British Journal of Pharmacology (2001) 133, 739–745

www.nature.com/bjp

Antioxidant activity of amiodarone on human lipoprotein oxidation

*,[1]Domenico Lapenna, [1]Giuliano Ciofani, [1]Cristiana Bruno, [1]Sante D. Pierdomenico & [1]Franco Cuccurullo

[1]Dipartimento di Medicina e Scienze dell'Invecchiamento, Università degli Studi G. d'Annunzio, Facoltà di Medicina e Chirurgia, 66100 Chieti, Italy

1 Lipoprotein oxidation is crucial in atherogenic processes. Amiodarone is a lipophilic antiarrhythmic/antianginal drug which is able to influence the physicochemical status of biological lipid components. Since oxidation of lipids is affected by their physicochemical state and amiodarone binds to lipoproteins, we hypothesized that the drug may exert an antioxidant activity on human lipoprotein oxidation.

2 Dose-dependent effects of therapeutically achievable amiodarone concentrations (1.5, 3, 5, 7 and 10 μM) were studied on copper-catalysed oxidation of the non-HDL fraction *in vitro*. Amiodarone inhibited oxidation as judged by generation of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH) and fluorescent products of lipoperoxidation (FPL) as well as from the kinetics of conjugated diene formation. This antioxidant activity was significant at 1.5 μM with total inhibition at 10 μM and an IC_{50} of 4 μM. The primary *in vivo* metabolite of amiodarone, namely desethylamiodarone, also exhibited specific antioxidant properties although it was less effective than amiodarone with an IC_{50} of 7 μM.

3 In further *in vivo* experiments, susceptibility to copper-mediated oxidation of the non-HDL fraction was investigated before and 4 weeks after oral amiodarone administration to humans. Following treatment, significant inhibition of TBARS, LOOH and FPL generation was observed in comparison with baseline levels and a placebo-treated control group, highlighting an effective antioxidant capacity of amiodarone *in vivo*.

4 Amiodarone did not change lipoprotein vitamin E and phospholipid content *in vivo* and did not show scavenging effects on oxidizing species involved in lipoprotein oxidation, such as peroxyl radicals, nor metal-binding/inactivating properties, suggesting that physicochemical modifications of lipoprotein lipids induced by the lipophilic drug may be involved in its antioxidant activity.

5 In conclusion, amiodarone, and its primary metabolite desethylamiodarone, show previously unrecognized antioxidant activity on human lipoprotein oxidation. This effect is also evident *in vivo* and at therapeutically achievable drug concentrations. Thus, amiodarone may act as an antioxidant/antiatherosclerotic agent in humans, although this issue warrants further clinical study.
British Journal of Pharmacology (2001) **133,** 739–745

Keywords: Amiodarone; antioxidants; atherosclerosis; lipoprotein oxidation

Abbreviations: AAPH, 2,2′-azobis (amidinopropane) dihydrochloride; CD, conjugated dienes; DPH, 1,1-diphenyl-2-pycrylhydrazyl; EDTA, ethylenediaminetetraacetic acid; FPL, fluorescent products of lipoperoxidation; LOOH, lipid hydroperoxides; PBS, phosphate buffered saline; TBARS, thiobarbituric acid reactive substances; TNB, 5-thio-2-nitrobenzoic acid

Introduction

In recent years evidence has accumulated indicating that lipoprotein oxidation is an important aspect of atherogenesis which may be counteracted by compounds acting as antioxidants of lipoprotein oxidative processes (Witzum & Steinberg, 1991; Esterbauer *et al*., 1992; Berliner & Heinecke, 1996). Amiodarone is a widely used antiarrhythmic/antianginal drug which binds to proteins and, especially, lipids due to its hydrophobic properties (Heger *et al*., 1984; Lalloz *et al*., 1984; Adams, 1985; Jandreski & Vanderslice, 1993; Zipes, 1997). There is experimental evidence that amiodarone can inhibit oxygen radical generation in neutrophils and hepatic cells (Wysocka *et al*., 1989; Ruch *et al*., 1991). Moreover, amiodarone has been shown to reduce rat liver microsomal lipid peroxidation, and this antioxidant effect is apparently related to its lipophilicity (Rekka *et al*., 1990). In this regard, hydrophobic compounds are known to exert an inhibitory activity on lipoperoxidation as a result of their high lipophilicity, thereby influencing the physicochemical status of the lipid phase (Nagatsuka & Nakazawa, 1982; Halliwell & Gutteridge, 1989; Breugnot *et al*., 1990; McLean & Hagaman, 1990; Rekka *et al*., 1990; Breugnot *et al*., 1991; Mazière *et al*., 1992; Wiseman *et al*., 1993; Lapenna *et al*., 1998; Mak *et al*., 1998; Mason *et al*., 1999). Given these observations and bearing in mind that amiodarone is also bound to lipoproteins in the plasma environment (Lalloz *et al*., 1984; Jandresky & Vanderslice, 1993) we hypothesized that amiodarone may exert an antioxidant effect on lipoprotein oxidation. We have now investigated this possibility in the present paper using both *in vitro* and *ex vivo* experiments.

*Author for correspondence at: Patologia Medica, Policlinico di Colle dell' Ara, Via dei Vestini, 66013 Chieti Scalo, Italy

Methods

Lipoprotein isolation and oxidation

The non-HDL fraction was obtained from EDTA plasma of healthy adults (age 30–60 years) as previously reported (Phelps & Harris, 1993; Zhang *et al*., 1994; Lapenna *et al*., 1997), using dextran sulphate (mol. wt. 500,000) plus 0.1 M $MgCl_2$ to precipitate the fraction itself and remove EDTA.

In a first set of experiments, the non-high-density lipoprotein (non-HDL) fraction (0.1 mg non-HDL protein ml^{-1}) was oxidized with 5 μM $CuCl_2$ in PBS (pH 7.4), over a period of 3 h at 37°C. The effect of amiodarone on this process was studied using therapeutically achievable drug concentrations (1.5, 3, 5, 7 and 10 μM; Holt *et al*., 1983; Heger *et al*., 1984; Adams *et al*., 1985; Zipes, 1997). Lipoprotein oxidation was evaluated by assay of thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LOOH) and fluorescent products of lipoperoxidation (FPL).

TBARS were measured spectrophotometrically at 532 nm according to the procedure of Ohkawa *et al*. (1979), as previously reported (Lapenna *et al*., 1997). Results were calculated as nmol TBARS mg^{-1} non-HDL protein, using a molar extinction coefficient of 154,000. Drugs used in this study did not form TBARS directly, nor alter lipoperoxidation-related pink chromogen formation when included only during the heating test phase.

LOOH were assessed spectrophotometrically at 580 nm by the xylenol orange-ferrous oxidation assay essentially as described by Hermes-Lima *et al*. (1995). Reaction mixtures contained 0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM xylenol orange and 0.12 ml of the lipoprotein sample. Results were calculated as nmol LOOH mg^{-1} non-HDL protein, using a molar extinction coefficient for linoleic acid hydroperoxide of 47,000 (Jiang *et al*., 1992).

FPL were measured at 430 nm emission after excitation at 360 nm (Esterbauer *et al*., 1992) using a Kontron SFM 25 spectrofluorimeter standardized with quinine sulphate (1.0 μg ml^{-1} in 0.1 N H_2SO_4) to give a fluorescence intensity of 200 at 430 nm with excitation at 360 nm. Results are expressed as units of relative fluorescence mg^{-1} non-HDL protein.

In other experiments, drug effects on the kinetics of copper-catalysed lipoprotein oxidation were investigated through continuous spectrophotometric monitoring of the increase in absorbance at 234 nm, reflecting conjugated diene (CD) formation during lipid peroxidation (Esterbauer *et al*., 1992; Lapenna *et al*., 1997). Incubation was at 37°C in quartz cuvettes containing 0.1 mg non-HDL protein ml^{-1} and 5 μM $CuCl_2$, with and without amiodarone, in PBS (pH 7.4). Reference cuvettes contained lipoprotein or lipoprotein plus amiodarone where appropriate. The molar extinction coefficient of CD was considered to be 29,500 at 234 nm (Esterbauer *et al*., 1992; Lapenna *et al*., 1997).

The specific antioxidant activity of probucol, propranolol, vitamin E and the primary amiodarone metabolite, desethylamiodarone, was also evaluated and compared with that of amiodarone. For these experiments, the non-HDL fraction (0.1 mg non-HDL protein ml^{-1}) was oxidized with 5 μM $CuCl_2$ for 3 h at 37°C in PBS (pH 7.4), and lipoprotein oxidation was assessed using the TBA-test, as described above. Minimal drug concentration inhibiting significantly lipoprotein oxidation (IC_{min}), and drug concentrations inhibiting by 50 and 100% lipoprotein oxidation (IC_{50} and IC_{100}, respectively), were determined.

Non-HDL proteins were measured by the method of Lowry *et al*. (1951).

Radical scavenging activity of amiodarone

Possible scavenging effects of amiodarone on peroxyl radicals, which are involved in lipoprotein oxidation, were investigated by assessing the ability of the drug to counteract peroxyl radical-induced oxidation of 5-thio-2-nitrobenzoic acid (TNB), resulting in decrease in TNB-related absorbance at 412 nm (Lapenna *et al*., 1998). TNB was prepared by reduction of 5,5′-dithiobis (2-nitrobenzoic acid) with 2-mercaptoethanol and its concentration was calculated using a molar extinction coefficient of 13,600 at 412 nm (Lapenna *et al*., 1998). 2,2′-azobis (amidinopropane) dihydrochloride (AAPH) was used to generate thermally peroxyl radicals. Reaction mixtures contained 10 μM TNB, 0.1 mM diethylenetriaminepentaacetic acid and 1 mM AAPH, with or without amiodarone, in PBS (pH 7.4). After 45 min incubation at 37°C, the absorbance at 412 nm (A_{412}) was recorded spectrophotometrically against appropriate drug-containing blanks.

We also evaluated drug capacity to scavenge the stable free radical 1,1-diphenyl-2-pycrylhydrazyl (DPH), resulting in DPH bleaching (Blois, 1958; Lapenna *et al*., 1998). Reaction mixtures contained 85 μM DPH (previously dissolved in ethanol) in PBS (pH 7.4), with or without amiodarone. After 5 min, DPH-related absorbance values at 517 (A_{517}) were recorded spectrophotometrically against appropriate blanks. Reactions were sometimes carried out directly in organic solvents (e.g. ethanol or chloroform).

Drug-copper interaction

Initially we studied whether amiodarone was able to prevent lipoprotein copper binding which is a process essential for lipoprotein oxidation (Kuzuya *et al*., 1992). For these specific experiments, the non-HDL fraction (0.5 mg non-HDL protein ml^{-1}) was incubated for 30 min at 37°C with 20 μM $CuCl_2$, with and without amiodarone, in PBS (pH 7.4); to obtain the same drug : metal molar ratios of the copper-mediated lipoprotein oxidation experiments, amiodarone was here used at concentrations of 6, 12, 20, 28 and 40 μM. After overnight dialysis to remove unbound metal, 0.5% deoxycholic acid and 0.2 mM ascorbic acid were added to the samples, followed by addition of 1.3 ml chloroform containing 0.2 mM bathocuproine as the copper (I) colorimetric detector. Absorbance values at 478 nm were then recorded spectrophotometrically using the organic layer against appropriate blanks and results were calculated as nmol copper mg^{-1} non-HDL protein using a molar extinction coefficient for the bathocuproine-copper (I) complex of 12,700 at 478 nm.

Drug-metal interaction was also studied using the copper-mediated ascorbate oxidation test (Empson *et al*., 1991). Reaction mixtures contained 70 μM ascorbic acid and 5 μM $CuCl_2$, with and without amiodarone, in 0.15 M NaCl. After 5 min incubation at 25°C, ascorbate-related absorbance at 265 nm was recorded spectrophotometrically against appro-

priate drug-containing blanks. Results were calculated as nmol ascorbic acid oxidized ml^{-1} min^{-1} using a molar extinction coefficient of 15,000.

Given the relevance of metal reduction in biomolecule oxidant damage, we also evaluated whether amiodarone decreased the capability of copper (II) to undergo reductive processes with copper (I) formation. Amiodarone was incubated for 30 min at 37°C with 5 μM $CuCl_2$, in PBS (pH 7.4) and then 0.5 mM ascorbate was added as reductant, followed by treatment with the chloroform–bathocuproine reagent as reported above.

Finally, to further determine a possible interaction of amiodarone with copper (II) and copper (I), a spectral study from 600 to 200 nm was performed using 5 μM $CuCl_2$ and 10 μM amiodarone, in PBS (pH 7.4), as well as 5 μM CuCl and 10 μM amiodarone in argon-purged acetonitrile.

Lipoprotein susceptibility to oxidation after amiodarone administration to humans

Seven subjects (5 males and 2 females, age 35–68 years) were administered orally 400 mg amiodarone twice a day for 1 week, followed by 200 mg once a day for another 3 weeks. Three subjects had paroxysmal idiopathic atrial fibrillation with spontaneous return to sinus rhythm. These subjects had no evidence of organic disease and were not taking other drugs. The other four subjects were healthy volunteers chosen from our medical or technical staff. Another seven matched control subjects (five males and two females, age 37–66 years) were placebo-treated for the same period. All subjects were from the same geographic area (Chieti, Abruzzo, Italy), and they maintained the same lifestyle during the drug supplementation period. No subject took antioxidant compounds. The study received approval by a local Ethics Committee. The non-HDL fraction was isolated before and after amiodarone or placebo administration and subjected to oxidation with 5 μM $CuCl_2$ for 3 h at 37°C in PBS (pH 7.4); TBARS, LOOH and FPL were evaluated as described above. No adverse effect nor significant changes of clinical chemistry analytes were observed after amiodarone intake. In four subjects the lipoprotein content of vitamin E and phospholipid was measured before and after the period of drug administration. Vitamin E was assayed by the spectrofluorometric method of Hansen & Warwick (1969), using α-tocopherol as standard, whilst phospholipids were measured spectrophotometrically using ammonium ferrothiocyanate to form a 485 nm-absorbing complex with phospholipids themselves and egg yolk phosphatidilcholine as the standard (New, 1990). In addition, in the three subjects with atrial fibrillation and in one volunteer, serum total amiodarone concentrations were measured at 240 nm wavelength by high-pressure liquid chromatography essentially according to the procedure by Heger *et al.* (1984).

Data analysis and statistics

Data were calculated as mean ± s.d. Drug effects were evaluated by one-way analysis of variance (ANOVA) plus Student–Newman–Keuls test (Glantz, 1987). When amiodarone was administered to humans, results were studied by paired or unpaired Student's *t*-test where appropriate (Glantz, 1987). $P<0.05$ was regarded as statistically significant.

Materials

Drugs and reagents were from Sigma Aldrich s.r.l., Milano, Italy, except for desethylamiodarone, which was a generous gift of Dr Roberto Latini, Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy, and AAPH, which was from Polisciences, Warrington, USA. Drug stock solutions were prepared daily in ethanol using the same aliquots of ethanol in control experiments.

Results

Drug effects on lipoprotein oxidation

When the non-HDL fraction was oxidized for 3 h with 5 μM $CuCl_2$, amiodarone inhibited lipoprotein oxidation in a dose-related manner (Figure 1). The lowest drug concentration to inhibit significantly TBARS, LOOH and FPL formation was 1.5 μM. A maximal effect (i.e. total inhibition of lipoprotein oxidation) was achieved at 10 μM with all peroxidative indices (Figure 1). The IC_{50} of amiodarone was estimated to be 4 μM (Table 1). Desethylamiodarone was less effective than amiodarone with an IC_{50} of 7 μM (Table 1). Comparing IC_{50} values (Table 1), amiodarone was 50-fold more potent than propranolol, but was considerably less effective than probucol, which was the most powerful inhibitor of copper-mediated lipoprotein oxidation used in this study. The antioxidant potency of amiodarone was however close to that of vitamin E, which had an IC_{50} value of 2.5 μM (Table 1).

As depicted in Figure 2, amiodarone also showed antioxidant activity on the kinetics of copper-mediated lipoprotein oxidation. Since the highest therapeutically relevant drug concentration (i.e. 10 μM) gave interference problems in the continuous spectrophotometric monitoring of

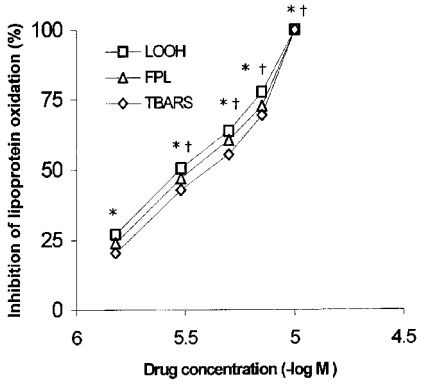

Figure 1 Concentration-dependent inhibition of copper-mediated lipoprotein oxidation by amiodarone. The non-HDL fraction was oxidized by 5 μM $CuCl_2$ for 3 h at 37°C, in PBS (pH 7.4), with and without 1.5, 3, 5, 7 and 10 μM amiodarone. LOOH, FPL and TBARS were, respectively, 297.5 ± 48 nmol LOOH mg^{-1} non-HDL protein, 92.5 ± 12 units of relative fluorescence mg^{-1} non-HDL protein and 58.5 ± 8 nmol TBARS mg^{-1} non-HDL protein in control experiments. The results represent the means of percentage inhibition of lipoprotein oxidation calculated from seven independent experiments (the s.d. is less than 10% and was omitted for clarity). *$P<0.05$ vs control; †$P<0.05$ vs the lower drug concentration (ANOVA plus Student–Newman–Keuls test).

Table 1 Drug inhibitory activities on the oxidation of human non-HDL fraction induced by copper

	IC_{min} (μM)	IC_{50} (μM)	IC_{100} (μM)
Amiodarone	1.5	4	10
Desethylamiodarone	3	7	16
Propranolol	100	200	450
Probucol	0.2	0.4	0.8
Vitamin E	1	2.5	6

IC_{min}: minimal drug concentration inhibiting significantly copper-mediated lipoprotein oxidation; IC_{50}: drug concentration inhibiting by 50% lipoprotein oxidation; IC_{100}: drug concentration inhibiting by 100% (i.e. totally) lipoprotein oxidation. Oxidant damage of the non-HDL fraction was evaluated by the TBA-test, as reported in the Methods section.

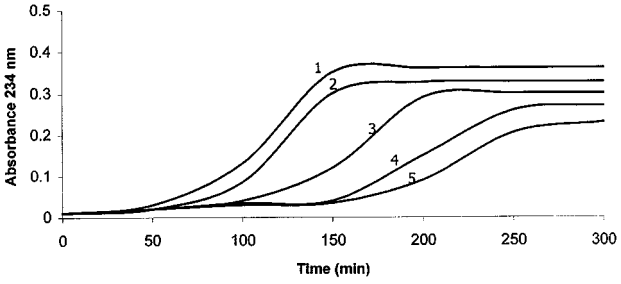

Figure 2 Antioxidant activity of amiodarone on the kinetics of copper-mediated oxidation of the non-HDL fraction evaluated by continuous spectrophotometric monitoring of absorbance increase at 234 nm due to conjugated diene formation. Trace 1: control; traces 2, 3, 4 and 5: 1.5, 3, 5 and 7 μM amiodarone, respectively. The results shown are representative of seven similar experiments. See Methods and Results sections for further explanations.

UV absorbance at 234 nm, it was not used in these experiments. The lag time of oxidation was 64 ± 6.7 min in control experiments, and it was significantly prolonged by amiodarone in a dose-dependent fashion (88 ± 12.5, 117 ± 23, 148 ± 18.7 and 177 ± 19.5 min with 1.5, 3, 5 and 7 μM, respectively, all $P<0.05$ vs control; 1.5 vs 3 μM, 3 vs 5 μM and 5 vs 7 μM, $P<0.05$; $n=7$). Oxidation rate was instead significantly decreased by amiodarone (from 1.35 ± 0.3 nmol CD min^{-1} mg^{-1} non-HDL protein of control experiments to 0.93 ± 0.2, 0.75 ± 0.25, 0.45 ± 0.23 and 0.35 ± 0.15 nmol CD min^{-1} mg^{-1} non-HDL protein using 1.5, 3, 5 and 7 μM amiodarone, respectively, all $P<0.05$ vs control; 5 vs 1.5 and 3 μM, and 7 vs 1.5 and 3 μM, $P<0.05$; 1.5 vs 3 μM, and 5 vs 7 μM, $P>0.05$; $n=7$).

Radical scavenging activity of amiodarone

Amiodarone, even at the highest therapeutically achievable concentration (i.e. 10 μM), did not antagonize peroxyl radical-induced TNB oxidation. In fact, incubation with AAPH caused a marked decrease in TNB-related A_{412} values, which was similar with and without 10 μM amiodarone (from 0.136 ± 0.005 to 0.032 ± 0.004 and 0.030 ± 0.003 absorbance units, respectively, with and without the drug, $P>0.05$; $n=4$). Thus, amiodarone would appear to have no direct scavenging activity on peroxyl radicals. Moreover, amiodarone did not scavenge the stable free radical DPH. Indeed, DPH-related A_{517} values were 0.507 ± 0.015 in control experiments, not significantly different from those observed in the presence of 10 μM amiodarone (0.512 ± 0.018; $n=4$). Notably, amiodarone was also ineffective when the reaction was carried out directly in organic solvents (e.g. chloroform), using lower DPH concentration (30 μM) and a more prolonged incubation time (0.147 ± 0.007 vs 0.150 ± 0.005 absorbance units of control experiments after 120 min incubation, $P>0.05$; $n=4$).

Drug-copper interaction

The amount of copper bound to the non-HDL fraction was 6.6 ± 0.7 nmol mg^{-1} non-HDL protein, not different from that measured in the presence of the highest amiodarone concentration used in this specific experiment, i.e. 40 μM (6.8 ± 0.9 nmol mg^{-1} non-HDL protein; $P>0.05$, $n=4$); the drug was therefore incapable of preventing lipoprotein metal binding. Moreover, copper-driven ascorbate oxidation was similar in the presence and absence of 10 μM amiodarone (7.7 ± 0.35 and 7.9 ± 0.3 nmol ml^{-1} min^{-1}, respectively, $P>0.05$; $n=4$), suggesting that metal redox potential and catalytic activity are unaffected by the drug. In line with these experiments, amiodarone did not influence the capability of copper (II) to undergo ascorbate-mediated reduction with copper (I) formation. In fact, absorbance values at 478 nm related to the bathocuproine-copper (I) complex were 0.060 ± 0.003 and 0.058 ± 0.002 with and without 10 μM amiodarone, respectively ($P>0.05$; $n=4$). Finally, as shown in Figure 3, the spectral characteristics of amiodarone were substantially identical in the absence or presence of $CuCl_2$; similarly, the drug spectrum was unaffected by CuCl (not shown), further indicating no apparent interaction between amiodarone and copper both in the oxidized and reduced form.

Effect of amiodarone administration on lipoprotein susceptibility to oxidation

Serum amiodarone concentrations were 1.52 ± 0.4 μM in the subjects analysed after the period of drug administration. The content of vitamin E in the non-HDL fraction of amiodarone-treated subjects was similar before and after the period of pharmacological treatment (1.8 ± 0.43 vs 1.85 ± 0.37 μg mg^{-1}

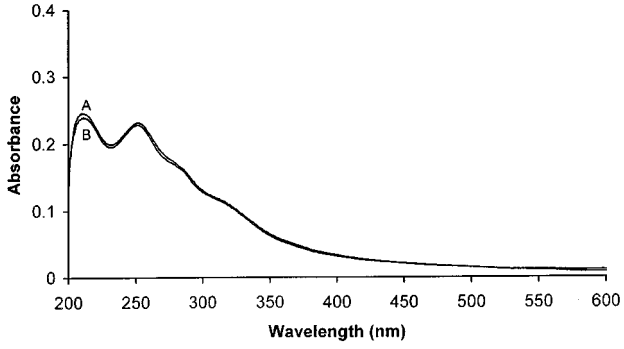

Figure 3 Spectral characteristics of 10 μM amiodarone in the absence (trace A) and presence (trace B) of 5 μM $CuCl_2$ in PBS (pH 7.4). See Methods and Results sections for further explanations.

non-HDL protein, $P>0.05$), as was that of phospholipids (125.2 ± 16.3 vs 123 ± 12.5 $\mu g\ mg^{-1}$ non-HDL protein, $P>0.05$). As a result of amiodarone administration, a decreased susceptibility of the non-HDL fraction to copper-mediated oxidation was observed. At the end of 3 h incubation with 5 μM $CuCl_2$, TBARS, LOOH and FPL were indeed significantly lowered with respect to both baseline levels and the values detected in the placebo-treated controls, who showed instead a lipoprotein vulnerability to oxidation similar to that observed before placebo administration (Table 2). It is therefore worth emphasizing that amiodarone also exhibits antioxidant activity on human lipoprotein oxidation in man *in vivo*.

Discussion

The present study shows that amiodarone has significant antioxidant effects on copper-catalysed human lipoprotein oxidation. A number of mechanisms may be involved including (1) metal chelation-inactivation; (2) chain-breaking antioxidant activity, namely scavenging of radical species involved in specific oxidative processes; (3) stabilization of the lipoprotein lipid moiety by drug hydrophobic groups, resulting in physicochemical modifications of the lipid phase with decreased propensity to undergo oxidative radical-chain reactions; and/or (4) changes in the endogenous lipoprotein antioxidant and/or lipid status (this mechanism may be operative essentially *in vivo* after chronic drug intake). Our data indicate that amiodarone has no copper chelating-inactivating properties; moreover, the drug does not act as a chain-breaking antioxidant since it is incapable of scavenging peroxyl radicals and the free radical DPH. The possibility that amiodarone may scavenge some lipophilic (e.g. lipid) radicals generated during oxidative processes directly within the hydrophobic lipoprotein core cannot be excluded. However, such a mechanism appears unlikely, considering that the drug is incapable of scavenging DPH even in hydrophobic environment like chloroform. Such a lack of effect of amiodarone to scavenge these specific radicals is apparently in contrast to a recent report by Ide *et al*. (1999) showing a protective effect of the drug on cardiac myocytes by a free radical scavenging activity. However, this activity was reported to be exerted specifically on hydroxyl radical ($OH^{\cdot}$) generated by chelated iron/H_2O_2 (Ide *et al*., 1999). Free $OH^{\cdot}$ can promote peroxidation of some lipid systems but does not seem to be involved in lipoprotein oxidation, which is instead driven essentially by oxidant species such as peroxyl (and equivalent) radicals (Esterbauer *et al*., 1992; Kalyanaraman *et al*., 1993; Noguchi *et al*., 1993; Abuja & Esterbauer, 1995). Indeed, lipoprotein oxidation is not counteracted by $OH^{\cdot}$ scavengers, such as mannitol (Steinbrecher, 1988; Heinecke *et al*., 1993; Lynch & Frei, 1993), whereas it is inhibited by metal chelators and chain-breaking antioxidants, such as phenolic compounds, which are able to scavenge peroxyl radicals (Leake & Rankin, 1990; Esterbauer *et al*., 1992; Kalyanaraman *et al*., 1993; Pryor *et al*., 1993). Thus, it is possible that amiodarone, which does not act as a peroxyl radical scavenger, exerts an inhibitory effect on lipoprotein oxidation unrelated to $OH^{\cdot}$-scavenging effects, further suggesting that other mechanisms are operative in its specific antioxidant activity. Moreover, phospholipid and vitamin E levels of the non-HDL fraction are apparently unaffected by amiodarone administration *in vivo*, indicating that drug antioxidant properties are not associated with quantitative changes of lipid or antioxidant lipoprotein content. On the other hand, the efficiency of amiodarone *in vitro* argues against the possibility that its antioxidant activity might be related to drug-induced changes in phospholipid unsaturation *in vivo*. In model lipid systems, amiodarone has been shown to penetrate deeply into the lipid matrix and markedly decrease lipid motional freedom and fluidity (Chatelain *et al*., 1986), which may hamper propagation of lipoperoxidative radical-chain reactions. Thus, given these aspects and the capacity of amiodarone to bind to lipoproteins (Lalloz *et al*., 1984; Jandresky & Vanderslice, 1993), it is conceivable that the antioxidant effect of the drug on lipoprotein oxidation are directly related to its lipophilicity, resulting in stabilization of lipoprotein lipids with a decreased propensity to undergo oxidant damage and propagate radical-chain reactions to the particle core. Remarkably, a direct relationship between the lipophilicity and the antilipoperoxidative activity has been shown for various pharmacological compounds (Rekka *et al*., 1990; Mazière *et al*., 1992; Mak *et al*., 1998; Mason *et al*., 1999), and specific protective effects on lipoprotein oxidation have been reported with lipophilic drugs, such as ticlopidine, phenothiazines, calcium antagonists, beta-blockers, aci-reductones and tamoxifen (Breugnot *et al*., 1990; 1991; Mazière *et al*., 1992; Wiseman *et al*., 1993; Lapenna *et al*., 1998; Mak *et al*., 1998).

The primary metabolite of amiodarone is desethylamiodarone which is formed *in vivo* through N-dealkylation processes and has pharmakodynamic properties similar to those of amiodarone (Zipes, 1997). Our data show that desethylamiodarone also has antioxidant effects on lipoprotein oxidation, although it is less effective than the parent compound. Notably, amiodarone differs from desethylamiodarone by the removal of a single ethyl group in its chemical structure; the carbon-hydrogen bonds of ethyl group are strongly apolar and removal of such a group by N-dealkylation augments

Table 2 Effect of amiodarone administration to humans on the susceptibility of the non-HDL fraction to copper-mediated oxidation

	Control group	*Amiodarone group*
	Baseline	*Baseline*
TBARS	57.5 ± 8.7	60 ± 9.2
LOOH	298 ± 47	300 ± 44.7
FPL	91.3 ± 11.5	86.7 ± 10.5
	After placebo	*After amiodarone*
TBARS	59.3 ± 9.5	34 ± 8*†
LOOH	296 ± 52	128 ± 39.5*†
FPL	88 ± 12.3	37.7 ± 11*†

The non-HDL fraction was isolated in seven subjects before and 4 weeks after oral amiodarone administration, as well as in other seven placebo-treated controls, and oxidized by 5 μM $CuCl_2$ for 3 h at 37°C, in PBS (pH 7.4). TBARS: thiobarbituric acid reactive substances (nmol TBARS mg^{-1} non-HDL protein); LOOH, lipid hydroperoxides (nmol LOOH mg^{-1} non-HDL protein); FPL: fluorescent products of lipoperoxidation (units of relative fluorescence mg^{-1} non-HDL protein). Means$\pm$s.d. *$P<0.0001$ vs baseline values (paired Student's *t*-test); †$P<0.0001$ vs control subjects (unpaired Student's *t*-test). See Methods section for further explanations.

drug polarity with decreased lipophilicity apparently resulting in a lower antioxidant activity. Although less efficient than amiodarone, desethylamiodarone could however act as a specific antioxidant *in vivo*, considering that it may reach mean plasma levels of about 5 μM, with concentrations even higher in tissue compartments (Adams *et al*., 1985; Zipes, 1997). Our data also show that amiodarone is more potent than propranolol as a lipoprotein antioxidant, conceivably as a result of its higher lipophilicity, but it is less effective than probucol, which is not only a lipophilic agent with marked lipid stabilizing effects (McLean & Hagaman, 1990), but also has significant peroxyl radical scavenging properties (Pryor *et al*., 1993). It is however of note that the antioxidant potency of amiodarone is close to that of vitamin E, which, in concentration terms, is the major lipoprotein physiological antioxidant (Esterbauer *et al*., 1992).

The therapeutic level of serum amiodarone concentrations appears to range between 1.5 and 5 μM, but some patients show values of 10 μM or more after long-term drug administration (Heger *et al*., 1984). Side effects of amiodarone could however represent a clinical problem, especially when serum concentrations are higher than 5 μM (Heger *et al*., 1984; Zipes, 1997). Remarkably, the specific antioxidant activity of amiodarone is significant at concentrations well below 5 μM, appearing therefore to be potentially of therapeutic relevance. It is indeed worth emphasizing that lipoproteins isolated from subjects taking amiodarone at a dosage resulting in mean serum drug concentrations of 1.5 μM, exhibit higher resistance to oxidative processes, indicating a specific antioxidant capacity of the drug *in vivo*. In this regard, it is noteworthy that inhibition of lipoprotein oxidation is higher *ex vivo* than *in vitro* at the same drug concentration (1.5 μM). In fact, taking TBARS generation as an example, its pharmacological inhibition is about 43 and 20% in *ex vivo* and *in vitro* experiments, respectively (Table 2 and Figure 1). It is possible that optimal drug-lipoprotein lipids interactions occur *in vivo*. Moreover, metabolites of amiodarone, such as desethylamiodarone, may contribute to the total drug antioxidant potential. Thus, the specific antioxidant effect of amiodarone may be especially relevant *in vivo*. These novel pharmakodynamic properties should be beneficial in the clinical setting, which is usually characterized by vascular atherosclerotic involvement (amiodarone is also considered an antianginal drug). A lipoprotein-associated lipophilic antioxidant such as amiodarone may delay lipoprotein oxidation especially in the arterial wall, where iron and copper are present in a catalytically-active form (Smith *et al*., 1992; Evans *et al*., 1995) and thus the development of atherosclerosis. Indeed, experimental studies have shown a strong inverse relationship between the lipoprotein content of lipophilic pharmacological antioxidants, such as probucol, and the extent of vascular atherosclerotic involvement (O'leary *et al*., 1996). Evidence for the relevance of delaying lipoprotein oxidation is also provided by clinical studies indicating an association between the lipoprotein susceptibility to oxidation and both the severity and the progression of atherosclerotic lesions (Rengström *et al*., 1992; Andrews *et al*., 1995; Salonen *et al*., 1997).

In conclusion, amiodarone, and its primary metabolite desethylamiodarone, are characterized by a previously unrecognized antioxidant effect on human lipoprotein oxidation, which is evident also *in vivo* and at therapeutically achievable drug concentrations. Thus, the drug could effectively act as an antioxidant/antiatherosclerotic agent in the clinical setting. This issue warrants further investigation. In any event, the antioxidant activity of amiodarone should be taken into account when investigating lipoprotein susceptibility to oxidative damage in human beings.

The authors are indebted to Dr Roberto Latini, Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy, for his generous gift of desethylamiodarone.

References

ABUJA, P.M. & ESTERBAUER, H. (1995). Simulation of lipid peroxidation in low density lipoprotein by a basic skeleton of reactions. *Chem. Res. Toxicol.,* **8,** 753 – 763.

ADAMS, P.C., HOLT, D.W., STOREY, G.C.A., MORLEY, A.R., CALLAGHAN, J. & CAMPBELL, R.W.F. (1985). Amiodarone and its desethyl metabolite: tissue distribution and morphologic changes during long-term therapy. *Circulation,* **72,** 1064 – 1075.

ANDREWS, B., BURNAND, K., PAGANGA, G., BROWSE, N., RICE-EVANS, C., SOMMERVILLE, K., LEAKE, D. & TAUB, N. (1995). Oxidisability of low density lipoprotein in patients with carotid or femoral artery atherosclerosis. *Atherosclerosis,* **112,** 77 – 84.

BERLINER, J.A. & HEINECKE, J.W. (1996). The role of oxidized lipoprotein in atherogenesis. *Free Radic. Biol. Med.,* **20,** 707 – 727.

BLOIS, M.S. (1958). Antioxidant determination by the use of a stable free radical. *Nature,* **158,** 1199 – 1200.

BREUGNOT, C., MAZIÈRE, C., AUCLAIR, M., MORA, L., RONVEAUX, M.F., SALMON, S., SANTUS, R., MORLIÈRE, P., LENAERS, A. & MAZIÈRE, J.C. (1991). Calcium antagonists prevent monocyte and endothelial cell-mediated modification of low density lipoproteins. *Free Radic. Res. Commun.,* **15,** 91 – 100.

BREUGNOT, C., MAZIÈRE, C., SALMON, S., AUCLAIR, M., SANTUS, R., MORLIERE, P., LENAERS, A. & MAZIÈRE, J.C. (1990). Phenothiazines inhibit copper- and endothelial cell-induced peroxidation of low density lipoprotein. *Biochem. Pharmacol.,* **40,** 1975 – 1980.

CHATELAIN, P., FERREIRA, J., LARUEL, L. & RUYSSCHAERT, J.M. (1986). Amiodarone induced modifications of the phospholipid physical state. A fluorescence polarization study. *Biochem. Pharmacol.,* **35,** 3007 – 3013.

EMPSON, K.L., LABUZA, T.P. & GRAF, E. (1991). Phytic acid as a food antioxidant. *J. Food Sci.,* **56,** 560 – 563.

ESTERBAUER, H., GEBICKI, J., PUHL, H. & JÜRGENS, G. (1992). The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic. Biol. Med.,* **13,** 341 – 390.

EVANS, P.J., SMITH, C., MITCHINSON, M.J. & HALLIWELL, B. (1995). Metal ion release from mechanically-disrupted human arterial wall. Implication for the development of atherosclerosis. *Free Radic. Res. Commun.,* **23,** 465 – 469.

GLANTZ, S.A. (1987). *Primer of Biostatistics*. New York: McGraw-Hill.

HALLIWELL, B. & GUTTERIDGE, J.M.C. (1989). *Free Radicals in Biology and Medicine*. Oxford: Clarendon Press.

HANSEN, L.G. & WARWICK, W.J. (1969). A fluorometric micromethod for serum vitamin A and E. *Am. J. Clin. Pathol.,* **51,** 538 – 541.

HEGER, J.J., SOLOW, E.B., PRYSTOWSKY, E.N. & ZIPES, D.P. (1984). Plasma and red blood cell concentrations of amiodarone during chronic therapy. *Am. J. Cardiol.,* **53,** 912 – 917.

HEINECKE, J.W., KAWAMURA, M., SUZUKI, L. & CHAIT, A. (1993). Oxidation of low density lipoprotein by thiols: superoxide-dependent and -independent mechanisms. *J. Lipid Res.,* **34,** 2051 – 2061.

HERMES-LIMA, M., WILLMORE, W.G. & STOREY, K.B. (1995). Quantitation of lipid peroxidation in tissue extracts based on Fe (III) xylenol orange complex formation. *Free Radic. Biol. Med.,* **19,** 271 – 280.

HOLT, D.W., TUCKER, G.T., JACKSON, P.R. & STOREY, G.C.A. (1983). Amiodarone pharmacokinetics. *Am. Heart J.,* **106,** 840 – 847.

IDE, T., TSUTSUI, H., KINUGAWA, S., UTSUMI, H. & TAKESHITA, A. (1999). Amiodarone protects cardiac myocytes against oxidative injury by its free radical scavenging action. *Circulation,* **100,** 690 – 692.

JANDRESKI, M.A. & VANDERSLICE, W.E. (1993). Clinical measurement of serum amiodarone and desethylamiodarone by using solid-phase extraction followed by HPLC with a high-carbon reversed-phase column. *Clin. Chem.,* **39,** 496 – 500.

JIANG, Z.Y., HUNT, J.V. & WOLFF, S.P. (1992). Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal. Biochem.,* **202,** 384 – 389.

KALYANARAMAN, B., JOSEPH, J. & PARTHASARATHY, S. (1993). Site-specific trapping of reactive species in low-density lipoprotein oxidation: biological implications. *Biochim. Biophys. Acta,* **1168,** 220 – 227.

KUZUYA, M., YAMADA, K., HAYASHI, T., FUNAKI, C., NAITO, M., ASAI, K. & KUZUYA, F. (1992). Role of lipoprotein-copper complex in copper catalized-peroxidation of low-density lipoprotein. *Biochim. Biophys. Acta,* **1123,** 334 – 341.

LALLOZ, M.R.A., BYFIELD, P.G.H., GREENWOOD, R.M. & HIMSWORTH, R.L. (1984). Binding of amiodarone by serum proteins and the effects of drugs, hormones and other ligands. *J. Pharm. Pharmacol.,* **36,** 366 – 372.

LAPENNA, D., DE GIOIA, S., CIOFANI, G., BRUNO, C., PORRECA, E., PIERDOMENICO, S.D. & CUCCURULLO, F. (1998). Antioxidant properties of ticlopidine on human low density lipoprotein oxidation. *FEBS Lett.,* **193,** 357 – 360.

LAPENNA, D., DE GIOIA, S., CIOFANI, G. & CUCCURULLO, F. (1997). Antioxidant activity of allopurinol on copper-catalysed human lipoprotein oxidation. *FEBS Lett.,* **409,** 265 – 268.

LEAKE, D.S. & RANKIN, S.M. (1990). The oxidative modification of low-density lipoproteins by macrophages. *Biochem. J.,* **270,** 741 – 748.

LOWRY, O.H., ROSENBURGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with Folin phenol reagent. *J. Biol. Chem.,* **193,** 265 – 275.

LYNCH, S.M. & FREI, B. (1993). Mechanisms of copper- and iron-dependent oxidative modification of human low density lipoprotein. *J. Lipid Res.,* **34,** 1745 – 1753.

MAK, I.T., MURPHY, A., HOPPER, A., WITIAK, D., ZIEMNIAK, J. & WEGLICKI, W.B. (1998). Potent inhibitory activities of hydrophobic aci-reductones (2-hydroxytetronic acid analogs) against membrane and human low-density lipoprotein oxidation. *Biochem. Pharmacol.,* **55,** 1921 – 1926.

MASON, R.P., AK, I.T., TRUMBORE, M.W. & MASON, P.E. (1999). Antioxidant properties of calcium antagonists related to membrane biophysical interactions. *Am. J. Cardiol.,* **84,** 16L – 22L.

MAZIÈRE, C., AUCLAIR, M. & MAZIÈRE, J.C. (1992). Lipophilic β-blockers inhibit monocyte and endothelial cell-mediated modification of low density lipoprotein. *Biochim. Biophys. Acta,* **1126,** 314 – 318.

MCLEAN, L.R. & HAGAMAN, K.A. (1990). Antioxidant activity of probucol and its effects on phase transitions in phosphatidylcholine liposomes. *Biochim. Biophys. Acta.,* **1029,** 161 – 166.

NAGATSUKA, S. & NAKAZAWA, T. (1982). Effects of membrane-stabilizing agents, cholesterol and cepharantin, on radiation-induced lipid peroxidation and permeability in liposomes. *Biochim. Biophys. Acta,* **691,** 171 – 177.

NEW, R.R.C. (1990). Characterization of liposomes. In: *Liposomes: A practical approach*. ed. New RRC. pp. 105 – 161. Oxford: IRL Press.

NOGUCHI, N., GOTOH, N. & NIKI, E. (1993). Dynamics of the oxidation of low density lipoprotein induced by free radicals. *Biochim. Biophys. Acta.,* **1168,** 348 – 357.

OHKAWA, H., OHISHI, N. & YAGI, K. (1979). Assay for lipid peroxidation in animal tissue by thiobarbituric acid reaction. *Anal. Biochem.,* **95,** 351 – 358.

O'LEARY, V.J., TILLING, L., FLEETWOOD, G., STONE, D. & DARLEY-USMAR, V. (1996). The resistance of low density lipoprotein to oxidation promoted by copper and its use as an index of antioxidant therapy. *Atherosclerosis,* **119,** 169 – 179.

PHELPS, S. & HARRIS, W.S. (1993). Garlic supplementation and lipoprotein oxidation susceptibility. *Lipids,* **28,** 475 – 477.

PRYOR, W.A., CORNICELLI, J.A., DEVALL, L.J., TAIT, B., TRIVEDI, B.K., WITIAK, D.T. & WU, M. (1993). A rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants. *J. Org. Chem.,* **58,** 3521 – 3532.

REKKA, E., MANNHOLD, R.M., BAST, A. & TIMMERMAN, H. (1990). Molecular pharmacological aspect of antiarrhythmic activity. Class I and class III compounds and lipid peroxidation. *Biochem. Pharmacol.,* **39,** 95 – 100.

RENGSTRÖM, J., NILSSON, J., TORNVALL, P., LANDOU, C. & HAMSTEN, A. (1992). Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet,* **339,** 1183 – 1186.

RUCH, R.J., BANDYOPADHYAY, S., SOMANI, P. & KLAUNIG, J.E. (1991). Evaluation of amiodarone free radical toxicity in rat hepatocytes. *Toxicology Lett.,* **56,** 117 – 126.

SALONEN, Y., NYYSSÖNEN, K., SALONEN, R., PORKKALA-SARATAHO, E., TUOMAINEN, T.-P., DICZFALUSY, U. & BJÖRKHEM, I. (1997). Lipoprotein oxidation and progression of carotid atherosclerosis. *Circulation,* **95,** 840 – 845.

SMITH, C., MITCHINSON, M.J., ARUOMA, O.I. & HALLIWELL, B. (1992). Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. *Biochem. J.,* **286,** 901 – 905.

STEINBRECHER, U.P. (1988). Role of superoxide in endothelial cell modification of low-density lipoprotein. *Biochim. Biophys. Acta,* **959,** 20 – 30.

WISEMAN, H., PAGANGA, G., RICE EVANS, C.A. & HALLIWELL, B. (1993). Protective actions of tamoxifen and 4-hydroxytamoxifen against oxidative damage to human low-density lipoproteins: a mechanism accounting for cardioprotective action of tamoxifen? *Biochem. J.,* **292,** 635 – 638.

WITZUM, J.L. & STEINBERG, D. (1991). Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.,* **88,** 1785 – 1792.

WYSOCKA, E., WYSOCKI, H., SIMINIAK, T. & SZCZEPANIK, A. (1989). Effect of selected antiarrhythmic drugs on the superoxide anion production by polymorphonuclear neutrophils in vitro. *Cardiology,* **76,** 264 – 269.

ZHANG, A., VERTOMMEN, J., VAN GAAL, L. & DE LEEUW, I. (1994). A rapid and simple method for measuring the susceptibility of low-density-lipoporotein and very-low-density-lipoprotein to copper-catalized oxidation. *Clin. Chim. Acta,* **227,** 159 – 173.

ZIPES, D.P. (1997). Management of cardiac arrhythmias: Pharmacological, electrical, and surgical techniques. In: *Heart Disease. A Textbook of Cardiovascular Medicine*. ed. Braunwald E. pp. 593 – 639. Philadelphia: Saunders Co.

(Received October 4, 2000
Revised April 19, 2001
Accepted April 19, 2001)