tetrazolium (NTB) were from Sigma Chemical Co. (St. Louis, MO). Alkaline phosphatase was from Boehringer Mannheim (Mannheim, Germany). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan). 3-Morpholinosydnonimine N-ethylcarbamide (SIN-1) was a gift from Cassella AG, Germany. Rat brain homogenate was obtained from Fischer 344 rats (Harlan-Sprague-Dawley, Indianapolis, IN) and used immediately after homogenization.

Reaction with hypochlorous acid

Reaction of DDC with HOCl was studied as described by Wasil *et al.*[21] Hypochlorous acid damages alpha 1-antitrypsin so that it can no longer inhibit elastase; scavenging of HOCl by DDC was evaluated by its protection of the elastase-inhibitory capacity of alpha 1-antitrypsin.

Effect on hydroxyl radicals generated with Fenton reagents

Hydroxyl radicals were generated with Fenton reagents and examined with an electron spin resonance (ESR) spectrometer.[27] The reaction mixture contained 325 μM each of ferrous sulfate and hydrogen peroxide, 90 mM DMPO. Concentrations of DDC used in these experiments are shown in the results. ESR spectra were recorded at room temperature 40 sec. after the addition of DMPO. The ESR instrumental settings were as follows: magnetic field intensity, 335 ± 5 mT; microwave frequency, 9.42 GHz; modulation width, 0.1 mT at 100 kHz modulation frequency; sweep time, 30 sec/10 mT, and microwave power, 8 mW.

Measurement of hydroxyl radical scavenging and iron binding

The inhibitory effect of DDC on Fenton reagent-generated hydroxyl radicals may be due either to its capacity to scavenge hydroxyl radicals or chelate iron ions. We attempt to distinguish between these two possibilities by examining the effects of DDC on inhibition of deoxyribose decomposition.[22,23] The reaction mixture contained the following reagents at the final concentration stated in 0.5 ml solution: 7.0 mM sodium phosphate-saline buffer (pH 7.4), 1.0 mM hydrogen peroxide, 2.5 mM deoxyribose, with or without 0.55 mM EDTA, and varying concentrations of DDC or other inhibitors. The reaction was initiated on addition of 0.1 mM $FeSO_4$. The samples were incubated at 37°C for 1 h. TBA-reactivity was measured spectrometrically at 532 nm.

Reaction with SIN-1-generated oxidants

SIN-1 generates superoxide and NO, which react to form peroxynitrite, a nitrating and oxidizing agent with the reactivity of hydroxyl radical.[24] The reaction mixture contained the following reagents at the final concentration stated: 9.0 mM phosphate-saline buffer (pH 7.4), 1.0 mM SIN-1, 0.1 mM diethylenetriaminepentaacetic acid (DTPA), 2.0 mM deoxyribose, and varying concentrations of DDC or other inhibitors. The reaction mixture was incubated at 37°C for 2 h. The TBA-reactivity was measured as described above.

Reducing properties

a). The reduction of ferric to ferrous ion was measured by chelation with bathophenanthroline sulfonate (BPS).[25] The reaction mixture (1.0 ml)

contained BPS (100 μg/ml), ferric chloride (50 μM) in phosphate-saline buffer (pH 7.4), and varying concentrations of DDC. The reaction mixture was incubated at room temperature for 10 min prior to the addition of 0.1 ml 6% (v/v) orthophosphoric acid and 0.5 ml distilled water. The absorbance maximum for the BPS-Fe^{++} complex was measured at 532 nm. b). Reduction of nitroblue tetrazolium (NBT) was examined by adding 0.1 ml EDTA (0.1 mM), 0.1 ml Triton X-100 (16%, v/v), and 0.1 ml NBT (1.0 mM) into 0.6 ml phosphate-saline buffer (pH 7.4). After mixing, 0.1 ml DDC solution of varying concentrations were added. The reaction mixture was incubated at 37°C for 15 min followed by addition of 2.0 ml phosphate-saline buffer (pH 7.4). The absorbance was measured at 540 nm.[25] c). Reduction of cytochrome c was examined by adding the DDC sample to a solution containing 100 μM cytochrome c in phosphate-saline buffer (pH 7.4) and the OD_{550} was read in a spectrophotometer.[26]

Measurement of oxidative damage in rat brain tissue

Rat brain tissue was treated with 325 μM of H_2O_2 and ferrous sulfate[27] and various concentrations of DDC were added. Incubation proceeded at 37°C for 30 min. Peroxidation was determined by the thiobarbituric acid assay,[28] and protein damage in the brain tissue was assessed by measurement of protein carbonyl.[29]

DNA damage measurements

DNA damage induced by oxidants was examined by measuring 8-hydroxy-2'-deoxyguanosine (oxo^8dG)[30,31], and by the bleomycin assay.[32] Double-strand calf thymus DNA was treated with Chelex-100 resin, neutralized with HCl, precipitated by the addition of acetate and ethanol, and dried on a vacuum pump before use. For the measurement of oxo8dG, an HPLC assay was employed in which solutions of DNA (0.5 mg/ml) in phosphate buffered solution (25 mM, pH 7) were subjected to the same conditions as above for the brain tissues. The samples were subjected to a incubation at 37°C for 1 hour. After the reaction, DNA was purified twice by ethanol precipitation, and then subjected to enzymatic hydrolysis and measured by HPLC-EC. In the bleomycin assay, the reaction mixture contained, 0.5 ml DNA (1 mg/ml), 0.05 ml bleomycin sulfate (1.5 units/ml), 0.1 ml MgCl2 (50 μM), 0.05 ml phosphate-saline buffer (pH 7.4), 0.1 ml ferric chloride (0.5 mM), with or without 0.1 ml ascorbic acid (5 mM) and appropriate concentrations of DDC or other inhibitors. The reaction mixtures were incubated at 37° C for 1 h, followed by the addition of 1 ml 25% (v/v) HCl and 1 ml TBA (1% w/v in 50 mM NaOH). After heating at 100°C for 10 min, the MDA-TBA chromogen was read at 532 nm.

Oxidation of human low-density lipoproteins (LDL)

LDL was isolated from human plasma and oxidation was induced with $CuSO_4$. The effects of DDC on the oxidation of the polyunsaturated fatty acid side-chains of the LDL were followed

TABLE 1 Effect of DDC as scavenger of hypochlorous acid

Reaction	Elastase activity (A_{410}/min)	Inhibition (%)
a. elastase	0.14	—
b. elastase + antitrypsin	<0.0	100
c. b + HOCl	0.13	9
d. c + 50 μM DDC	0.11	19
e. c + 100 μM DDC	0.09	35
f. c + 250 μM DDC	0.07	48
g. c + 1.0 mM DDC	0.02	88

Alpha 1-antitrypsin (0.2 mg/ml), HOCl (100 μM) and DDC were incubated in a final volume of 1.0 ml in phosphate-buffered saline, pH 7.4 at room temperature for 1 h. Then 1 ml of phosphated-buffered saline and 0.05 ml of elastase were added, followed by further incubation at room temperature for 30 min. The remaining elastase activity was then measured by adding elastase substrate. Concentrations of DDC added were those present in the first (1.0 ml) reaction mixture; DDC and alpha 1-antitrypsin were mixed together before adding HOCl. Control experiments showed that 1 mM DDC did not inhibit elastase activity or interfere with the ability of alpha 1-antitrypsin to inhibit it.

by continuous monitoring of conjugated-diene formation spectrophotometrically, assessing the increase in the absorbance between 200 nm and 270 nm with time.[33]

RESULTS

Table 1 shows the results of the reaction of DDC with HOCl. Alpha 1-antitrypsin inhibited the activity of pig pancreatic elastase *in vitro* and incubation of alpha 1-antitrypsin with HOCl led to loss of its elastase-inhibitory capacity. At 1 mM concentration, DDC was able to protect alpha 1-antitrypsin nearly completely against the effect of 100 μM-HOCl, an effect comparable to that of oxyphenbutazone (a powerful scavenger of hypochlorous acid).[26] Control experiments showed that DDC did not inhibit elastase directly or interfere with the ability of alpha 1-antitrypsin to inhibit elastase.

As shown in Figure 1, DDC showed a dose-dependent effect on inhibiting the generation of DMPO-hydroxyl radical adducts. At a concentration of 1 mM, DDC nearly completely inhibited hydroxyl radicals formation generated by 325 μM of ferrous sulfate and hydrogen peroxide (about 8.0×10^{16} spins/ml or 140 μM radical concentration). We have also compared the concentrations of DDC and other known radical scavengers required to inhibit by 50% the formation of the DMPO-OH adduct: DDC at a IC_{50} of 0.14 mM was

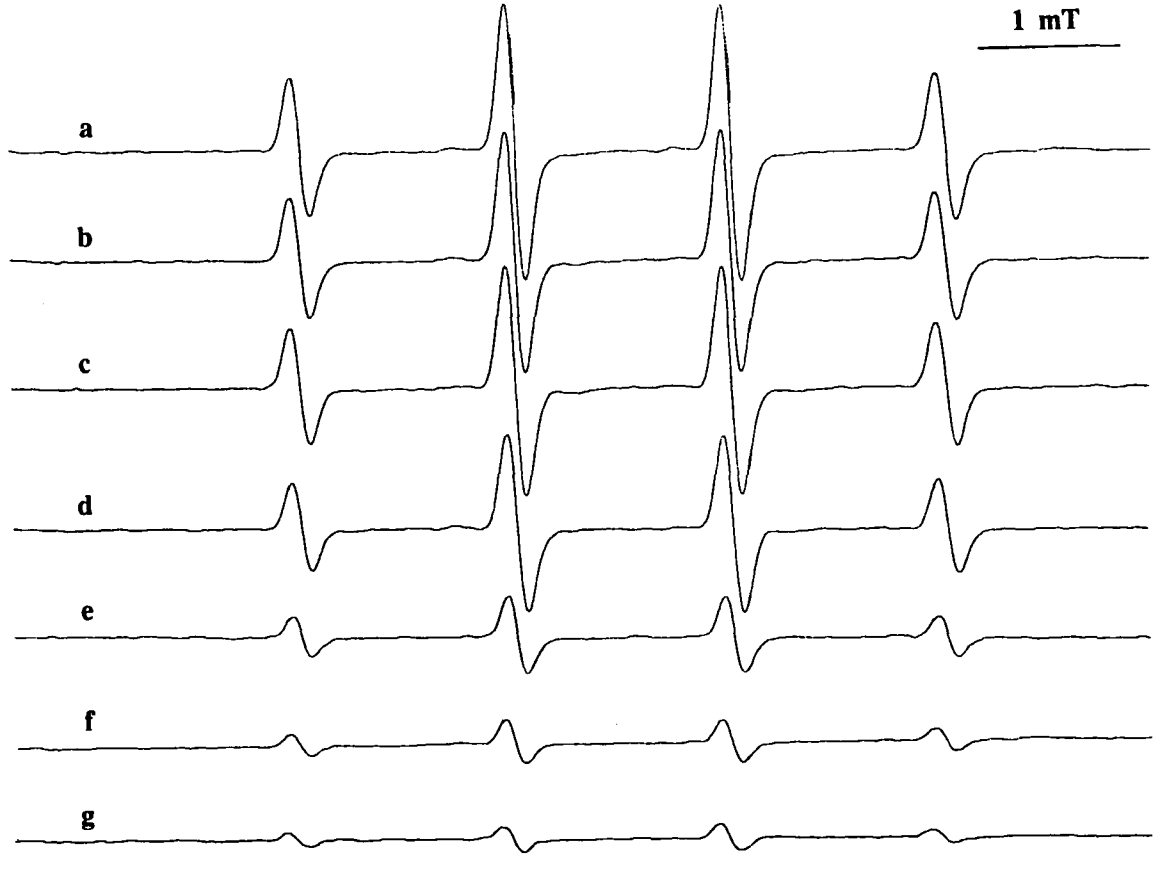

FIGURE 1 ESR spectra recorded from a hydroxyl radical-generating system (Fenton reagents) with various concentrations of DDC. *a*, Control containing 325 μM of each $FeSO_4$ and H_2O_2, and 90 mM of DMPO; *b, c, d, e, f,* and *g* are the same as *a* plus 0.025, 0.05, 0.10, 0.25, 0.5 and 1.0 mM DDC.

shown to be 10 to 100-fold more potent as a hydroxyl radical scavenger than ascorbic acid (0.98 mM), dimethyl sulfoxide (54.7 mM) or mannitol (87.5 mM).

Deoxyribose oxidation provides a simple assay for screening hydroxyl radical scavengers, and determining the iron-binding capacity of a radical scavenger. Table 2 shows that DDC exhibited dose-dependent inhibition of deoxyribose degradation both in the presence and absence of EDTA. This is comparable to the effect of deferoxamine, an efficient iron-chelator and hydroxyl radical scavenger. The rate constant for DDC reacting with hydroxyl radicals was calculated with this assay to be 1.1×10^{10} M^{-1} s^{-1}. The H_2O_2-induced enhancement of deoxyribose degradation was inhibited by 70% on adding catalase in the absence of EDTA and over 100% in the presence of EDTA. The extra inhibition of catalase with the EDTA iron chelate is not understood but a complex of catalase and the chelate is possible.

The incubation of SIN-1 and deoxyribose in the presence of diethylenetriaminepentaacetic acid produced considerable deoxyribose degradation (Table 3). DDC showed an effective, dose-dependent inhibition of deoxyribose damage which was induced by oxidants from SIN-1, which includes NO, superoxide and the reaction product peroxynitrite. The inhibitory effect of DDC was shown to be much greater than that of deferoxamine.

The antioxidant activity of DDC was also examined by its ability to protect against *in vitro* oxidative DNA damage. Oxidative DNA damage was induced with a ferric-H_2O_2 system and examined by measuring 8-hydroxy-2′-deoxyguanosine by HPLC with tandem electrochemical and UV detection (Table 4). DDC dose-dependently inhibited the formation of oxo^{8}dG, more effectively than deferoxamine and mannitol. Catalase significantly inhibited the oxidative DNA damage.

Bleomycin-Fe(III) induces DNA degradation in the presence of a suitable reductant such as ascorbic acid. Although DDC is a reductant (but not as good as ascorbic acid as shown in the reducing property assay), it did not enhance

TABLE 2 Effect of DDC on deoxyribose degradation by ferrous ion/hydrogen peroxide

	TBA reactive substance formed from deoxyribose/h			
	EDTA absence		EDTA presence	
	A_{532}	% inhibition	A_{532}	% inhibition
Blank	0.02	—	0.02	—
+ H_2O_2	0.02	—	0.02	—
+ $FeSO_4$	0.35	—	0.31	
Control	0.75		0.71	
+ 0.125 mM DDC	0.50	41	0.40	45
+ 0.25 mM DDC	0.48	43	0.36	53
+ 0.50 mM DDC	0.43	51	0.31	59
+ 1.00 mM DDC	0.38	59	0.29	61
+ 2.0 mM DDC	0.24	81	0.17	79
+ 4.0 mM DDC	0.16	93	0.08	92
+ 1.0 mM Deferoxamine	0.16	93	0.22	71
+ 250 mM Mannitol	0.23	82	0.14	80
+ 1000 U/ml Catalase	0.47	44	0.15	78

The blank contained only deoxyribose, and the control contained deoxyribose, hydrogen peroxide and ferrous salt. All concentrations shown are final reaction concentrations. The results are mean values obtained from a representative triplicate test of experiments, the SEM (omitted) was generally less than 10%. The % inhibition was calculated after subtraction of appropriate blanks and the inhibitions greater than 20% are considered significantly different ($p<0.01$). The final concentrations of ferrous ions, hydrogen peroxide and deoxyribose were 0.1, 1.0 and 2.5 mM, respectively.

TABLE 3 Effect of DDC on deoxyribose degradation by generation of nitric oxide, superoxide and peroxynitrite from SIN-1

	Rate of formation of TBA reactivity from deoxyribose/2h	
	A_{532}	% inhibition
Blank (Deoxyribose and DTPA)	0.01	—
Control (Deoxyribose + DTPA + SIN – 1)	0.07	—
+ 0.25 mM DDC	0.06	23
+ 0.50 mM DDC	0.05	30
+ 1.00 mM DDC	0.04	48
+ 2.00 mM DDC	0.03	60
+ 1.00 mM Deferoxamine	0.05	37
+ 20 mM Mannitol	0.04	48
+ 100 U/ml Catalase	0.07	3
+ 100 U/ml SOD	0.06	15

Concentrations are final reaction concentrations. The results are mean values obtained from a representative triplicate test of two experiments. The final concentrations of SIN-1, DTPA, deoxyribose were 1 mM, 0.1 mM and 2 mM, respectively.

bleomycin-Fe(III)-induced DNA damage (data not shown). On the other hand, DDC dose-dependently inhibited ascorbic acid-accelerated DNA damage in the bleomycin-Fe(III) system (Table 4). Deferoxamine, as expected, was more effective than DDC in inhibiting the DNA damage because bleomycin-induced DNA degradation is thought to involve a ferrous ion.

Ferric-ascorbate-induced oxidation of brain cellular molecules was detected by measuring TBA-reactive substances (damage to lipids and also some other cellular components) and protein carbonyl (damage to protein). As shown in Table 5, DDC dose-dependently inhibited formation of TBA-reactive substances and protein carbonyls and was as effective as deferoxamine. The effects of mannitol, catalase, and SOD on the formation of TBA-reactive substances were modest (less than 10%); and showed no inhibition on protein carbonyl formation. Protection to protein (BSA) oxidation by Fe(III)-H_2O_2 was examined and DDC showed a similar protective effect (Table 6).

To study mechanisms, the reducing properties of DDC were studied with three systems. As is

TABLE 4 Effect of DDC on DNA damage

	HPLC assay		Bleomycin assay	
	pmol oxo^{8}dG/mg DNA	% inhibition	A_{532}	% inhibition
DNA only	14.4	—	0.06	—
Control	103	—	1.36	—
+ 0.50 mM DDC	50.5	51	1.13	18
+ 1.00 mM DDC	18.7	82	0.76	46
+ 2.00 mM DDC	10.3	90	0.30	82
+ 1.0 mM deferoxamine	37.3	64	0.18	91
+ 125 mM Mannitol	86.0	16	1.32	3
+ 1000 U/ml Catalase	50.5	51	1.25	8

Concentrations are final reaction concentrations. The HPLC assays are mean values obtained from duplicate experiments. The control contained DNA + H_2O_2 + Fe(III) and the oxo8dG for the DNA + H_2O_2 and DNA + Iron(III) blanks are 47.1 and 89.9 pmol/mg DNA respectively. The bleomycin assays are mean values obtained from a triplicate test of two separate assays. The control contained DNA + bleomycin + $MgCl_2$ + Fe(III) + ascorbic acid and the A_{532} for the DNA + bleomycin and DNA + $MgCl_2$ + $FeCl_3$ blanks were 0.061 and 0.064.

TABLE 5 Effect of DDC on oxidative damage to brain induced by ferric ions in the presence of ascorbic acid

	MDA		Carbonyl	
	(nmole/mg)	% inhibition	(nmole/mg)	% inhibition
Blank	0.36	—	2.01	—
Control	2.49	—	4.03	—
+ 0.008 mM DDC	2.07	20	3.91	6
+ 0.015 mM DDC	1.17	62	3.50	26
+ 0.031 mM DDC	0.69	85	3.41	31
+ 0.062 mM DDC	0.61	88	3.18	41
+ 0.125 mM DDC	0.56	91	2.73	64
+ 0.25 mM DDC	0.56	91	2.68	66
+ 0.50 mM DDC	0.56	91	2.66	68
+ 1.0 mM DDC	0.35	100	2.60	71
+ 1.0 mM deferoxamine	0.39	99	2.68	67
+ 250 mM mannitol	2.30	9	4.50	—
+ 200 U/ml catalase	2.32	7	4.18	—
+ 100 U/ml SOD	2.28	10	4.32	—

The blank contained only brain homogenate or protein, and the control contained brain homogenate or protein, ferric ions and ascorbic acid. Concentrations are final reaction concentrations. The results are mean values obtained from a representative triplicate test of two experiments. The final concentration of ferric ions and ascorbic acid were 200 μM each.

expected for other reduced thiols, DDC effectively reduced both NBT and cytochrome c (Figure 2). As shown in Table 7, DDC also showed a strong reducing effect on ferric ions though much weaker than ascorbic acid, a powerful antioxidant with strong prooxidant activity *in vitro* due to this reducing effect.

Figure 3 shows that copper-ion-dependent conjugated-diene formation of LDL [0.25 mg/ml LDL + Cu(II)] was protected completely by 10 μM of DDC.

DISCUSSION

The antioxidant activities of DDC in different chemical and biological systems have been

TABLE 6 Effect of DDC on protein damage by an iron(III) salt in the presence of hydrogen peroxide

	Carbonyl concentration (nmole/mg protein)	% inhibition
BSA only	3.96	—
Blank (BSA + H_2O_2)	4.66	—
Control (BSA + H_2O_2 + Fe(III))	6.12	—
+ 0.25 mM DDC	5.42	32
+ 0.50 mM DDC	5.03	50
+ 1.00 mM DDC	4.40	79
+ 2.00 mM DDC	4.23	87
+ 1.0 mM deferoxamine	4.49	75
+ 250 mM mannitol	4.60	71
+ 200 U/ml catalase	5.05	49
+ 100 U/ml SOD	5.38	12

Concentrations are final reaction concentrations. The results are mean values obtained from a representative triplicate test of two experiments. The final concentrations of BSA, hydrogen peroxide and ferric ions in the reaction mixture were 2 mg/ml, 1 mM and 0.1 mM, respectively.

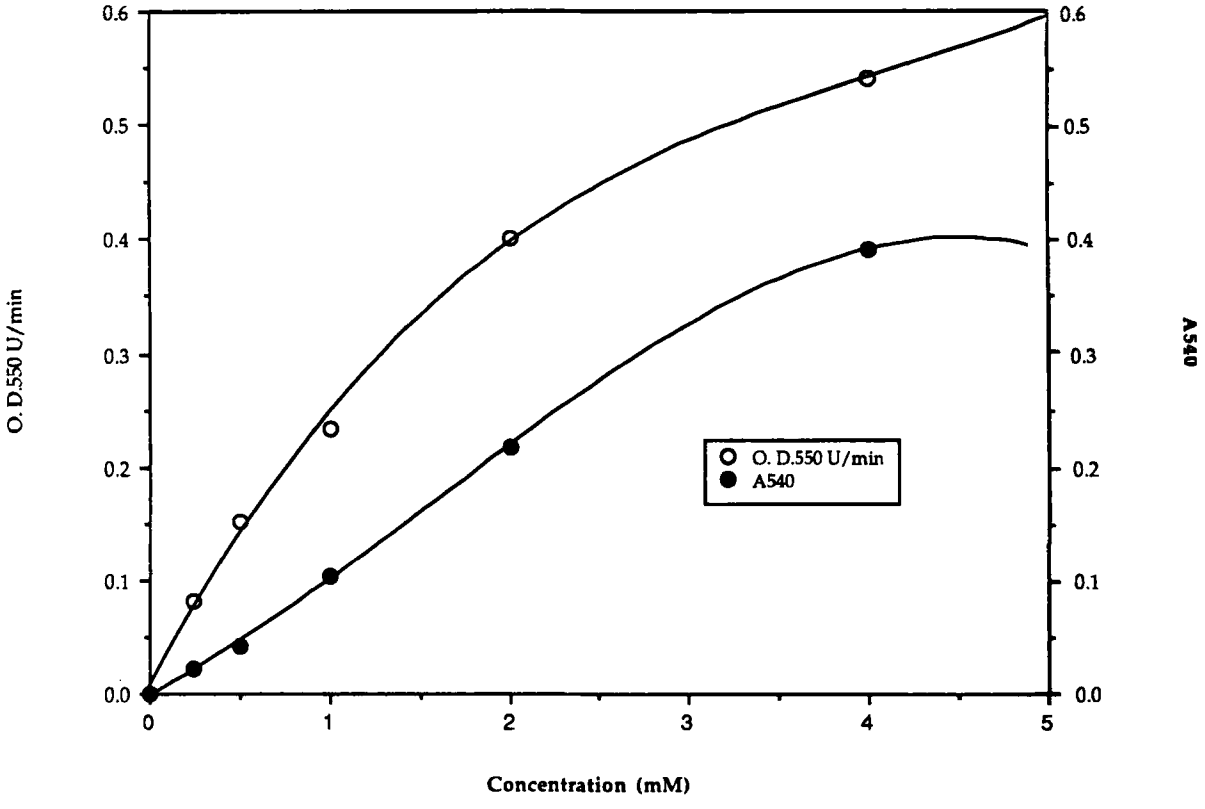

FIGURE 2 The reduction of cytochrome c (O.D.$_{550}$ U/min) and NBT (A_{540}) by DDC. For details, see Materials and Methods.

TABLE 7 Iron-reducing properties of DDC, compared with those of deferoxamine and ascorbic acid

	A_{532}
Control (BPS + ferric chloride)	0.078
+ 0.015 mM DDC	0.12
+ 0.031 mM DDC	0.16
+ 0.062 mM DDC	0.24
+ 0.125 mM DDC	0.31
+ 0.250 mM DDC	0.34
+ 0.500 mM DDC	0.37
+ 1.000 mM DDC	0.39
+ 1.000 mM DFX	0.12
+ 0.012 mM AA	0.28
+ 0.100 mM AA	0.37

BPS, DFX and AA represent bathophenanthroline sulfonate, deferoxamine and ascorbic acid, respectively. The concentrations of BPS and ferric chloride were 100 μg/ml and 50 μM in a phosphate-saline buffer.

examined and compared with those of some other known antioxidants.

Hypochlorous acid is produced by oxidation of chlorine ions at sites of inflammation by the neutrophil enzyme myeloperoxidase. One of the major extracellular targets of HOCl is alpha 1-antiproteinase which is the major circulating inhibitor of serine proteinases such as elastase.[22] Our results show that DDC effectively protects alpha 1-antitrypsin against HOCl inactivation, suggesting that DDC is a good scavenger of HOCl, an effect that may be related to its anti-inflammatory activity.

The ESR results show that DDC has a powerful inhibiting effect on the generation of DMPO-OH adducts by Fenton reagents. These results suggest that DDC is either a hydroxyl radical scavenger or

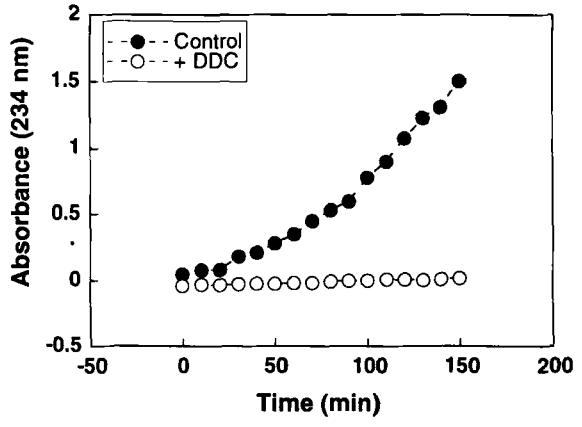

FIGURE 3 Time-course of $CuSO_4$-induced conjugated diene formation in human LDL. The reaction mixture contains, in final concentration, 0.25 mg/ml of LDL, 10 μM of $CuSO_4$ and 10 μM of DDC. Conjugated dienes were measured spectrophotometrically as described in Materials and Methods and is expressed as absorbance at 234 nm in the control (filled circle) or in the presence of 10 μM DDC (open circle).

an iron-chelator that inhibits hydroxyl radical generation by binding iron ions or both.

The deoxyribose assay not only provides a simple assay for screening hydroxyl radical scavengers, but can also be used to determine the iron-binding capacity of a scavenger. The ability of a substance to inhibit deoxyribose degradation induced by iron-EDTA is a measure of its scavenging ability for hydroxyl radicals formed in free solution while the ability to inhibit deoxyribose degradation induced by iron alone is a measure of its iron-binding affinities.[34] The results obtained in the ferrous-hydrogen peroxide-induced deoxyribose assay both in the presence and absence of EDTA indicate that DDC is not only a hydroxyl radical scavenger, but also an efficient chelator of ferrous ions.

Dithiocarbamate has several resonance structures[35]; therefore, it can form complexes with metal ions in different ways.[36] In addition, dithiocarbamate may stabilize high oxidation states of metals as in $[Fe^{IV}(\text{dithiocarbamate})_3]^+$. The tris-chelates are supposed to have intramolecular, metal-centered dynamic processes proceeding via a trigonal twist mechanism.[35] More importantly, $Fe(R_2\text{dithiocarbamate})_2$ is oxidized rapidly in air to $Fe(R_2\text{dithiocarbamate})_3$.[37] This metal chelation and oxidation property is probably the most important aspect of the antioxidant effect of DDC in the iron-containing system. This is further supported by our results from the $CuCl_2$-induced human LDL peroxidation. Metal chelation is not always protective, some metal complexes may be more pro-oxidant and toxic, e.g., ferric nitrilotriacetate is a pro-oxidant and carcinogen.[38] The antioxidant effect of DDC in the LDL peroxidation suggest that DDC can keep the metal ions at higher oxidation states. Iron-oxidizing has been considered as an important antioxidant activity in biological systems[39] and Halliwell[40] has suggested that the most promising agents for brain damage therapy are iron chelators. This is further confirmed by our examination of the iron-dependent peroxidation of rat brain and DNA.

The studies with DNA damage, rat brain peroxidation and protein damage further demonstrate that DDC plays its antioxidant role by hydroxyl radical scavenging and iron-binding and has no prooxidant effect even in the presence of oxidized transition metal ions. The *in vitro* DNA damage study indicates that the protection of DNA damage by DDC may be dependent on both its iron-binding and hydroxyl radical scavenging properties as neither a hydroxyl radical scavenger nor an iron-chelator could effectively compete with DDC in inhibiting the iron-dependent oxidative damage to DNA. The effective inhibition of the ferric-ascorbate-induced brain tissue peroxidation and protein damage suggest that the observed inhibitory effects are mainly produced by iron-chelation rather than radical scavenging as this effect is quite similar to that of deferoxamine. This idea is further supported by the bleomycin assay.

The bleomycin-Fe(III)-ascorbic acid-induced DNA damage is thought to occur by the iron-catalyzed Haber-Weiss reaction. Free radicals generated in close proximity to the DNA are involved in specific site damage leading to release

of pyrimidine bases, strand scission and oxidation of the deoxyribose moiety. Attempts to inhibit this reaction with specific and non-specific radical scavengers have been unsuccessful, with only metal chelators preventing the DNA damage[25] and with phenolics accelerating the DNA damage.[27,32] Our results suggest the inhibition of DNA damage by DDC in this system may be only from its ferrous ion binding and oxidizing effect because the damage occurs at a site-specific manner as mannitol did not inhibit and deferoxamine had the greatest inhibition. Therefore, this finding clearly shows that DDC can, as the iron-chelator deferoxamine, effectively chelate ferrous ions to block the site-specific damage to DNA by chelating the ferrous ions and the oxidizing ferrous to ferric ions which will not catalyze the radical generation reaction. Thus, DDC can be contrasted to antioxidants such as flavonoids, which inhibit lipid peroxidation but aggravate iron-dependent radical damage in non-lipid peroxidation by reducing ferric to ferrous thereby enhancing Fenton chemistry.[40]

NO is attracting great attention in neurobiology as it is a novel neurotransmitter and plays an important role in modulating oxygen toxicity. SIN-1 simultaneously generates NO and superoxide, which react to form peroxynitrite. Peroxynitrite is then converted to peroxynitrous acid which spontaneously decomposes to intermediates with the reactivity of hydroxyl radical and nitrogen dioxide. NO traps, SOD or hydroxyl radical scavengers all inhibit SIN-1-caused deoxyribose degradation.[24] It is likely that the inhibition of damage by DDC may be attributed to its NO scavenging and hydroxyl radical scavenging though the possibility that DDC reacts with peroxynitrite can not be excluded. DDC is a specific lipophilic trap of NO and can form a rather stable adduct *in vivo*.[41,42] Therefore, DDC, as its iron-complex, has been used in the ESR study to monitor the *in vivo* NO formation.[41,42] This suggests that DDC could modulate NO toxicity by scavenging NO and hydroxyl radicals.

One of the most important properties of an antioxidant molecule is the ability to act as a reducing agent to donate electrons to other oxidants. This may result in pro-oxidant activity under some conditions, especially in the presence of transition metals. It is well-known that ascorbic acid and thiols can induce peroxidation by reducing iron. DDC does not show any pro-oxidant activity. The reason appears to be that DDC can effectively chelate both ferrous and ferric ions, forming a stable complex with ferrous which will not participate in radical generation. Ascorbic acid also reduces ferric to ferrous but does not bind it tightly. Deferoxamine also may have some reducing effect. This may explain some pro-oxidant or detrimental effects of deferoxamine. Gutteridge *et al.*[43] recently found that aged or heated deferoxamine transfers an electron to the ferric ions it binds to form a ferrous-deferoxamine complex, a potential prooxidant.

The mechanism of the antioxidant effect of DDC has been examined both in a system employing superoxide generation[20] and in a system in which lipid peroxides are formed.[19] In all of the experiments described in our systems, solutions at physiological pH have been employed. Since DDC has a pKa of 3.4,[19] this compound is present as a monothiolate anion species in the solutions. Thus, the effects observed in the radical systems appears not to involve hydrogen abstraction such as those of many thiols, and may possibly involve an electron transfer mechanism, similar to that proposed for the reaction of dibutyldithiocarbamate-nickel complexes with alkyl peroxyl radical[44] or in the lipid peroxidation system[19]:

$$R\bullet + Et_2N(CS)S^- + H^+ \rightarrow RH + Et_2N(CS)S\bullet \quad [1]$$

followed by

$$2Et_2N(CS)S\bullet \rightarrow Et_2N(CS)S-S(CS)NEt_2 \text{ (disulfiram)} \quad [2]$$

The disulfiram may then be reduced to DDC. This idea is supported by the metabolic study that

DDC is the major metabolite of disulfiram *in vivo*.[45] The higher protection of DDC against peroxidation in isolated rat liver mitochondria[46] may be attributable to the regeneration of DDC through this mechanism.

DDC shows a stronger protective effect than deferoxamine in a ferric system (Table 6) while it shows a weaker effect than deferoxamine in a ferrous system (Table 2). The results from the DNA damage in two different systems further confirmed this conclusion. In the Fe(III)-H_2O_2-induced DNA damage, DDC exhibits stronger inhibition than deferoxamine; and in the bleomycin-Fe(III)-ascorbate-induced DNA degradation, DDC shows weaker inhibition than deferoxamine because ferric ions are reduced to ferrous ions immediately in the presence of ascorbate and it is the ferrous ions that play a major role in the bleomycin-induced DNA degradation.[32]

Our results demonstrate that DDC can scavenge hypochlorous acid and hydroxyl radicals, protect against the damage inflected by reactive by-products of nitrogen oxide intermediates, bind ferrous iron ions to block hydroxyl radical generation, and effectively protect against *in vitro* oxidative damage to tissues, proteins and DNA. Together with other reports,[18–20] we consider that DDC is a powerful antioxidant but poor prooxidant even in the presence of transition metal ions. The understanding of the antioxidant activities of DDC may explain the experimental and clinical observations of DDC on oxidative stress-related diseases.

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References

1. R.E. Heikkila, F.S. Cabbat and G. Cohen (1976) In vivo inhibition of superoxide dismutase in mice by diethyldithiocarbamate. *Journal of Biological Chemistry*, **251**, 2182–2185.
2. L.A. Bynoe, J.D. Gottsch, S. Pou and G.M. Rosen (1992) Light-dependent generation of superoxide from human erythrocytes. *Photochemistry and Photobiology*, **56**, 353–356.
3. M.D. Faiman, R.G. Mehl and F.W. Oehme (1971) Protection with disulfiram from central and pulmonary oxygen toxicity. *Biochemical Pharmacology*, **20**, 3059–3067.
4. L. Frank, D.L. Wood and R.J. Roberts (1978) Effect of DDC on oxygen toxicity and lung enzyme activity in immature and adult rats. *Biochemical Pharmacology*, **27**, 251–254.
5. S.M. Deneke, S.P. Bernstein and B.L. Fanburg (1979) Enhancement by disulfiram (Antabuse) of toxic effects of 95 and 97% O_2 on the rat lung. *Journal of Pharmacology and Experimental Therapy*, **208**, 377–380.
6. L.S. Kaminsky, J.M. Fraser, M. Seaman and D. Dunbar (1992) Rat liver metabolism and toxicity of 2, 2, 2-trifluoroethanol. *Biochemical Pharmacology*, **44**, 1829–1837.
7. P. Puccini, S. Menicagli, V. Longo, A. Santucci and P.G. Gervasi (1992) Purification and characterization of an acetone-inducible cytochrome P450 from hamster liver microsomes. *Biochemical Journal*, **287**, 863–870.
8. E.D. Kharasch, K.E. Thummel, J. Mhyre and J.H. Lillibridge (1993) Single-dose disulfiram inhibition of chlorzoxazone metabolism: a clinical probe for P450 2E1. *Clinical Pharmacology and Therapy*, **53**, 643–650.
9. J. Pan, J.Y. Hong, D. Li, E.G. Schuetz, P.S. Guzelian, W. Huang and C.S. Yang (1993) Regulation of cytochrome P450 2B1/2 genes by diallyl sulfone, disulfiram, and other organosulfur compounds in primary cultures of rat hepatocytes. *Biochemical Pharmacology*, **45**, 2323–2329.
10. C.W. Broner, J.L. Sheneep, D.C. Stokes, D. Fairclough, W.K. Hildner, S.A. Storgion and J.E. Rehg (1993) Reversal of dopamine-refractory septic shock by diethyldithiocarbamate, an inhibitor of endothelium-derived relaxing factor. *Journal of Infective Disease*, **167**, 141–147.
11. C.J. East and R.F. Borch (1992) The importance of schedule on diethyldithiocarbamate modulation of drug-induced myelosuppression. *Cancer Chemotherapy and Pharmacology*, **31**, 123–126.
12. L.M. Schuchter, W.E. Luginbuhl and N.J. Meropol (1992) The current status of toxicity protectants in cancer therapy. *Seminars in Oncology*, **19**, 742–751.
13. P. Dufour, J.M. Lang, C. Giron, B. Duclos, P. Haehnel, D. Jaeck, J.M. Jung and F. Oberling (1993) Sodium dithiocarb as adjuvant immunotherapy for high risk breast cancer: a randomized study. *Biotherapy*, **6**, 9–12.
14. E.M. Hersh, C.Y. Funk, E.A. Petersen and D.E. Mosier (1990) Biological activity of diethyldithiocarbamate (Dithiocarb, Imuthiol) in an animal model of retrovirus-induced immunodeficiency disease and in clinical trials in patients with HIV infection: The Dithiocarb Study Group. *Developmental Biology and Standardazation*, **72**, 355–363.
15. A. Coutellier, D. Bonnefont-Rousselot, J. Delattre, O. Lopez, M.C. Jaudon, S. Herson and J. Emerit (1992) Oxidative stress in 29 HIV seropositive patients: two-year results of a double-blind trial of diethyldithiocarbamate versus placebo. *Presse Medicine*, **21**, 1809–1812.
16. G.L. Vanham, L. Kestens, J. Van-Hoof, G. Penne, R. Colebunders, C. Giolav, M. Vandenbruaene, R. El-Habib and P. Gigase (1993) Immunological parameters during treatment with dithiocarb (Imuthiol). *AIDS*, **7**, 525–530.

17. R.E. Evans, C.R. Engel, C.L. Wheatly, J.R. Nielsen and L.J. Ciborowski (1983) An in vitro study of the radioprotective effect of diethyldithiocarbamate (DTC). *International Journal of Rad. Onc. Biol. Phys*, **9**, 1635–1640.
18. G. Bartoli, A. Muller, E. Cadenas and H. Sies (1983) Antioxidant effect of diethyldithiocarbamate on microsomal lipid peroxidation assessed by low-level chemiluminescence and alkane production. *FEBS Letters*, **164**, 371–374.
19. A.L. Zanocco, R. Pavez, A. Videla and E.A. Lissi (1989) Antioxidant capacity of diethyldithiocarbamate in a metal independent lipid peroxidative process. *Free Radical Biology and Medicine*, **7**, 151–156.
20. S. Mankhetkorn, Z. Abedinzadeh and C. Houee-Levin (1994) Antioxidant action of sodium diethyldithiocarbamate: reaction with hydrogen peroxide and superoxide radical. *Free Radical Biology and Medicine*, **17**, 517–527.
21. M. Wasil, B. Halliwell, D.C.S. Hutchison and H. Baum (1987) The antioxidant action of human extracellular fluids. *Biochemical Journal*, **243**, 219–223.
22. B. Halliwell (1991) Drug antioxidant effects—a basis for drug selection. *Drugs*, **42**, 569–605.
23. J.M.C. Gutteridge, L. Maidt and L. Poyer (1990) Superoxide dismutase and Fenton chemistry: reaction of ferric-EDTA complex and ferric-bipyridyl complex with hydrogen peroxide without the apparent formation of iron(II). *Biochemical Journal*, **269**, 169–174.
24. N. Hogg, V.M. Darley-Usmar, M.T. Wilson and S. Moncada (1992) Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochemical Journal*, **281**, 419–424.
25. J.M.C. Gutteridge and X.C. Fu (1981) Enhancement of bleomycin-iron free radical damage to DNA by antioxidants and their inhibition of lipid peroxidation. *FEBS Letters*, **123**, 71–74.
26. M. Paya, B. Halliwell and J.R.S. Hoult (1992) Interaction of a series of coumarins with reactive oxygen species: scavenging of superoxide, hypochlorous acid and hydroxyl radicals. *Biochemical Pharmacology*, **44**, 205–214.
27. J. Liu and A. Mori (1993) Monoamine metabolism provides an antioxidant defense in the brain against oxidant- and free radical-induced damage. *Archives of Biochemistry and Biophysics*, **302**, 118–127.
28. H. Ohkawa, N. Ohishi and K. Yagi (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, **95**, 351–358.
29. C.N. Oliver, B-W. Ahn, E.J. Moerman, S. Goldstein and E.R. Stadtman (1987) Age-related changes in oxidized proteins. *Journal of Biological Chemistry*, **262**, 5488–5491.
30. J.R. Wagner, C-C. Hu and B.N. Ames (1992) Endogenous oxidative damage of deoxycytidine in DNA. *Proceedings of National Academy of Sciences of USA*, **89**, 3380–3384.
31. M.K. Shigenaga, E.N. Aboujaoude, Q. Chen and B.N. Ames (1994) Assays of oxidative DNA damage biomarkers 8-oxo-2′-deoxyguanosine and 8-oxoguanine in nuclear DNA and biological fluids by high-performance liquid chromatography with electrochemical detection. *Methods in Enzymology*, **234**, 16–33.
32. J.M.C. Gutteridge and B. Halliwell (1987) Radical-promoting loosely-bound iron in biological fluids and the bleomycin assay. *Life Chemistry Reports*, **4**, 113–142.
33. H. Wiseman, G. Paganga, C. Rice-Evans and B. Halliwell (1993) Protective actions of tamoxifen and 4-hydroxytamoxifen against oxidative damage to human low-density lipoproteins: a mechanism accounting for the cardioprotective action of tamoxifen? *Biochemical Journal*, **292**, 635–638.
34. O.I. Aruoma and B. Halliwell (1988) The iron-binding and hydroxyl radical scavenging action of anti-inflammatory drugs. *Xenobiotica*, **18**, 459–470.
35. R.C. Fay, J.R. Weir and A.H. Bruder (1984) Nuclear magnetic resonance studies of stereochemical rearrangements in pentagonal-bipyramidal. *Inorganic Chemistry*, **23**, 1079–1089.
36. B.J. McCormick (1984) Monothio- and monoseleno-carbamate complexes. *Coordination Chemistry Reviews*, **54**, 99–130.
37. A.M. Bond (1984) Electrochemistry and redox behavior of transition metal dithiocarbamates. *Coordination Chemistry Reviews*, **54**, 23–98.
38. S. Okada, Y. Minamiyama, S. Hamazaki, S. Toyokuni and A. Sotomatsu (1993) Glutathione cycle dependency of ferric nitrilotriacetate-induced lipid peroxidation in mouse proximal renal tubules. *Archives of Biochemistry and Biophysics*, **301**, 138–142.
39. J.M.C. Gutteridge and G.J. Quinlan (1993) Antioxidant protection against organic and inorganic oxygen radicals by normal human plasma: the important primary role for iron-binding and iron-oxidizing proteins. *Biochimica et Biophysica Acta*, **1156**, 144–150.
40. B. Halliwell (1989) Oxidants and the central nervous system: some fundamental questions. *Acta Neurologica Scandinavica*, **126**, 23–33.
41. A.F. Vanin, P.I. Mordvintcev, S. Hauschildt and A. Mulsch (1993) The relationship between L-arginine-dependent nitric oxide synthesis, nitrite release and dinitrosyl-iron complex formation by activated macrophages. *Biochimica et Biophysica Acta*, **1177**, 37–42.
42. S. Sato, T. Tominaga, T. Ohnishi and S.T. Ohnishi (1993) EPR spin-trapping study of nitric oxide formation during bilateral carotid occlusion in the rat. *Biochimica et Biophysica Acta*, **1181**, 195–197.
43. J.M.C. Gutteridge, G.J. Quinlan, J. Swain and J. Cox (1994) Ferrous ion formation by ferrioxamine prepared from aged desferrioxamine: a potential prooxidant property. *Free Radical Biology and Medicine*, **16**, 733–739.
44. J.A. Howard and J.H.B. Chemier (1976) Metal complexes as antioxidant II. Reaction of nickel di-n-butyldithiocarbamate and nickel diisopropyl-dithiophosphate with alkyl peroxy radicals. *Canadian Journal of Chemistry*, **54**, 382–389.
45. T. Gessner and M. Jakubowski (1972) Diethyldithiocarbamic acid methyl ester: a metabolite of disulfiram. *Biochemical Pharmacology*, **21**, 219–230.
46. V. Narayanaswami and H. Sies (1990) Oxidative damage to mitochondria and protection by ebselen and other antioxidants. *Biochemical Pharmacology*, **40**, 1623–1629.