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LIPOIC AND DIHYDROLIPOIC ACIDS AS ANTIOXIDANTS. A CRITICAL EVALUATION

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A detailed evaluation of the antioxidant and pro-oxidant properties of lipoic acid (LA) and dihydrolipoic acid (DHLA) was performed. Both compounds are powerful scavengers of hypochlorous acid, able to protect α_1-antiproteinase against inactivation by HOCl. LA was a powerful scavenger of hydroxyl radicals ($OH^{\cdot}$) and could inhibit both iron-dependent $OH^{\cdot}$ generation and peroxidation of ox-brain phospholipid liposomes in the presence of $FeCl_3$-ascorbate, presumably by binding iron ions and rendering them redox-inactive. By contrast, DHLA accelerated iron-dependent $OH^{\cdot}$ generation and lipid peroxidation, probably by reducing Fe^{3+} to Fe^{2+}. LA inhibited this pro-oxidant action of DHLA. However, DHLA did not accelerate DNA degradation by a ferric bleomycin complex and slightly inhibited peroxidation of arachidonic acid by the myoglobin-H_2O_2 system. Under certain circumstances, DHLA accelerated the loss of activity of α-antiproteinase exposed to ionizing radiation under a N_2O/O_2 atmosphere and also the loss of creatine kinase activity in human plasma exposed to gas-phase cigarette smoke. Neither LA nor DHLA reacted with superoxide radical ($O_2^{\cdot -}$) or H_2O_2 at significant rates, but both were good scavengers of trichloromethylperoxyl radical ($CCl_3O_2^{\cdot}$). We conclude that LA and DHLA have powerful antioxidant properties. However, DHLA can also exert pro-oxidant properties, both by its iron ion-reducing ability and probably by its ability to generate reactive sulphur-containing radicals that can damage certain proteins, such as α_1-antiproteinase and creatine kinase.

KEY WORDS: Lipoic acid, dihydrolipoic acid, antioxidant, superoxide, hydroxyl radical, lipid peroxidation, hypochlorous acid, trichloromethylperoxyl radical.

INTRODUCTION

Lipoic acid (LA) is a derivative of the fatty acid octanoic acid containing a disulphide bond (Figure 1). In its reduced form (DHLA), two thiol groups are present (Figure 1). Lipoic acid plays an essential metabolic role as a component of α-keto acid dehydrogenases, in which the $-SH$ groups of its reduced form serve to carry acyl groups. In addition, however, there is considerable interest in the possibility of using LA/DHLA in the treatment of diabetes[1,2] and neurodegenerative disorders[3,4] and as a radioprotective agent.[5] Some or all of the beneficial effects claimed may be due to the antioxidant ability of LA/DHLA, since reactive oxygen species are implicated in most, if not all, human diseases.[6,7]

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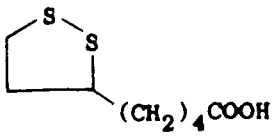

α-LIPOIC ACID

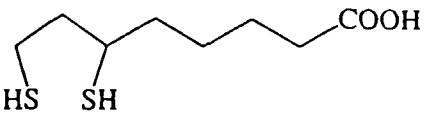

FIGURE 1 *Structures of lipoic acid (LA) and dihydrolipoic acid (DHLA).*

The antioxidant properties of LA/DHLA have been described in several papers. Both LA and DHLA are powerful scavengers of hypochlorous acid, able to protect α-antiproteinase against inactivation.[8] DHLA was reported[9] to scavenge $O_2^{\cdot -}$ with a rate constant of $3.3 \times 10^5\ M^{-1}s^{-1}$ and also to scavenge hydroxyl radicals, $OH^{\cdot}$. However, reports of the effects of LA and DHLA on lipid peroxidation are contradictory. Thus, DHLA inhibited H_2O_2-induced peroxidation in rat heart mitochondria[10] and rat liver microsomes[11,12] but LA had no protective effect in the latter system.[11] By contrast, another group[13] reported that DHLA had a pro-oxidant effect on microsomal peroxidation, apparently by reducing Fe^{3+} to Fe^{2+}. Indeed, any protective effects of DHLA against peroxidation may involve "recycling" of other antioxidants (such as GSH, ascorbate or vitamin E) rather than direct antioxidant effects of DHLA itself.[10-16] Bonomi *et al.*[17] found that DHLA could reductively release iron from ferritin, a potentially-worrying observation since iron is a powerful promoter of free radical damage[18] and is normally largely "sequestered" in safe forms *in vivo*.[6,7,19] The ability of dihydrolipoamide to accelerate the redox cycling of quinones has recently been reported.[20]

Before proposing antioxidants for therapeutic use in the treatment of human disease, it is important to assess their antioxidant ability in a wide range of different systems.[21,22] In the present paper, we present a detailed characterization of the antioxidant and potential pro-oxidant properties of LA and DHLA. In addition, we have examined the effects of LA and DHLA upon biological damage produced by certain drug-derived radicals, since it has been proposed that several drugs in therapeutic use can be converted *in vivo* to damaging drug-derived radicals that contribute to the side-effects of the drugs.[23,24]

MATERIALS AND METHODS

LA and DHLA were provided by Asta Medica, Frankfurt, Germany. The purity of DHLA was checked by measurement of −SH groups using DTNB and found to be always >95%. All other reagents were of the highest quality available from Sigma Chemical Company (Poole, Dorset, UK) or the BDH Chemical Company (Gillingham, Kent, UK). α-Antiproteinase was Sigma type A9024 and elastase was type E0258. Superoxide dismutase was the bovine erythrocyte copper-zinc enzyme.

Deoxyribose Assay

This was carried out essentially as described in[25] with some modifications. Reaction mixtures contained, in a total volume of 1.2 ml, the following reagents at the final concentrations stated: deoxyribose (2.8 mM), $FeCl_3$ (50 μM), EDTA (100 μM), KH_2PO_4-KOH buffer pH 7.4 (10 mM), H_2O_2 (2.8 mM) and ascorbate (100 μM). The $FeCl_3$ and EDTA were pre-mixed before addition to the reaction mixture. After incubation at 37°C for 1h, the products of attack of $OH^{\cdot}$ on deoxyribose were measured by the thiobarbituric acid (TBA) test.[25]

Liposomal Lipid Peroxidation

Ox-brain phospholipid liposomes were prepared[26], and induced to peroxidize by addition of $FeCl_3$ (100 μM) and ascorbate (100 μM). Peroxidation was measured by the TBA test as described in[26], except that 0.1% butylated hydroxyluene was added with the TBA reagents to inhibit peroxidation during the TBA test itself.[27]

Reaction with Trichlomethylperoxyl Radical

$CCl_3O_2^{\cdot}$ was generated using the Linear Accelerator Facility at the Paterson Institute, Christie Hospital, Manchester, by courtesy of Dr. John Butler. Reaction mixtures contained 1% (v/v) CCl_4, 50% (v/v) isopropyl alcohol and 49% (v/v) 10 mM KH_2PO_4-KOH buffer pH 7.4.

Bleomycin-dependent DNA Damage

The bleomycin assay[28] was carried out using minor modifications as described in.[29]

Reaction with Hypochlorous Acid

Reaction of LA and DHLA with HOCl was studied essentially as described by Wasil *et al.*[30] Hypochlorous acid damages α_1-antiproteinase so that it can no longer inhibit elastase. A compound that scavenges HOCl protects the α_1-AP, so its elastase-inhibitory capacity is retained.

Reaction with Superoxide

Superoxide was generated by a mixture of hypoxanthine and xanthine oxidase and detected by its ability to reduce cytochrome *c* (as ΔA_{550}) or nitro-blue tetrazolium (as ΔA_{560}).[31] Final concentrations of cytochrome *c* or NBT were 100 μM. Xanthine oxidase activity was measured by omitting cytochrome *c* or NBT from the reaction mixtures and measuring the rise in absorbance at 290 nm.

Reaction with Hydrogen Peroxide

Reaction of LA with H_2O_2 was studied using a peroxidase-based assay system.[32] Because of the possibility that DHLA could interfere with such assay systems,[33] the reaction of DHLA with H_2O_2 was studied by incubating DHLA and H_2O_2 at pH 7.4 and measuring the loss of −SH groups using Ellmans reagent.[32] We also looked for spectral changes accompanying the conversion of DHLA to LA in the presence of H_2O_2.[34]

Exposure to Cigarette Smoke

Human plasma prepared from blood drawn from healthy adult male volunteers was exposed to gas-phase cigarette smoke at 37°C as described in.[35,36] University of Kentucky research-grade RI cigarettes were used together with a Cambridge filter to remove particulates.[35] Creatine kinase activity was measured by hospital auto-analyzer.[36] This uses a coupled enzyme assay in which the creatine phosphate-dependent formation of ATP from ADP is linked to the reduction of $NADP^+$ in the presence of hexokinase and glucose-6-phosphate dehydrogenase. Results are presented as international units/litre (normal range 22–250 units/litre).

Peroxidation by Haem Protein/H_2O_2 Systems

Peroxidation of arachidonic acid was studied in reaction mixtures containing myoglobin and H_2O_2, essentially as described in.[37] Reaction mixtures contained final concentrations of 500 μM arachidonic acid, 50 μM myoglobin, 25 mM NaH_2PO_4-NaOH buffer pH 7.4 and 0.5 mM H_2O_2 in a final volume of 1 ml. They were incubated at 37°C for 10 min. Then 0.5 ml 1% (w/v) thiobarbituric acid (TBA) in 50 mM NaOH was added, followed by 0.1 ml of 0.22% (w/v) butylated hydroxytoluene (in ethanol) and 0.5 ml of 2.8% (w/v) trichloroacetic acid. Tubes were heated at 80°C for 20 min., cooled, and the chromogen extracted into 2.5 ml of butan-1-ol prior to measuring absorbance at 532 nm.

RESULTS

Scavenging of Hydroxyl Radicals

A mixture of ascorbate, H_2O_2 and $FeCl_3$-EDTA was used[25] to generate $OH^\cdot$ at pH 7.4.

$$\text{Fe(III)} - \text{EDTA} + \text{ascorbate} \rightarrow \text{ascorbate}^\cdot + \text{Fe(II)} - \text{EDTA} \quad (1)$$

$$\text{Fe (II)} - \text{EDTA} + H_2O_2 \rightarrow \text{Fe(III)} - \text{EDTA} + OH^\cdot + OH^- \quad (2)$$

The $OH^\cdot$ reacts with deoxyribose, which is degraded. The reaction of LA and DHLA with $OH^\cdot$ was measured by assessing their ability to inhibit deoxyribose degradation. Lipoic acid was a powerful inhibitor of deoxyribose degradation, competitive with deoxyribose (Figure 2). From the slope of the competition plots, a rate constant for the reaction of LA with $OH^\cdot$ of $4.71 \times 10^{10} M^{-1}s^{-1}$ was calculated, essentially a diffusion-controlled rate.

If ascorbate is omitted from the reaction mixture, a low rate of $OH^\cdot$ formation is still observed, since $FeCl_3$-EDTA reacts slowly[38] with H_2O_2 to generate $OH^\cdot$.

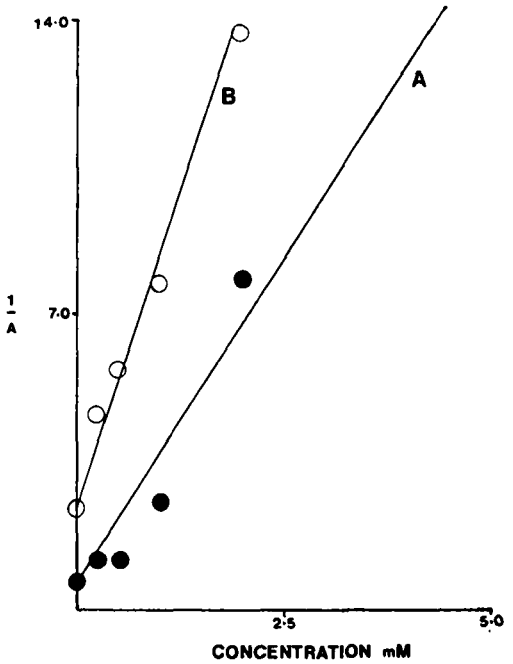

FIGURE 2 *Scavenging of hydroxyl radicals by lipoic acid.* Experiments were carried out as described in the materials and methods section. The second-order rate constant for reaction of LA with $OH^{\cdot}$ (line A) was calculated[25] from the equation

$$k_2 = \text{gradient} \times k_{DR} \times A^{\circ} \times [DR] \text{ where } k_{DR} \text{ is } 3.1 \times 10^9\ M^{-1}s^{-1},$$

A° is the absorbance in the absence of LA and [DR] is the deoxyribose concentration in the reaction mixture. *Line A* Iron present in reaction mixture as $FeCl_3$-EDTA *Line B* No EDTA present (iron added as $FeCl_3$).

Lipoic acid did not increase this low rate of deoxyribose degradation, suggesting that it cannot reduce $FeCl_3$-EDTA (which would have accelerated $OH^{\cdot}$ generation). In fact, LA inhibited, presumably due to $OH^{\cdot}$ scavenging.

By contrast, DHLA had no inhibitory effect on deoxyribose degradation by the ascorbate/H_2O_2/$FeCl_3$-EDTA system. If ascorbate was omitted from the reaction mixture, DHLA showed a pro-oxidant action, accelerating deoxyribose degradation (Figure 3). It seems that, although DHLA, being a thiol, is probably a powerful scavenger[9,39] of $OH^{\cdot}$, this effect is more than counterbalanced under our reaction conditions by its ability to reduce $FeCl_3$-EDTA and accelerate $OH^{\cdot}$ production (equation 2). Another possibility is that DHLA was recycling ascorbic acid[16] in reaction mixtures containing both ascorbate and DHLA.

If EDTA is omitted from the deoxyribose assay reaction mixture, iron ions bind to deoxyribose and any $OH^{\cdot}$ produced attacks this sugar in a site-specific manner.[40,41] The only molecules that can inhibit site-specific $OH^{\cdot}$-dependent deoxyribose degradation are those that can bind iron ions, withdraw them from the deoxyribose and render them inactive or poorly active in reacting with H_2O_2 to form $OH^{\cdot}$.[40,41] Lipoic acid was again a good inhibitor of deoxyribose degradation (Figure 2), suggesting

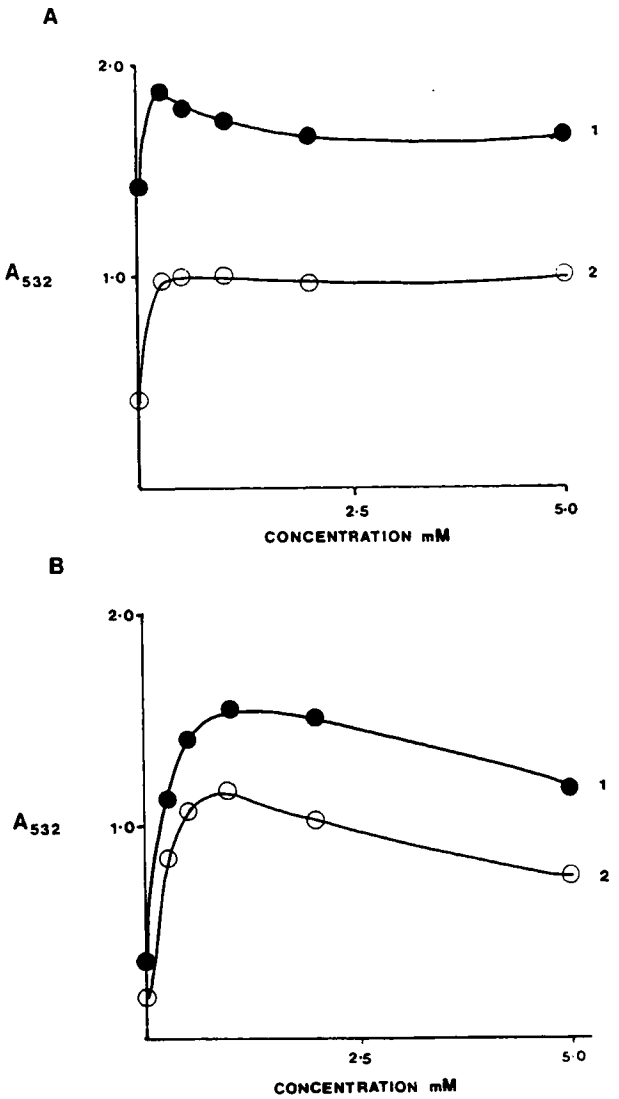

FIGURE 3 *The effects of dihydrolipoate on deoxyribose degradation by hydroxyl radicals.* **A** Reaction mixtures contained H_2O_2, ascorbate and $FeCl_3$-EDTA at pH 7.4 as described in the materials and methods section, together with DHLA at the final concentration stated (Line 1). Line 2 – ascorbate omitted from the reaction mixture. **B** Reaction mixtures did not contain EDTA. Line 1 , ascorbate present, Line 2 – ascorbate omitted.

that it can bind iron and render it redox-inactive.[40,41] By contrast, DHLA was still pro-oxidant in the absence of EDTA (Figure 3B).

Control experiments showed that neither LA nor DHLA interfered with the assay of deoxyribose degradation (they had no effect when added with the TBA reagents) nor did either compound generate any chromogen when deoxyribose was omitted from the reaction mixture.

TABLE 1
Action of lipoic and dihydrolipoic acids on reduction of nitro-blue tetrazolium by $O_2^{\cdot -}$ generated by hypoxanthine-xanthine oxidase

Addition to reaction mixture	ΔA_{560}/sec
Complete (HX/XO)	0.0099 ± 0.0013
Omit enzyme or hypoxanthine	0
+10 units SOD	0.0006 ± 0.0003
5 mM DHLA, no enzyme	0.0039 ± 0.0020
5 mM DHLA	0.01168 ± 0.0018
ΔA_{560} corrected for direct reduction by DHLA	0.0100 ± 0.0018

Units of SOD are as defined in the cytochrome c assay. Results are mean ± SEM of four different experiments.

Scavenging of Hypochlorous Acid

Both LA and DHLA were found to be powerful scavengers of HOCl, able to protect α_1-antiproteinase against inactivation by this molecule (data not shown as essentially confirmatory of a previous report[8]).

Scavenging of Superoxide

Superoxide, generated by a mixture of hypoxanthine and xanthine oxidase, was detected by reduction of cytochrome *c* or nitro-blue tetrazolium. Even at 10 mM concentrations, lipoic acid had no effect on $O_2^{\cdot -}$-dependent cytochrome *c* reduction, nor did it reduce cytochrome *c* itself. By contrast, superoxide dismutase inhibited reduction almost completely.

In agreement with its expected reducing capacity, DHLA was found to reduce cytochrome *c* directly and rapidly, and so could not be tested in the cytochrome *c* assay. However, its rate of reduction of NBT was slow. When this background rate was accounted for, DHLA had no effect on $O_2^{\cdot -}$ dependent NBT reduction (Table 1), suggesting that DHLA has no significant ability to scavenge $O_2^{\cdot -}$.

Scavenging of Hydrogen Peroxide

A peroxidase-based assay system[32] was used to show that LA, tested at concentrations up to 6 mM, did not react with H_2O_2 at a significant rate. By contrast, DHLA decreased the absorbance changes in this assay. However, because it is possible that thiols can act as substrates for peroxidases,[33] the reaction of DHLA with H_2O_2 was studied directly by measuring loss of the −SH group. No loss of −SH groups was observed when 2 mM DHLA was incubated with H_2O_2 at concentrations up to 2 mM for up to 2 h. Conversion of DHLA to LA also causes an absorbance change at 333 nm:[34] no such change was observed during incubations of DHLA with H_2O_2.

Action in the Bleomycin Assay

Bleomycin is an antitumour antibiotic that degrades DNA in the presence of iron ions and a reducing agent,[28] and the ability of a compound to accelerate DNA degradation by Fe(III)-bleomycin is often used as a measure of its iron reducing pro-oxidant ability.[22,29,42] Table 2 shows the effects of LA and DHLA in this system. In the absence of a reducing agent, there is little DNA degradation. Addition of ascorbic

TABLE 2

Effects of lipoic acid and dihydrolipoic acid on bleomycin-dependent DNA degradation. DNA degradation in the presence of bleomycin and $FeCl_3$ was measured as described in the materials and methods section in the presence of compounds at the final concentrations stated below

Test compound added	DNA degradation A_{532}
None	0
Lipoic acid (1 mM)	0
Dihydrolipoic acid (1 mM)	0
Ascorbic acid (0.2 mM)	1.03
plus DHLA (0.1 mM)	1.37
(0.2 mM)	1.37
(0.3 mM)	1.31
(0.5 mM)	1.16
(1.0 mM)	0.87
Ascorbic acid (0.1 mM)	0.27
plus DHLA (0.1 mM)	0.42
(0.2 mM)	0.51
(0.3 mM)	0.53
(0.5 mM)	0.53
(1.0 mM)	0.46

acid greatly accelerates DNA breakdown. However, LA and DHLA had no pro-oxidant effect in this assay. By contrast, when DHLA was added together with ascorbate, it accelerated bleomycin-dependent DNA degradation, especially at low ascorbate concentrations (Table 2).

Effects on Lipid Peroxidation

Previous studies of the effects of LA and DHLA have used microsomes as a substrate for peroxidation, which can cause problems because of variations in the levels of endogenous antioxidants (e.g. tocopherols) that could interact with DHLA to give variable results.[11-13] To avoid this problem, we used ox-brain phospholipid liposomes as a substrate, and peroxidation was started by adding $FeCl_3$ and ascorbate. Figure 4 shows that LA was an inhibitor of peroxidation in this system, whereas DHLA was not. If ascorbate was omitted from the reaction mixture, the rate of peroxidation was greatly decreased and LA had little effect. By contrast, DHLA exerted a pro-oxidant action, in that it accelerated peroxidation (Figure 4). LA inhibited the pro-oxidant actions of DHLA (data not shown).

Experiments were also performed using liposomes containing dl-α-tocopherol incorporated during their preparation. The extent of peroxidation was lower, but LA and DHLA had essentially the same effects.

Scavenging of a Peroxyl Radical

The trichlomethyl peroxyl radical, $CCl_3O_2^{\cdot}$ is a reactive peroxyl radical often used as a model to study the reaction of antioxidants with peroxyl radicals.[29,43,44] It was generated by radiolysis of an aqueous solution containing CCl_4 and propan-2-ol

$$e_{aq}^- + CCl_4 \rightarrow {}^{\cdot}CCl_3 + Cl^- \quad (3)$$

$$^{\cdot}OH + CH_3CHOHCH_3 \rightarrow H_2O + CH_3\dot{C}OHCH_3 \quad (4)$$

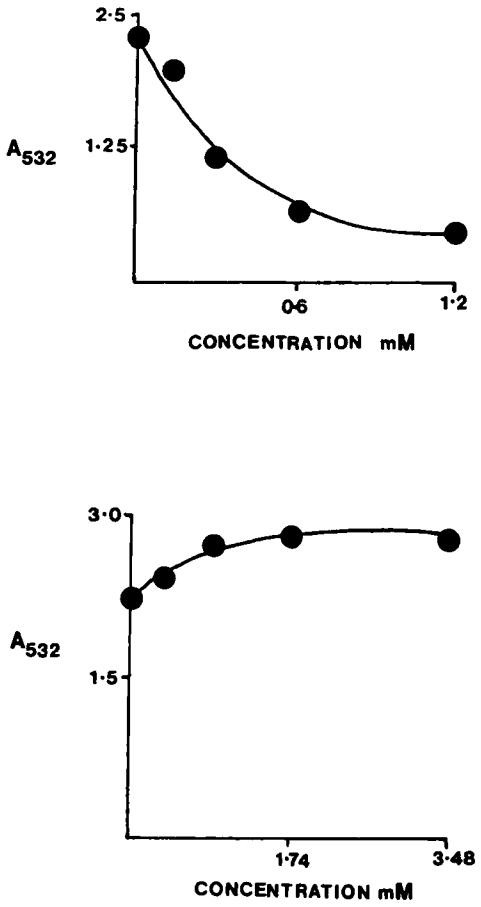

FIGURE 4 *The effects of lipoic and dihydrolipoic acid on lipid peroxidation in ox-brain phospholipid liposomes.* Peroxidation was studied as described in the materials and methods section. Results are presented as A_{532}. Top graph: lipoic acid: bottom graph, DHLA. All reaction mixtures contained $FeCl_3$ and ascorbic acid.

$$CH_3\dot{C}OHCH_3 + CCl_4 \rightarrow CH_3COCH_3 + H^+ + {}^{\cdot}CCl_3 \quad (5)$$

$${}^{\cdot}CCl_3 + O_2 \rightarrow CCl_3O_2^{\cdot} \quad (6)$$

Rates of reaction were measured at 440 nm. Both LA and DHLA were good scavengers of $CCl_3O_2^{\cdot}$, with rate constants of 1.8×10^8 and 2.3×10^7 $M^{-1}s^{-1}$ respectively.

Effects on Oxidative Damage to Plasma by Cigarette Smoke

Exposure of human blood plasma to gas phase cigarette smoke causes lipid peroxidation, oxidative protein damage and depletions of −SH groups, ascorbate and α-tocopherol.[35,36] Dihydrolipoate, but not LA, offers some protection against all of these events.[45] Table 3 shows the effect of DHLA on the activity of plasma creatine kinase, an enzyme that has an essential −SH group at its active site.[46] Creatine

TABLE 3

Effect of lipoic and dihydrolipoic acids on creatine kinase activity in human plasma. Freshly-prepared human plasma was incubated at 37°C under air or exposed to 9 puffs of gas-phase cigarette smoke (1 per 20 min, 3h total incubation) as described in.[35,36] Creatine kinase activity was measured by a spectrophotometric method. Where indicated, thiols were added to the plasma at a final concentration of 1 mM. Results are mean ± SD, n in parentheses. DTT-dithiothreitol

Treatment of plasma	Thiol added	Enzyme activity units/litre
Air-exposed	–	77 ± 5 (8)
Smoke exposed	–	56 ± 6 (8)
Smoke exposed	DHLA	34 ± 4 (6)
Smoke exposed	GSH	28 ± 2 (3)
Smoke exposed	DTT	22 (1)

kinase activity is slowly lost when plasma is incubated at 37°C under air[60], and DHLA prevents this loss (data not shown). By contrast, smoke greatly accelerates the loss of enzyme activity and DHLA produced a further acceleration of this loss, perhaps suggesting that products generated when DHLA interacts with cigarette smoke can aggravate damage to creatine kinase. Dithiothreitol and GSH similarly accelerated the loss of enzyme activity (Table 3).

Effects on Protein Damage by Drug-derived Radicals

The anti-inflammatory drugs phenylbutazone and penicillamine can generate free radicals which can damage proteins.[37,47] Such damage is often detected using α_1-antiproteinase, a biologically-important inhibitor of serine protease enzymes that is highly sensitive to inactivation by reactive oxygen species.[30,37,47] For example, exposure of α_1-AP to $OH^{\cdot}$ generated by pulse radiolysis causes some loss of activity. Addition of either penicillamine or phenylbutazone can accelerate this inactivation, because both react with $OH^{\cdot}$ to form drug-derived radicals that are efficient at inactivating α_1AP.[37,47]

Table 4 shows that both LA and DHLA, at low concentrations, exacerbated inactivation of α_1AP but at higher concentrations they were protective. Neither LA nor DHLA prevented phenylbutazone from accelerating α_1AP inactivation. Indeed both worsened its effect. However, mixtures of penicillamine with LA, and especially with DHLA, were highly-protective towards α_1AP. These variable results are probably due to the extremely complex radical interactions that occur in irradiated thiol solutions.[39,48,49]

Effects of DHLA and LA on Peroxidation Stimulated by Myoglobin

Mixtures of haem proteins with H_2O_2 generate ferryl species and amino acid side-chains radicals that can stimulate lipid peroxidation[51,52] and such reactions may play deleterious roles *in vivo*.[22,50–53] The effects of LA and DHLA on peroxidation of arachidonic acid by the myoglobin/H_2O_2 system are shown in Table 5. Lipoic acid had little effect. DHLA decreased peroxidation partially but reproducibly: control experiments showed that this was not due to interference with the TBA test. As observed previously, phenylbutazone accelerated arachidonic acid peroxidation[36]: DHLA again reproducibly decreased peroxidation in reaction mixtures containing phenylbutazone.

TABLE 4

Effects of lipoic acid and dihydrolipoic acid on inactivation of irradiated α_1-antiproteinase. Solutions were bubbled with N_2O/O_2 (80:20) prior to irradiation and then exposed to a total amount of 180 μM $OH^{\cdot}$ using a linear accelerator. They contained 10 mM KH_2PO_4-KOH buffer pH 7·4, α_1-antiproteinase (5 mg/ml) and other compounds at the final concentrations stated. After irradiation, aliquots of the assay mixture were withdrawn and tested for elastase-inhibitory capacity. Control experiments showed that compounds irradiated in the absence of α_1-antiproteinase had no effect on elastase directly. A representative experiment is shown

Addition to reaction mixture	Elastase activity $\Delta A_{410} \times 10^3$/sec	α_1-Antiproteinase activity (as 100-% elastase activity)
No α_1 AP	26.8	0
α_1 AP, not irradiated	0.40	99
α_1 AP, irradiated	9.94	63
- plus LA (50 μM)	15.49	42
(100 μM)	12.87	52
(200 μM)	10.45	61
(500 μM)	6.60	75
- plus DHLA (50 μM)	12.62	53
(100 μM)	0.52	98
(200 μM)	0.77	97
(500 μM)	0.08	99
- plus phenylbutazone (200 μM)	18.63	30
- plus phenylbutazone (200 μM) and LA (100 μM)	23.13	14
(200 μM)	20.37	24
(500 μM)	23.18	14
- plus phenylbutazone (200 μM) and DHLA (100 μM)	20.56	23
(200 μM)	23.18	14
(500 μM)	25.53	5
- plus DL-penicillamine (100 μM)	13.35	50
- plus penicillamine (100 μM) and LA (100 μM)	9.27	65
(200 μM)	7.16	73
(500 μM)	7.45	72
- plus penicillamine (100 μM) and DHLA (100 μM)	3.80	86
(200 μM)	0.99	96
(500 μM)	0.07	99

TABLE 5

Effects of lipoic and dihydrolipoic acids on arachidonic acid peroxidation by the myoglobin/H_2O_2 system. Reaction mixtures were as described in the materials and methods section. All contained arachidonic acid, myoglobin, and H_2O_2 and, where indicated, LA, DHLA or phenylbutazone at the concentrations stated. Peroxidation was measured by the TBA test and expressed as absorbance at 532 nm. Results are the means of two determinations

Addition to reaction mixture	Peroxidation (A_{532})
-	0.45
Lipoic acid (1 mM)	0.50
DHLA (1 mM)	0.34
Phenylbutazone (0.5 mM)	0.72
Phenylbutazone + LA (1 mM)	0.67
Phenylbutazone + DHLA (1 mM)	0.46

DISCUSSION

Dihydrolipoic acid has been claimed to have substantial antioxidant properties *in vitro*[8-16] and these may be relevant to its effects *in vivo*.[1-5] However, caution has previously been expressed that DHLA may be able to exert pro-oxidant effects by interaction with metal ions.[13,17] We therefore undertook a detailed characterization[22] of the antioxidant and potential pro-oxidant effects of DHLA and lipoic acid.

Lipoic acid was a good scavenger of $OH^{\cdot}$, reacting at essentially a diffusion-controlled rate (rate constant 4.71×10^{10} $M^{-1}s^{-1}$ at 37°C). Results of the deoxyribose assay indicated that it could also bind iron ions and diminish their activity in *forming* $OH^{\cdot}$, detectable by deoxyribose, from H_2O_2. Of course, it may be that the iron-LA complex still forms $OH^{\cdot}$, which reacts with the LA and is not detected in free solution. By contrast, DHLA had pro-oxidant effects in the deoxyribose assay. Although it almost certainly[9,39] reacts fast with $OH^{\cdot}$, its ability to interact with iron (either directly or by recycling ascorbic acid) and reduce it to Fe^{2+} gave it an overall pro-oxidant effect under our reaction conditions.

Despite these results in the deoxyribose assay, DHLA did not accelerate bleomycin-induced DNA degradation, suggesting that it cannot reduce the ferric bleomycin complex to ferrous bleomycin. However, it potentiated the action of ascorbate, presumably by recycling this molecule.

We confirmed a previous report[8] that both DHLA and LA are excellent scavengers of HOCl. However, we found no evidence for any significant reaction of either compound with $O_2^{\cdot -}$ or H_2O_2. This is not perhaps surprising, since thiols are fairly poor scavengers of either of these molecules[32,54].

The effects of LA and DHLA on lipid peroxidation depended on the assay system used. At fairly high concentrations, DHLA decreased peroxidation induced by the myoglobin-H_2O_2 system, as previously reported for other thiols.[55-57] By contrast, it accelerated peroxidation of ox-brain phospholipid liposomes in the presence of Fe^{3+} and ascorbate: this system was chosen to avoid complications due to the alleged ability of DHLA to recycle endogenous antioxidants in natural membranes.[11] Even when α-tocopherol was incorporated into the liposomes, DHLA was still pro-oxidant. Our data are consistent with earlier studies of Bast and Haenen on microsomes.[13] By contrast, LA *inhibited* liposomal peroxidation, presumably by binding iron ions and rendering them redox-inactive, as observed also in the deoxyribose assay (see above). Both LA and DHLA scavenged trichlomethylperoxyl radicals at significant rates.

One potential problem with thiol compounds is that free radical scavenging by these molecules could generate damaging thiyl and oxysulphur radicals.[48-49] This has been demonstrated for penicillamine, which generates a radical capable of inactivating α_1-antiproteinase.[47] At low concentrations, LA and DHLA accelerated α_1-antiproteinase inactivation in the presence of $OH^{\cdot}$. An especially surprising result was that both LA and DHLA decreased damage to α_1-antiproteinase by penicillamine radicals, but not by phenylbutazone radicals. Sulphur radical chemistry is extremely complex[39,49,50] and the overall effect of thiols is highly concentration-dependent (as may be seen from the data in Table 4).

Another indication that there may be problems with radicals derived from DHLA is the data in Table 3. DHLA prevented the slow loss of creatine kinase activity in human plasma incubated under air. This enzyme has an −SH group essential for activity, and DHLA presumably prevented its oxidation. By contrast, in plasma exposed to gas-phase cigarette smoke, DHLA reproducibly accelerated loss of CK

activity. A possible explanation of these results is that scavenging of radicals in smoke by DHLA generates sulphur-containing radicals that can damage creatine kinase. The dithiol dithiothreitol and the monothiol GSH also accelerated loss of enzyme activity.

Overall, LA and DHLA are interesting compounds with good antioxidant properties. Most, but not all (Tables 3,4) of the pro-oxidant actions of DHLA *in vitro* involve interaction with metal ions, either directly or by the recycling of other metal ion-reducing agents such as ascorbate. In health, metal ions catalytic for free radical reactions are safely sequestered.[19,58] However, in tissue damage by disease or other mechanisms, transition metal ions can become more available.[6,7] For example, advanced human atherosclerotic lesions contain metal ions "catalytic" for free radical reactions.[59] Any effects of DHLA in the treatment of human disease may then represent a balance between antioxidant and pro-oxidant properties. However, it should be noted that LA is a powerful *inhibitor* of iron-dependent lipid peroxidation and $OH^{\cdot}$ generation (Figure 2, Figure 4). Perhaps LA should be the therapeutic agent of choice. The ability of LA to diminish some of the pro-oxidant effects of DHLA is also worthy of note.

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