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Amiodarone inhibits tocopherol-mediated human lipoprotein peroxidation

Domenico Lapenna *, *Giuliano Ciofani*, *Sante Donato Pierdomenico*, *Matteo Neri*, *Maria Adele Giamberardino*, *Franco Cuccurullo*

Dipartimento di Medicina e Scienze dell'Invecchiamento and Centro di Scienze dell'Invecchiamento-Fondazione Università G. d'Annunzio, Facoltà di Medicina e Chirurgia, Chieti, Italy

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ABSTRACT

It is unknown whether lipoprotein tocopherol-mediated peroxidation (TMP) is influenced by peculiar drug physicochemical properties such as hydrophobicity. Thus, we studied the effect of the extremely hydrophobic agent amiodarone on human non-HDL TMP. The drug, albeit devoid of specific radical-scavenging effects, inhibited TMP at therapeutic concentrations and with an efficiency similar to that of the physiological co-antioxidant ascorbic acid, showing indeed an IC_{50} of 5 μM. A comparable efficiency was observed with human LDL, and with a pure LDL-VLDL mixture. TMP was also inhibited by other hydrophobic cationic amphiphiles without radical-scavenging activity, namely desethylamiodarone, chlorpromazine, clomipramine, promethazine, promazine, verapamil, bromhexine, propranolol, mepivacaine, metoprolol, tramadol and ranitidine, whose anti-TMP potency was far lower than that of amiodarone and related to drug hydrophobicity degree. Further, TMP was strongly inhibited by butylhydroxytoluene, a lipophilic radical scavenger. Hydrophobic acidic (diclofenac, indomethacin, ibuprofen and ketoprofen) or neutral (*n*-hexane, anthracene, *o*-xylene and toluene) compounds could not instead inhibit TMP, indicating a stringent requirement for drug basicity in the pharmacological inhibition of TMP. Amiodarone effectiveness was lowered by lipoprotein α-tocopherol enrichment, suggesting some drug-α-tocopherol interaction and less lipid pharmacological protection at higher α-tocopheroxyl radical generation. Drug anti-TMP activity may so be related to electrostatic and hydrophobic interactions with lipoprotein α-tocopherol and lipid moiety, resulting in decreased radical phase transfer and lipid propensity to undergo radical-driven peroxidation. In conclusions, primarily drug basicity and then hydrophobicity are solely relevant to TMP inhibition. Amiodarone, at therapeutic concentrations, has anti-TMP properties, which could occur in the clinical setting.

* *Corresponding author at*: Policlinico "SS Annunziata", Patologia Medica, Via dei Vestini, 66013 Chieti Scalo, Italy. Tel.: +39 0871 358098; fax: +39 0871 551615.
E-mail address: piersd@tiscalinet.it (D. Lapenna).

Abbreviations: AAPH, 2,2'-azobis(amidinopropane) dihydrochloride; α-TOH, α-tocopherol; α-TO, α-tocopheroxyl radical; BHT, butylhydroxytoluene; CDH, conjugated diene hydroperoxides; DPH, 1,1-diphenyl-2-pycrylhydrazyl; DTPA, diethylenetriaminepentaacetic acid; FOX, ferrous oxidation in xylenol orange assay; IC_{min}, minimal drug concentration inhibiting significantly TMP; IC_{50}, drug concentration inhibiting by 50% TMP; log *P*, octanol–water partition coefficient; LOOH, lipid hydroperoxides; PBS, phosphate buffered saline; TMP, tocopherol-mediated peroxidation; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TNB, 5-thio-2-nitrobenzoic acid

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1. Introduction

Oxidative modification of lipoproteins has been increasingly implicated in the pathogenesis of atherosclerosis [1–4]. Lipoprotein oxidation is usually studied using high oxidant fluxes imposed by micromolar concentrations of redox-active transition metals, such as copper. In these experimental conditions, α-tocopherol (α-TOH), in concentration terms the major endogenous lipoprotein antioxidant, exerts antilipoperoxidative effects through scavenging of peroxyl (and equivalent) radicals [1–4]. In recent years, however, it has been realized that under more physiological conditions of low oxidant fluxes α-TOH can foster lipoprotein peroxidation [4–10]; this phenomenon is known as ''tocopherol-mediated peroxidation'' (TMP). The TMP model predicts that α-TOH favors the entry of a radical from the aqueous phase into lipoproteins with generation of hydrophobic α-tocopheroxyl radical (α-TO$^{\bullet}$), which can behave as a radical chain transfer agent able to trigger lipid peroxidation; primary lipoperoxidative products, namely lipid hydroperoxides (LOOH), are so formed in the presence of α-TOH. Since lipoprotein α-TOH is located on the phospholipid surface coat, α-TO$^{\bullet}$ is here formed before cruising throughout the particle polar coat and the nonpolar core with possible TMP initiation [4,10]. Thus, when present at lipoprotein surface level, α-TO$^{\bullet}$ may make contact with aqueous antioxidants such as ascorbic acid eventually resulting in TMP inhibition. Indeed, TMP can be inhibited by reducing and radical-scavenging compounds named co-antioxidants (e.g. ascorbic acid itself), which act through α-TO$^{\bullet}$ reduction and export of the radical character from the lipoprotein compartment into the aqueous phase [4,5,7–9]. Such compounds, however, are not expected to inhibit TMP unless the radical formed can escape the lipoprotein particle favoring ''shuttling'' of radicals between particles with radical termination reactions [4,5,8–10]. Bulky hydrophobic agents could so be poorly effective or hamper even the radical escape process potentially favoring TMP. On the other hand, some lipophilic drugs such as amiodarone can counteract copper, high oxidant flux-dependent lipoprotein oxidation conceivably owing to their hydrophobic interactions with the lipid phase resulting in decreased lipid propensity to undergo peroxidative processes [11]. To date, it is unknown whether peculiar drug physicochemical properties such as hydrophobicity influences TMP. The present study was therefore designed to investigate the effect of the extremely hydrophobic agent amiodarone on human lipoprotein TMP. Since amiodarone is bound to β-lipoproteins in human plasma [12], experiments were basically performed using the non-high-density lipoprotein (non-HDL) fraction, namely LDL plus VLDL, which are Apo B100-containing atherogenic lipoproteins susceptible to TMP [4,9].

2. Materials and methods

2.1. *Reagents and lipoprotein preparation*

Drug and reagents were generally from Sigma–Aldrich Corp. (St. Louis, MO, USA). 2,2′-azobis(amidinopropane) dihydrochloride (AAPH) was from Polysciences, Warrington, USA; desethylamiodarone was a generous gift of Dr. Roberto Latini, Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy.

The non-HDL fraction was isolated from EDTA plasma of healthy normolipidemic adults (age 48–57 years) as previously reported [11,13,14], using dextrane sulfate (mol. wt. 500,000) plus $MgCl_2$ to selectively precipitate the apo B-containing lipoproteins, namely LDL plus VLDL. The healthy plasma donors were of the same geographic area (Chieti, Abruzzo, Italy), had similar nutritional intake and took no antioxidant compounds, including vitamin E. To 4.0 ml of twice diluted plasma, 0.4 ml of precipitation reagent (formed by mixing equal volumes of 20 g/l solution of dextrane sulfate with 2 M $MgCl_2$) was added, followed by vortexing and centrifugation at 1500 × *g* for 10 min. To wash away any HDL, residual plasma proteins and EDTA from the non-HDL fraction, the non-HDL pellet was suspended in 4.0 ml of 0.9% saline and reprecipitated twice by adding 0.2 ml precipitation reagent, vortexing and centrifuging [11,13,14]. The washed reprecipitated non-HDL pellet was dissolved in 4% saline; for all lipoperoxidative experiments, aliquots of this concentrated non-HDL solution were then added to Chelex 100-treated phosphate buffered saline (PBS), pH 7.4, at final content of 0.1 mg non-HDL protein/ml. Protein concentrations were measured by Lowry method as previously reported [11,13]. It is of note that the non-HDL fraction can be obtained rapidly thus avoiding possible artifactual lipid peroxidation, and has oxidative properties similar to those of lipoproteins obtained by density gradient ultracentrifugation [11,13,14]. In this regard, in some experiments we also used pure LDL, which were isolated from EDTA plasma of the aforementioned healthy subjects by density gradient ultracentrifugation according to Kleinveld et al. [15], as well as a mixture of pure LDL and VLDL after extraction of the latter lipoprotein class by density gradient ultracentrifugation basically as reported by Mohr and Stocker [9]. The two separately purified lipoproteins were recombined to obtain a molar ratio resembling that present in normal human plasma, i.e. LDL/VLDL = 18, corresponding to a LDL protein/VLDL protein ratio of about 1.7 [9]. In preliminary experiments, adequacy of separation and purity of lipoprotein preparations was further checked by agarose gel electrophoresis, which showed indeed no band in the chylomicron and α region nor bands of plasma proteins including albumin, with normal electrophoretic mobility of ApoB100-containing lipoproteins; to remove dextran sulfate, addition of $BaCl_2$ to the non-HDL fraction followed by centrifugation was performed before electrophoresis [14].

Since amiodarone is not only hydrophobic but also basic (cationic) and amphiphilic in nature, we also tested various other agents with similar physicochemical properties covering a broad range of hydrophobicity, i.e. of octanol–water partition coefficient (log *P*) values; importantly, these agents had also to be devoid of specific radical-scavenging properties (see below). Thus, after appropriate selection, the following drugs belonging to various therapeutic classes could be tested: amiodarone, desethylamiodarone, chlorpromazine, clomipramine, promethazine, promazine, verapamil, bromhexine, propranolol, mepivacaine, tramadol, metoprolol and ranitidine, whose log *P* values are, respectively, 8.6 [16], 6.4 [16], 5.35 [17], 5.2 [17], 4.65 [17], 4.55 [17], 4.4 [18], 4.3 [19], 3.5 [17], 2.7 [20], 2.3 [21], 1.5 [18]

and 0.63 [18]. Moreover, we evaluated on non-HDL TMP other hydrophobic compounds with acidic properties, namely diclofenac, indomethacin, ibuprofen and ketoprofen, whose log *P* values are, respectively, 4.4, 4.2, 3.6 and 3 [17,21]; the hydrophobic neutral compounds anthracene, *n*-hexane, *o*-xylene and toluene, having log *P* values of, respectively, 4.5, 4.1, 3.1 and 2.7 [22] were also tested. Finally, anti-TMP activity of butylhydroxytoluene (BHT), a lipophilic antioxidant with specific radical-scavenging properties [8], and of the physiological co-antioxidant ascorbic acid was also assessed.

Hydrophobic drugs such as amiodarone are expected to be incorporated into the lipoprotein compartment *in vivo*. To favor a similar incorporation *in vitro*, amiodarone and the other hydrophobic agents dissolved in ethanol were pre-incubated in the dark for 60 min at 37 °C with the lipoprotein sample before adding AAPH to start lipoperoxidation, using the same alcohol aliquots and pre-incubation in control experiments (final ethanol content was generally 0.005%). No lipid peroxidation nor changes in endogenous α-TOH content occurred as a result of the pre-incubation procedure. Amiodarone was used at therapeutically achievable concentrations, namely from 0.7 to 16 μM [23–25], which were totally incorporated into lipoproteins. Indeed, after dialysis all the drug pre-incubated with the lipoprotein sample was here recovered; moreover, oxidation experiments carried out with both undialyzed and dialyzed amiodarone-treated lipoproteins gave substantially the same results, further indicating total drug incorporation into the lipoprotein particle. Similar to amiodarone, also desethylamiodarone and BHT were totally incorporated into the lipoprotein compartment, while incorporation of chlorpromazine, clomipramine, promethazine, promazine, bromhexine, verapamil, propranolol, mepivacaine, tramadol, metoprolol and ranitidine corresponded to, respectively, about 32, 45, 57, 28, 62, 56, 43, 52, 56, 45 and 54% of the initial pre-incubated amount. Incorporation of diclofenac, ibuprofen, ketoprofen and indomethacin was estimated to be, respectively, about 85, 90, 80 and 90%. The cationic drugs were assessed on the basis of their absorbance at: 242 nm for amiodarone-desethylamiodarone, 254 nm for chlorpromazine, clomipramine, promethazine and promazine, 310 or 252 nm for bromhexine, 280 nm for verapamil, 288 nm for propranolol, 226 nm for mepivacaine, 275 nm for metoprolol, 272 nm for tramadol and 314 or 236 nm for ranitidine. Diclofenac, ibuprofen, ketoprofen and indomethacin were assayed on the basis of their absorbance at, respectively, 276, 226, 261 and 267 or 230 nm (binding of the neutral compounds anthracene, *n*-hexane, *o*-xylene and toluene could not be determined due the lack of their specific absorbance peaks). BHT was assessed fluorometrically at 280/310 nm excitation/emission [26]. For drug lipoprotein binding assessment, appropriate blanks formed by lipoprotein samples were always considered to exclude any lipoprotein-related absorbance; in this regard, spectral characteristics of drug alone and of drug bound to lipoproteins were the same after subtraction of lipoprotein blank, indicating no lipoprotein interference. Notably, in some experiments performed with amiodarone this methodological approach gives results similar to those obtained with complex HPLC-based procedures.

2.2. *Lipoprotein TMP*

Drug effects on lipoprotein TMP were at first evaluated through continuous spectrophotometric monitoring of absorbance increase at 234 nm due to formation of conjugated diene hydroperoxides (CDH) during lipid peroxidation [1,3,7,11,13,15,27]. Experiments were carried out in quartz cuvettes containing PBS, pH 7.4, and the non-HDL fraction (0.1 mg non-HDL protein/ml), with or without varying drug concentrations; the oxidative reaction was started by 300 μM AAPH, which generates thermally in the aqueous phase peroxyl radicals able to promote lipoprotein TMP [4–8], allowing 5 h incubation at 37 °C. For proper recording of absorbance increase at 234 nm, reference cuvettes had to contain the non-HDL fraction with or without the same drug concentrations, as appropriate, and absorbance at 234 nm was recorded every min; moreover, an AAPH-containing reagent blank was always considered to correct for the low level of lipid peroxidation-independent, AAPH-related absorbance increase at 234 nm (Fig. 1). Under these experimental conditions, only concentrations of CDH were measured and calculated using a molar extinction coefficient of 29,500 at 234 nm [11,13,27]. The adequacy of such method was confirmed by the strict concordance between the levels of CDH accumulated at the end of the 5 h incubation period with AAPH and those of authentic LOOH measured spectrophotometrically after the same period by the methanolic ferrous oxidation in xylenol orange (FOX) assay [28], which was also used to assess specifically lipoprotein TMP and its pharmacological inhibition. In this regard, data were calculated as nmol LOOH/mg non-HDL protein using a molar extinction coefficient for linoleic acid hydroperoxide of 47,000 [11,28]. Moreover, drug effects on lipoprotein TMP were assessed by a thiobarbituric acid (TBA)-test, which detects mainly

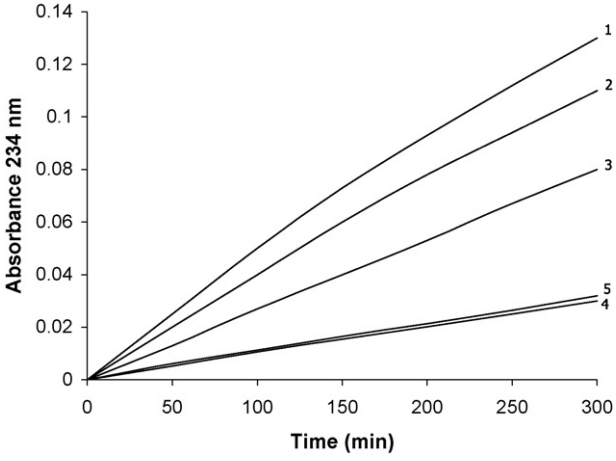

Fig. 1 – Antilipoperoxidative activity of amiodarone on the kinetics of non-HDL TMP evaluated through continuous spectrophotometric monitoring of absorbance increase at 234 nm due to CDH formation during lipid peroxidation. Trace 1: control; trace 2, 3 and 4: 0.85, 5 and 14 μM amiodarone, respectively; trace 5: 300 μM AAPH alone. The results shown are representative of 6 similar experiments. See Sections 2 and 3 for further explanations.

breakdown aldehydic products of LOOH [1,11,13,14,29] and was carried out after the 5 h incubation period with AAPH basically according to Buege and Aust procedure as previously reported [29]. Data were calculated as nmol TBA-reactive substances (TBARS)/mg non-HDL protein, using a molar extinction coefficient of 154,000 [11,13,29]. No agent tested nor AAPH, at the concentrations used, gave interference problems in the FOX and TBA assays.

Based primarily on inhibition of LOOH and TBARS generation, minimal drug concentration inhibiting significantly non-HDL TMP (IC_{min}) and, especially, drug concentration inhibiting by 50% non-HDL TMP (IC_{50}) were determined.

At AAPH concentrations and radical fluxes low as those used in the present study, α-TOH may behave as a prooxidant and promote TMP favoring the entry of radicals from the aqueous phase into the lipoprotein compartment [4–10]. To confirm the occurrence of TMP in our experimental model, we used α-TOH-enriched lipoproteins with the expectation to observe an increase in AAPH-mediated lipoprotein peroxidation. α-TOH enrichment of the non-HDL fraction *in vitro* was accomplished basically as previously reported [6,7], namely by incubating the EDTA plasma of healthy normolipidemic donors with 0.5 mM α-TOH (pre-dissolved in ethanol) for 3 h at 37 °C; lipoproteins were then isolated and dialyzed for 3 h at 4 °C in the dark against PBS plus 10 μM EDTA, pH 7.4, to remove possible α-TOH unincorporated into the lipoprotein particle. A blank with plasma and a corresponding amount of ethanol alone (1%, v/v) was simultaneously prepared in the same way. Non-HDL α-TOH content, as assessed by the spectrofluorometric method of Hansen and Warwick [30], was significantly increased by such enrichment procedure (22.5 ± 2.7 versus 10.8 ± 1.2 nmol α-TOH/ml non-HDL of controls, $p < 0.0001$; $n = 6$). Control and α-TOH-enriched lipoproteins (0.1 mg non-HDL protein/ml) were oxidized for 5 h at 37 °C by 300 μM AAPH in PBS, pH 7.4, and primary lipoperoxidative products resulting from TMP, i.e. CDH and LOOH, assessed as reported above.

Finally, the effects of amiodarone were also tested using human LDL, which were oxidized under the same experimental conditions employed for the non-HDL fraction. However, conceivably for the extreme clearness of pure LDL related to the absence of triglyceride-rich VLDL, kinetic assessment of CDH formation at 234 nm could be performed with reference cuvettes containing either AAPH plus the drug, whose absorbance was therefore automatically excluded, or lipoproteins plus the drug as reported for the non-HDL fraction. These methodological approaches gave similar results, further pointing out the adequacy of non-HDL TMP evaluation. Regarding the lipoprotein mixture obtained recombining pure LDL and VLDL, its TMP assessment was carried out as for the non-HDL fraction.

2.3. Drug radical-scavenging activity

Possible drug scavenging effects against peroxyl radicals and α-TO$^{\bullet}$, which are involved in the TMP model, were specifically investigated. For peroxyl radicals, we also used the 5-thio-2-nitrobenzoic acid (TNB)-test [11], which is based on the capacity of peroxyl radicals generated by AAPH to oxidize the yellow compound TNB with loss of its absorbance at 412 nm [11]. Reaction mixtures contained 10 μM TNB and 1 mM AAPH, with or without varying drug concentrations, in PBS, pH 7.4, plus 100 μM diethylenetriaminepentaacetic acid (DTPA); after 45 min incubation at 37 °C, absorbance values at 412 nm (A_{412}) were recorded spectrophotometrically against appropriate blanks. TNB concentrations were calculated using a molar extinction coefficient of 13,600 at 412 nm [11]. Moreover, drug effects on peroxyl radical-induced polyunsaturated fatty acid peroxidation were tested. An emulsion of 2 mM linolenic acid (pre-dissolved in ethanol) was prepared in PBS, pH 7.4, containing 100 μM DTPA, and then oxidized for 5 h at 37 °C by 300 μM AAPH. Given the DTPA interference in the FOX assay, lipoperoxidation was here assessed by the TBA-test as reported above. A blank without AAPH resulted in undetectable TBARS formation, indicating that only AAPH-generated peroxyl radicals did induce linolenic acid peroxidation.

We then evaluated drug capacity to scavenge the stable free radical 1,1-diphenyl-2-pycrylhydrazyl (DPH), resulting in 1,1-diphenyl-2-picrylhydrazine formation and DPH bleaching [11,31]. DPH (previously dissolved in ethanol) was incubated for 15 min in chloroform at 16 μM final concentration with or without varying drug concentrations. DPH-related absorbance at 517 (A_{517}) was then recorded spectrophotometrically against appropriate drug-containing blanks.

α-TO was generated by reaction of α-TOH with DPH [32,33], and detected by appearance of its specific absorbance at 435 nm [34]. The reaction system contained 0.64 mM α-TOH and 0.3 mM DPH in chloroform, with and without varying drug concentrations; after 20 s, α-TO-related absorbance at 435 nm (A_{435}) was recorded spectrophotometrically against an appropriate blank.

2.4. Statistics

Data were calculated as means ± S.D. Specific drug effects were evaluated by one-way analysis of variance (ANOVA) plus Student–Newman–Keuls test [11,35]. Data relative to lipoprotein α-TOH enrichment were analyzed by paired Student's *t*-test [35]. Correlations were studied by linear regression analysis with Pearson's *r* calculation [35]. $p < 0.05$ was regarded as statistically significant [35].

3. Results

3.1. Drug effects on lipoprotein TMP

As depicted in Fig. 1, incubation of the non-HDL fraction with 300 μM AAPH resulted in a linear increase of CDH formation indicative of specific lipoprotein peroxidation. Such oxidative pattern, which has been already reported [7], differs from that typically occurring in metal-mediated, high radical flux models, which are characterized by a lag phase followed by uninhibited propagation of lipid peroxidation [1,3,11,13,15,27]. The occurrence of TMP in our model system was confirmed in the experiments performed with α-TOH-enriched lipoproteins, which were significantly more susceptible to lipid peroxidation mediated by 300 μM AAPH. Indeed, as a result of TMP such lipoproteins generated 0.146 ± 0.017 versus 0.105 ± 0.012 nmol CDH/min/mg non-HDL protein of controls ($p < 0.0001$; $n = 6$). These values correspond to a total generation

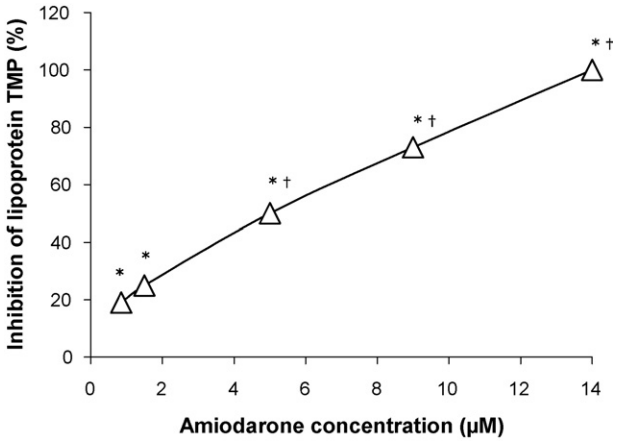

Fig. 2 – Concentration-dependent inhibition of lipoprotein TMP by amiodarone. Non-HDL TMP was assessed measuring LOOH by the FOX assay. The results represent the means of percentage inhibition of lipoprotein TMP calculated from 6 control experiments giving LOOH values of 34.9 ± 3.7 nmol/mg non-HDL protein (standard deviations are less than 7% and were omitted as they do not exceed the size of the symbols). *$p < 0.05$ vs. control; †$p < 0.05$ vs. the lower drug concentration (ANOVA plus Student–Newman–Keuls test). See Sections 2 and 3 for further explanations.

after 5 h incubation with 300 μM AAPH of, respectively, 43.8 ± 5.1 and 31.5 ± 3.6 nmol CDH/mg non-HDL protein, which, as noted before, is strictly concordant with that of LOOH detected by the FOX assay in α-TOH-enriched and control lipoproteins, i.e. 45 ± 5.4 and 32.2 ± 3.8 nmol LOOH/mg non-HDL protein, respectively (α-TOH-enriched versus control lipoproteins: $p < 0.0001$; $n = 6$). Overall, these results highlight the adequacy of our experimental model and of lipoprotein peroxidation assessment.

Fig. 1 also shows that amiodarone had a specific antilipoperoxidative activity. The drug counteracted in fact significantly CDH, LOOH and TBARS generated from non-HDL TMP with an IC_{min} of 0.85 μM, while at 5 μM halved lipoperoxide generation and at 14 μM afforded a total antioxidant protection (Figs. 1 and 2, Table 1).

Table 2 – IC_{50} and log *P* values of the cationic drugs tested

	IC_{50} (μM)	log *P*
Amiodarone	5	8.4
Desethylamiodarone	17	6.4
Chlorpromazine	50	5.35
Clomipramine	55	5.2
Promethazine	65	4.65
Promazine	70	4.55
Verapamil	75	4.4
Bromhexine	80	4.3
Propranolol	120	3.5
Mepivacaine	500	2.7
Tramadol	1200	2.3
Metoprolol	1600	1.5
Ranitidine	2700	0.63

Besides amiodarone, the other cationic agents incorporated into lipoproteins were also effective against non-HDL TMP, which was notably inhibited according to drug hydrophobicity, namely log *P* (Table 2). Indeed, there was a significant negative correlation between the values of log *P* and those of IC_{50} of the cationic drugs tested (Pearson's $r = -0.8$, $p = 0.001$). This means that to a higher hydrophobicity, i.e. log *P*, corresponds a higher specific drug antilipoperoxidative potency. On the other hand, there was no significant relationship between the pK_a and IC_{50} values of the cationic drugs (Pearson's $r = -0.2$, p = NS), indicating that the degree of basicity is not crucial for TMP inhibition. Accordingly, desethylamiodarone, which has a pK_a of 9 [16], is more effective than chlorpromazine, propranolol or tramadol, whose pK_a values are, respectively, 9.4, 9.45 and 9.6 [16]. In spite of their hydrophobicity, the acidic and neutral compounds tested could not inhibit non-HDL TMP (not shown), further highlighting the primary relevance of drug basicity in the pharmacological inhibition of TMP. Regarding BHT, it inhibited non-HDL TMP with an IC_{50} of 1.7 μM; this powerful antilipoperoxidative effect may be explained considering that BHT is a lipophilic antioxidant with specific radical-scavenging properties [8]. It is noticeable that ascorbic acid had an anti-TMP activity similar to that of amiodarone, showing indeed an IC_{50} of 4 μM.

To investigate whether the anti-TMP capacity of amiodarone is related to some interaction with lipoprotein α-TOH, drug effects were evaluated using the α-TOH-enriched

Table 1 – Inhibitory activity of amiodarone on human non-HDL TMP

	CDH	LOOH	TBARS
Control	0.112 ± 0.016	34.9 ± 3.7	4.8 ± 0.55
Control plus			
0.85 μM Amiodarone	0.09 ± 0.01*	28 ± 3.3*	3.8 ± 0.43*
5 μM Amiodarone	0.054 ± 0.008*†	17.2 ± 2*†	2.37 ± 0.3*†
14 μM Amiodarone	ND	ND	ND

TMP of the non-HDL fraction (0.1 mg non-HDL protein/ml) was induced by 5 h incubation at 37 °C with 300 μM AAPH, in PBS, pH 7.4. CDH: conjugated diene hydroperoxides (nmol CDH/min/mg non-HDL protein); LOOH: lipid hydroperoxides (nmol LOOH/mg non-HDL protein); TBARS: thiobarbituric acid reactive substances (nmol TBARS/mg non-HDL protein). Minimal drug concentration able to inhibit significantly non-HDL TMP (0.85 μM), and drug concentrations inhibiting by 50% (5 μM) and totally (14 μM) TMP are reported in the table. Means ± S.D. of 6 independent experiments. *$p < 0.05$ vs. control experiments; †$p < 0.05$ vs. the preceding values. ND: not detectable (total TMP inhibition). See Section 2 for further explanations.

non-HDL fraction always oxidized for 5 h at 37 °C by 300 μM AAPH (see Section 2). Indeed, if amiodarone interacted with non-HDL α-TOH, then a lower drug antilipoperoxidative capacity could be expected at higher lipoprotein α-TOH content. In these experiments performed with α-TOH-enriched lipoproteins, amiodarone had effectively a lower antilipoperoxidative potency showing an IC_{50} of 11 μM (22.7 ± 2.8 nmol LOOH/mg non-HDL protein and 0.07 ± 0.009 nmol CDH/min/mg non-HDL protein resulted from TMP with 11 μM amiodarone, in comparison with 45.3 ± 5.8 nmol LOOH/mg non-HDL protein and 0.143 ± 0.02 nmol CDH/min/mg non-HDL protein of specific control experiments, $p < 0.05$; $n = 5$).

Similar to the non-HDL fraction, oxidative kinetics of LDL and of the mixture of LDL plus VLDL was characterized by a linear increase of CDH formation indicative of TMP occurrence (not shown). Amiodarone could inhibit LDL TMP with an efficiency similar to that observed with the non-HDL fraction, showing indeed an IC_{50} of 6 μM (19.9 ± 2.7 versus 39.9 ± 5.5 nmol LOOH/mg LDL protein of control experiments, $p < 0.05$; $n = 5$); at the same drug concentration, the generation of CDH was also halved (0.064 ± 0.009 versus 0.13 ± 0.017 nmol CDH/min/mg LDL protein of control experiments, $p < 0.05$; $n = 5$), as was that of TBARS (2.8 ± 0.3 versus 5.5 ± 0.6 nmol TBARS/mg LDL protein of control experiments, $p < 0.05$; $n = 5$). In line with these results, amiodarone inhibited TMP of the mixture obtained recombining pure LDL and VLDL with an IC_{50} of 6 μM evident for LOOH (17.9 ± 2.4 versus 36.1 ± 4.7 nmol LOOH/mg protein of control experiments, $p < 0.05$; $n = 5$), CDH (0.06 ± 0.007 versus 0.12 ± 0.013 nmol CDH/min/mg protein of control experiments, $p < 0.05$; $n = 5$) and TBARS (2.5 ± 0.3 versus 5.1 ± 0.67 nmol T-TBARS/mg protein of control experiments, $p < 0.05$; $n = 5$). Besides the anti-TMP properties of amiodarone, these results further highlight the adequacy of the use of the non-HDL fraction to study lipoprotein oxidation and its pharmacological inhibition.

3.2. *Drug radical-scavenging activity*

Since in all the following tests amiodarone was ineffective, we will report only the results obtained with the highest drug concentration tested, i.e. 16 μM.

Amiodarone could not inhibit peroxyl radical-induced TNB oxidation; in fact, incubation with AAPH resulted in a marked decrement of TNB-related A_{412} values (from 0.138 ± 0.007 to 0.034 ± 0.005), which was similar with 16 μM amiodarone (0.032 ± 0.004; p = NS, $n = 5$). The drug was also ineffective on peroxyl radical-driven linolenic acid peroxidation, which resulted in 6.9 ± 0.7 and 7.1 ± 0.5 nmol TBARS/ml with and without 16 μM amiodarone, respectively (p = NS, $n = 5$). Moreover, amiodarone had no capacity to scavenge the stable free radical DPH; indeed, A_{517} values were 0.112 ± 0.006 in control experiments, not different from those observed with 16 μM amiodarone (0.114 ± 0.005, p = NS; $n = 5$). Finally, 16 μM amiodarone did not affect α-TO•-related A_{435} values, which were 0.120 ± 0.010 and 0.118 ± 0.013 with and without the drug, respectively (p = NS, $n = 5$), indicating no α-TO• scavenging activity.

The other cationic drugs tested, at concentrations able to counteract lipoprotein TMP, were also ineffective in all the aforementioned tests (not shown), namely had no significant scavenging activity against peroxyl radicals, α-TO• and DPH.

4. Discussion

The present study shows that lipoprotein TMP can be inhibited by amiodarone and other drugs which shares with it two fundamental physicochemical properties, i.e. at first basicity and then hydrophobicity. More specifically, these agents are hydrophobic cationic amphiphiles, containing indeed in close proximity a lipophilic aromatic ring system and a side chain with a nitrogen protonized at physiogical pH. The ineffectiveness of amiodarone and of the other cationic drugs tested as peroxyl radical and α-TO• scavengers indicates that they do not inhibit TMP through a specific radical-scavenging activity. Under a mechanistic profile, other aspects have to be considered such as: (1) inhibition of radical phase transfer, namely of the α-TOH-mediated transport of the aqueous radical character into the lipoprotein compartment; (2) pharmacological stabilization of lipoprotein lipids with their decreased propensity to undergo initiation of oxidation by α-TO• and propagation of peroxidative processes by lipid peroxyl radicals.

The finding that peroxyl radical-induced peroxidation of α-TOH-free linolenic acid is unaffected by amiodarone and related catamphiphiles suggests that these drugs have to interact with lipoprotein α-TOH, and, conceivably, with lipids structured in a lipoprotein form to inhibit TMP. Indeed, our experiments performed with α-TOH-enriched lipoproteins show that the antilipoperoxidative potency of amiodarone is lower at higher endogenous α-TOH content, suggesting some drug-α-TOH interaction. In such a context, it is possible that the phenolic hydroxyl group of α-TOH may form an hydrogen bond to the drug hydrogen bond acceptor amino group, hampering α-TOH capacity to import the radical character from the aqueous phase into the lipoprotein, as well as α-TOH-phospholipid interactions at lipoprotein surface. Regarding the experiments performed with α-TOH-enriched lipoproteins, it is also possible that amiodarone, which has profound lipid hydrophobic interactions [36], may protect less the lipoprotein lipid moiety from α-TO•-driven peroxidation at higher α-TO• generation related to endogenous α-TOH enrichment. Accordingly, cationic drugs like amiodarone are prone to interact specifically with biological lipids; in fact, the cationic nitrogen is attracted to the negatively charged phosphate of the phospholipid headgroup, while the aromatic ring system is directed towards the lipid hydrophobic interior [37–39]. Substances lacking these specific interactions with biological lipids and/or α-TOH such as the acidic and neutral ones here tested are remarkably ineffective against TMP. In this context, Yang et al. [40] have reported binding of the acidic drug ibuprofen to human lipoproteins, hypothesizing interaction of its negatively charged carboxyl group with the positively charged trimethyl phospholipid headgroups. It is evident that this type of ionic interaction involving the most outer part of the lipoprotein phospholipid surface does not result in TMP inhibition, which instead occurs for that slightly deeper involving the negatively charged phosphate of the phospholipid headgroup with drug cationic nitrogen. It is

worth noting that the chromanol-OH of α-TOH may prefer right the region of the phospholipid phosphate groups [10,41], suggesting that specific electrostatic interactions of cationic drugs occurring at this level are crucial for TMP inhibition. Also relevant to TMP inhibition are hydrophobic interactions occurring deeper within the lipoprotein between the cationic drug lipophilic aromatic ring system and the particle lipid interior. As a matter of fact, our results show for the cationic drugs tested a significant inverse relationship between log *P* and IC_{50} values, that is, at a higher hydrophobicity corresponds a higher anti-TMP potency; moreover, similar lipoprotein antilipoperoxidant protection is afforded by compounds somewhat structurally diverse, such as promazine, verapamil and bromhexine, that share a similar log *P* value. It is also of note that amiodarone, propranolol and chlorpromazine are more effective TMP inhibitors than, respectively, desethylamiodarone, metoprolol and promazine (or promethazine), which are structurally very similar to the former drugs but less hydrophobic. Thus, amiodarone and the other cationic agents may exert anti-TMP effects through their complex electrostatic and hydrophobic interactions with α-TOH and lipids of plasma lipoproteins. As a consequence of these interactions, phase transfer of aqueous radical character into the lipid environment via α-TOH at the lipoprotein surface may be hampered, leading to TMP inhibition; moreover, drug–lipid interactions could render lipoprotein lipids less susceptible to the oxidative attack of α-$TO^{\bullet}$ and to the propagation of lipid peroxidation. The present data may add new insight about the pharmacological inhibition of TMP, indicating that primarily drug basicity and then hydrophobicity are solely relevant to such inhibition; it is indeed worth emphasizing that amiodarone, albeit devoid of reducing and radical-scavenging properties, can inhibit non-HDL TMP with a potency similar to that of the physiological co-antioxidant ascorbic acid.

Physicochemical (especially ionic) interactions of amiodarone with lipoproteins may be specific and strong enough to represent a pharmacodynamic mechanism adequately translatable into an *in vivo* situation. Indeed, amiodarone is bound to β-lipoproteins in human plasma [12]. Moreover, the drug, whose therapeutic serum levels are 1.5–5 μM with values of 10 μM or more after long-term treatment [23,24], can counteract lipoprotein TMP at therapeutic concentrations, suggesting its specific antilipoperoxidative activity in the clinical setting. Relevant to the *in vivo* situation may be also the anti-TMP capacity of desethylamiodarone, which reaches micromolar levels in human plasma [23,25], and the possibility of optimal drug–lipoprotein interactions with localized high drug concentrations in the hydrophobic lipoprotein compartment. As a matter of fact, we have shown *ex vivo* inhibition of lipoprotein oxidation by amiodarone in subjects with mean serum drug concentration of 1.5 μM [11]. Overall, achieving lipoprotein antioxidant protection at therapeutically relevant drug concentrations based solely on specific physicochemical interactions with lipoproteins appears possible as a selective therapeutic strategy.

We previously reported that amiodarone is able to inhibit lipoprotein oxidation induced metal-dependently by high radical flux [11]. Metal-mediated radical burden and biomolecular oxidation occur especially in advanced atherosclerotic lesions, where redox-active transition metals, such as copper and iron, become pathologically available [42,43]. We have now demonstrated that amiodarone can counteract lipoprotein TMP, which may also be relevant to atherosclerosis. TMP occurrence *in vivo* is indeed suggested by the coexistence of specific LOOH configurational isomers (*cis*/*trans*) and α-TOH in human atherosclerotic lesions [44]; moreover, there is experimental evidence that dietary vitamin E supplementation of rabbits after arterial injury increases significantly both the aortic levels of α-TOH and the overall content of LOOH *cis*/*trans* isomers [44]. Albeit a potent antiarrhythmic agent like amiodarone cannot be considered as a potential experimental medication to achieve lipoprotein antioxidant protection in the general population, nonetheless it is possible that such protection occurs in the numerous patients already taking the drug for arrhythmic problems. In any event, the chemical structure of amiodarone may represent the basis for the synthesis of new compounds able to inhibit lipoprotein peroxidation; in particular, addition of radical-scavenging groups to amiodarone could lead to extremely effective antilipoperoxidative agents.

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