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TAMOXIFEN AND HYDROXYTAMOXIFEN AS INTRAMEMBRANEOUS INHIBITORS OF LIPID PEROXIDATION. EVIDENCE FOR PEROXYL RADICAL SCAVENGING ACTIVITY

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Abstract—Tamoxifen (TAM) is the antiestrogen most widely used in the chemotherapy and chemoprevention of breast cancer. It has been reported that TAM and its more active metabolite 4-hydroxytamoxifen (OHTAM) induce multiple cellular effects, including antioxidant actions. Here sarcoplasmic reticulum membranes (SR) were used as a simple model of oxidation to clarify the antioxidant action type and mechanisms of these anticancer drugs on lipid peroxidation induced by Fe^{2+}/ascorbate and peroxyl radicals generated by the water-soluble 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) and by the lipid-soluble 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN). Peroxidation was monitored by different assay systems, namely *cis*-parinaric acid (PnA) fluorescence quenching, production of thiobarbituric acid-reactive substances, polyunsaturated fatty acids (PUFA) degradation and oxygen consumption. TAM and OHTAM are efficient inhibitors of lipid peroxidation induced by Fe^{2+}/ascorbate and strong intramembraneous scavengers of peroxyl radicals generated either in the water or lipid phases by AAPH and AMVN, respectively. However, these drugs are not typical chain-breaking antioxidant compounds as compared with vitamin E. Additionally, their antioxidant effectiveness enhances the protective capacity of vitamin E against lipid peroxidation induced by AMVN. OHTAM is a more powerful intramembraneous inhibitor of lipid peroxidation as compared with TAM; this effectiveness not correlating with alterations on membrane fluidity may be due to the presence of a hydrogen-donating HO-group in the OHTAM molecule and its preferential location in the outer bilayer regions where it can donate the hydrogen atom to quench free radicals capable of initiating the membrane oxidative degradation. The stronger OHTAM intramembraneous scavenger capacity over TAM also correlates with its higher partition in biomembranes. Therefore, the strong peroxyl radical scavenger activity of OHTAM in the hydrophobic membrane phase may putatively contribute to the mechanisms of cytostatic and chemopreventive action of its promoter TAM on development of breast cancer.

Key words: tamoxifen; 4-hydroxytamoxifen; anticancer drugs; breast cancer; sarcoplasmic reticulum; lipid peroxidation; antioxidants; scavengers; peroxyl radicals

Free radicals and other oxygen reactive species, implicated in a wide range of cell and tissue damage and in the pathogenesis of several diseases [1, 2], are known to play important roles in neoplastic transformation of cells [3–5]. Concerning this, an apparent relationship has been observed between lipid peroxidation and the potential risk of later-developing breast cancer [6, 7]. Moreover, the inhibition of membrane peroxidation by antioxidants has been shown to have a protective effect in the initiation and promotion of certain cancers and in the side effects of several cytostatic drugs [8–12].

TAM§ (Fig. 1) is a synthetic nonsteroidal antiestrogen that has been successfully used in the chemotherapy and chemoprevention of breast cancer [13, 14]. However, the mechanisms by which TAM inhibits and prevents mammary cancer cell proliferation, and increases the incidence of certain tumors [15, 16] are not yet understood and cannot be explained solely by the competition for estrogen receptors.

It has been reported that TAM and its more active metabolite OHTAM (Fig. 1) [17] induce multiple cellular effects, including antioxidant actions [18, 19]. However, these actions, assessed on the basis of the classical TBARS-assay, a method that has been questioned owing to its proneness to interferences and lack of sensitivity and specificity [20, 21], have not yet been clarified. The proposed membrane-stabilizing effects of these drugs on the basis of lipid peroxidation inhibition [19, 22, 23] contradict our previous data on membrane fluidity [24, 25].

The hydrophobic nature of TAM and OHTAM,

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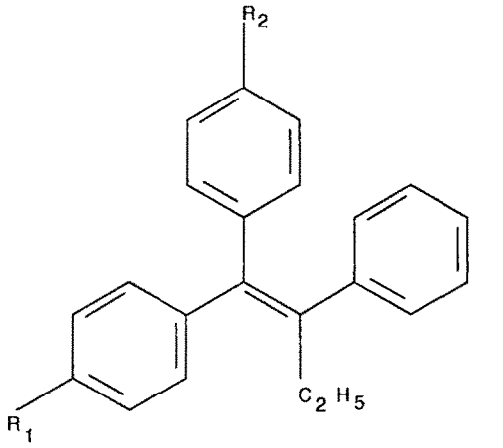
§ Abbreviations: TAM, tamoxifen; OHTAM, 4-hydroxytamoxifen; SR, sarcoplasmic reticulum; MDA, malonaldehyde; AAPH, 2,2′-azobis (2-amidinopropane)dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); TBA, thiobarbituric acid; PnA, *cis*-parinaric acid (9,11,13,15-*cis*,*trans*,*trans*,*cis*-octadecatetraenoic acid); PMSF, phenylmethanesulfonyl fluoride; TBARS, TBA-reactive substances; PUFA, polyunsaturated fatty acids; GLC, gas–liquid chromatography; DTT, dithiothreitol.



Tamoxifen: $R_1 = H$; $R_2 = OCH_2CH_2N(CH_3)_2$

Hydroxytamoxifen: $R_1 = OH$; $R_2 = OCH_2CH_2N(CH_3)_2$

Fig. 1. Chemical structure of TAM and OHTAM.

as demonstrated by their high partition coefficients in biomembranes [26], favors, in principle, a potential chain-breaking antioxidant activity of these drugs in the cell membranes. Therefore, it is of obvious interest to clarify TAM and OHTAM effects on membrane lipid oxidative degradation, in particular their peroxyl radical scavenger activities. Thus, the aim of this study is to define the antioxidant action mechanisms of TAM and OHTAM on membrane lipid peroxidation, and to examine potential relationships to membrane partition coefficients and localization owing to the different distribution of these drugs across the membrane thickness [24, 25].

Native SR vesicles and liposomes of SR total lipid extracts were used as models [27] to investigate the effects of TAM and OHTAM on lipid peroxidation induced either by Fe^{2+}/ascorbate system or by peroxyl radicals generated at a constant rate, in the aqueous or lipid phases, by the thermal decomposition of the azo-initiators AAPH and AMVN, respectively [28].

MATERIALS AND METHODS

Chemicals. PnA was purchased from Molecular Probes (Junction City, OR). Azocompounds, AAPH and AMVN were purchased from Polysciences, Inc. (Warrington, PA) TAM and 4-hydroxytamoxifen were obtained from Sigma Chemical Co. (Poole, U.K.) and Amersham (Amersham, U.K.), respectively. All the other chemicals were of research grade. Solutions were prepared in deionized ultrapure water to minimize metal contamination; those of $FeSO_4$, ascorbic acid, AAPH and AMVN were prepared freshly before use.

Preparation of membrane materials. Vesicles of fragmented SR were prepared from rabbit white muscles as described elsewhere [29], except that the isolation medium contained 2.5 mM DTT and 10 μM of the protease inhibitor PMSF. These conditions are very efficient to get preparations with high energetic coupling ratios [27, 30]. The microsomal fraction was suspended in the isolation medium without DTT and PMSF and kept at −80°. Protein content was estimated by the biuret assay [31], using bovine serum albumin as standard.

Lipids of SR membranes were extracted as described elsewhere [29] and determined by measuring the amount of inorganic phosphate [32] after hydrolysis at 180° in 70% $HClO_4$ [33]. Liposomes were prepared from mixtures of SR lipid extracts with AMVN and vitamin E in solvents, evaporated under a stream of nitrogen to dryness. The residues were dispersed in 20 mM Tris–HCl buffer, pH 7.4, hand-shaking in a water bath at 35° and vortexing for 1 min. The mixture was sonicated for 30 sec in a water bath to disperse large lipid aggregates and subsequently vortexed and sonicated for 1 min.

Parinaric acid assays. Experimental conditions for PnA oxidation were established as previously described [27] to ascertain that the fluorescence intensity of incorporated PnA is linear with its concentration and that membrane lipid concentration is high enough to incorporate most of the probe, avoiding the probe partition in the water phase.

The oxidation of 1.5 μM PnA incorporated in native SR membranes (200 μM in phospholipid or 0.23 mg/mL protein) suspended in 10 mM Tris–maleate, 150 mM NaCl, pH 7.0, was induced by Fe^{2+}/ascorbate (1 μM/2 μM) system at 25°. These concentrations of the oxidation inducers are significantly lower than those used for TBA test and fatty acid degradation measurements, owing to the higher sensitivity of the parinaric test over other methodologies (see below). The oxidative degradation of PnA (7.5 μM) incorporated in liposomes (800 μM) of SR lipid extracts containing AMVN (330 μM) or supplemented with vitamin E (1.3 μM), was induced by peroxyl radicals generated by thermal decomposition of the lipid-soluble AMVN at 50° [34, 35]. TAM and OHTAM drugs were preincubated with SR membranes and liposomes for 20 min at 25° and 37°, respectively, before starting the PnA assay. The incorporation of PnA, after 30 sec of recording the basal signal, was achieved by incubating an aliquot of an ethanolic solution of 1.5 mM PnA with the SR membranes or liposomes of their lipid extract by 2 min or 2.5 min, respectively, before starting the oxidation reaction in the spectrofluorometer cuvettes. PnA degradation was monitored by detecting the decay of fluorescence in a Perkin–Elmer LS-50 spectrofluorometer computer controlled provided with thermostated cuvettes and magnetic stirring. The excitation was set at 324 nm and the emission at 413 nm. The excitation and emission slit widths were 3 and 3.5 nm or 5 and 5.5 nm for monitoring PnA degradation in SR membranes and liposomes, respectively. Control experiments were performed in the absence of drugs; moreover, blank experiments of SR membranes plus PnA without addition of Fe^{2+}/ascorbate were performed to rule out that PnA fluorescence decay is the consequence of quenching mediated by TAM or OHTAM. The fluorescence intensity of PnA

was normalized as indicated by the unchanged fluorescence signal from 30 sec to 150 sec before induction of peroxidation and the rate of PnA fluorescence decay induced by pro-oxidants was taken as an index of the rate of oxidative degradation of PnA.

TBA test. Lipid peroxidation was induced in SR suspensions containing 2 mg protein per mL by Fe^{2+}/ascorbate (20 μM/100 μM), as described previously [27]. Accumulation of lipid peroxidation products reacting with TBA was measured spectrophotometrically at 535 nm, according to Buege and Aust [36]. The TBA reagent contained butylated hydroxytoluene to prevent the formation of additional chromophore during the assay procedure. Controls were carried out either in the absence of Fe^{2+}/ascorbic acid or TAM drugs to monitor the spontaneous peroxidation of the SR preparation and the effect of drugs in the absence of the inducer system.

Analysis of SR membrane lipids. Changes in the fatty acid contents of SR membranes during the peroxidation process, induced by Fe^{2+}/ascorbate as described for TBA-test, were evaluated by gas–liquid chromatography with flame ionization detection (Varian Series 1400). Membrane lipids were extracted from the reaction mixtures as described elsewhere [29] and fatty acid composition of the lipid extracts was analyzed after transesterification with H_2SO_4–methanol as previously described [27]. The injector and detector temperatures were 205° and 240°, respectively. The nitrogen flow rate in the column (SGE BP-20, 25 m, 0.53 mm) was 3 mL/min and the temperature program was started at 157°, kept for 8 min, and then increased to 205° at 4°/min and using 25 mL N_2/min as make-up gas in the detector system.

Oxygen consumption. The membrane lipid oxidation induced by peroxyl radicals generated in the aqueous phase by thermal decomposition of the azo-initiator AAPH was kinetically followed by the measurement of oxygen consumption using a Clark-type oxygen electrode (YSI model 5331, Yellow Spring Inst.), providing a kinetic monitoring of the rate of membrane oxidation [37]. Reactions were carried out in a closed glass vessel thermostated at 37°, and provided with magnetic stirring; reactions were initiated by addition of AAPH (10 mM final concentration) to 1 mL of medium [38] containing 20 mM phosphate buffer, 100 mM NaCl, pH 7.0, and 200 μM membrane phospholipid. Oxygen consumption was calculated assuming an oxygen concentration of 177 nmol/mL in the initial incubation mixture at 37°. The TAM drugs were incorporated in SR membrane suspensions for 20 min at 37° before starting the oxidative stress.

Blank experiments in the absence of SR membranes were performed to evaluate the oxygen consumption rate induced by 10 mM AAPH itself. To obviate misinterpretations, the drug effects are expressed as rates of 0_2 consumption and compared to control results (absence of drugs).

It should be pointed out that the efficiency of peroxyl radical production by AMVN is relatively low when compared with AAPH. Therefore, a more sensitive methodology to follow peroxidation kinetics induced by AMVN is required. Thus, parinaric acid methodology has been used to evaluate the kinetics of peroxidation induced by AMVN, owing to its higher sensitivity whereas the peroxidation initiated by AAPH was evaluated by oxygen uptake. Moreover, we found a good correlation between the parinaric acid and the oxygen consumption assays in the evaluation of antioxidant activity of several compounds [38].

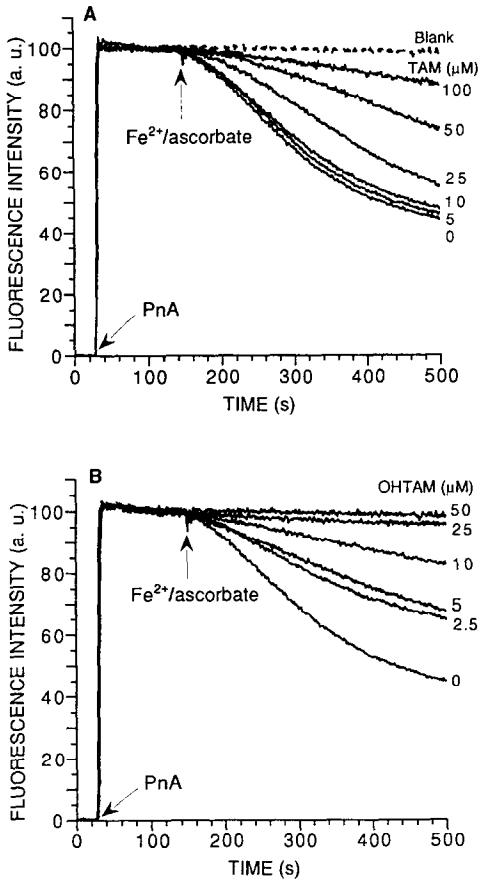


Fig. 2. Effect of TAM (A) and OHTAM (B) as protectors against the peroxidative degradation of *cis*-parinaric acid (PnA) incorporated into SR membranes. The fluorescence decay of PnA, after addition of Fe^{2+}/ascorbic acid system, was kinetically monitored at 324 nm excitation and 413 nm emission with a 3 and 3.5 nm slit, respectively. The dashed lines show the spontaneous fluorescence decay of PnA at 25° (line marked blank). The recordings are typical assays of three independent experiments.

RESULTS

Effects of TAM and OHTAM on peroxidation induced by Fe^{2+}*/ascorbate*

PnA as a polyunsaturated fatty acid has been used as a sensitive fluorescent probe to follow the initial stages of lipid peroxidation in several membrane systems [35, 39, 40] and low density lipoproteins [38]. More recently, PnA has been used to determine the susceptibility of SR membranes to lipid

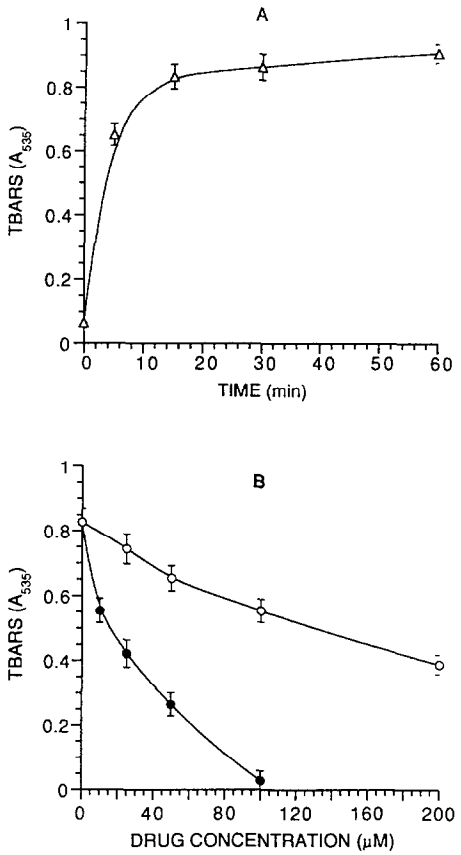


Fig. 3. TBARS formation in SR membranes (2 mg protein/mL) incubated with Fe^{2+}/ascorbic acid (20 μM/100 μM) in the absence of drugs at 25°, as a function of peroxidation time (A). Inhibition of lipid peroxidation by TAM (○) and OHTAM (●) as a function of drug concentration in SR membrane suspensions, as followed by the production of TBARS measured after 15 min of peroxidation and expressed as absorbance at 535 nm (B). Data are means of triplicate determinations.

peroxidation and to study the effects of drugs of pharmacological interest on membrane peroxidation [27, 34].

The effect of the anticancer drug TAM and of its active metabolite OHTAM on lipid peroxidation of SR membranes, as monitored by the fluorescence intensity decrease of PnA, is shown in Fig. 2. In the absence of drugs (control), the addition of Fe^{2+}/ascorbate to membrane suspensions induces a decrease of PnA fluorescence intensity. Under these assay conditions, the rate of PnA fluorescence decay parallels the rate of its oxidative degradation [27]. TAM and OHTAM effectively protect against the oxidative degradation of PnA incorporated into native SR membranes (Fig. 2) in a concentration-dependent way. It is clear that 25 μM OHTAM almost completely prevents the oxidation of PnA (Fig. 2B), whereas TAM is less effective at 100 μM (Fig. 2A), suggesting that OHTAM is significantly more efficient as an antioxidant than TAM.

The antioxidant effect of TAM and OHTAM in the membrane lipoperoxidation was also monitored by the production of TBARS. Lipid peroxidation is a free radical-mediated chain reaction occurring in the unsaturated fatty acids of membrane phospholipids which form lipid hydroperoxides [41]. These are further degraded into mixtures of secondary products (TBARS), which have been evaluated by the TBA assay to estimate the oxidative damage in biological systems [42].

Figure 3A shows that the time course of the accumulation of peroxidation products (TBARS) in native SR membranes by radical generation from the Fe^{2+}/ascorbic acid system, reaches an apparent maximum after 15 min of peroxidation, remaining almost constant thereafter. The amount of TBARS was negligible when Fe^{2+}/ascorbic acid was omitted in either the absence or the presence of drugs. Fig. 3B displays the inhibition of membrane lipid peroxidation by TAM and OHTAM at 15 min of peroxidation as a function of drug concentration. Although either TAM or OHTAM protect against the oxidative degradation of membrane lipids, OHTAM exhibits a significantly higher protection, as previously described [18]. Whereas 25 μM OHTAM decreases the amount of TBARS by about 50%, the same concentration of TAM exerts an effect of 10%. However, the TBA assay is known to be prone to errors, and lacking in sensitivity and specificity [20]. Therefore, possible artifacts and misinterpretations were ruled out by evaluating the degradation of fatty acyl chains of phospholipids monitored by gas–liquid chromatographic analysis, a conventional and specific technique for monitoring the oxidative degradation process [43].

Incubation of membrane suspensions for 15 min in the presence of Fe^{2+}/ascorbic acid at 25° decreases the relative amounts of PUFA with a consequent increase in the amount of saturated and mono-unsaturated fatty acids. The most significant changes were observed for PUFA with three or more double bonds: linolenic (18:3), arachidonic (20:4), eicosopentaenoic (20:5), docosopentaenoic (22:5) and docosohexaenoic (22:6) acids (Table 1). The less unsaturated acids, linoleic (18:2) and eicosodienoic (20:2), are not degraded, probably owing to the mild peroxidation conditions used in our study, according to previous published data [27].

The incorporation of 50 μM and 100 μM of TAM and OHTAM in SR membranes significantly decreases the oxidative degradation of PUFA in a concentration-dependent way (Table 1). Although both drugs significantly inhibit the membrane lipid peroxidation, OHTAM has a stronger antioxidant effect when compared with TAM. Concentrations of OHTAM (50 to 100 μM) in the range of those estimated in human peripheral tissues [24, 26, 44], almost completely suppress the peroxidative degradation of polyunsaturated fatty acyl chains in SR membranes. These results reasonably agree with data of PnA and TBA-assays and correlate with the different intramembraneous concentrations of these drugs [26]. Moreover, the different effects of the two drugs on lipid peroxidation may be a consequence of multiple factors, such as partitioning, localization across the bilayer thickness and H donation of the phenolic group of OHTAM (Fig. 1).

Table 1. Phospholipid fatty acyl composition of SR membranes (control), expressed as % of total fatty chains analysed, and after 15 min of peroxidation induced by Fe^{2+}/ascorbic acid at 25°, in the absence and in the presence of 50 μM and 100 μM TAM or OHTAM, monitored by gas–liquid chromatography

		Peroxidation				
			TAM		OHTAM	
	Control	No drug	50 μM	100 μM	50 μM	100 μM
16:0*ald*	5.31 ± 0.26	5.23 ± 0.05	5.03 ± 0.02	4.94 ± 0.18	5.71 ± 0.18	5.51 ± 0.06
16:0	26.47 ± 0.38	30.29 ± 0.21	29.78 ± 0.26	29.52 ± 0.09	27.12 ± 0.44	26.47 ± 0.04
18:0*ald*	2.11 ± 0.02	2.25 ± 0.07	1.97 ± 0.03	2.35 ± 0.18	2.44 ± 0.04	2.29 ± 0.05
18:0	9.40 ± 0.11	10.38 ± 0.07	10.43 ± 0.07	9.99 ± 0.12	9.46 ± 0.18	9.21 ± 0.14
18:1	16.16 ± 0.10	18.22 ± 0.08	18.00 ± 0.05	17.59 ± 0.04	16.58 ± 0.07	16.26 ± 0.04
18:2	21.98 ± 0.27	21.51 ± 0.11	21.93 ± 0.11	21.92 ± 0.16	21.85 ± 0.07	21.96 ± 0.08
18:3*	0.92 ± 0.04	0.66 ± 0.01	0.72 ± 0.03	0.72 ± 0.04	0.84 ± 0.03	0.84 ± 0.02
20:2	0.79 ± 0.16	0.94 ± 0.11	0.88 ± 0.06	0.82 ± 0.03	0.83 ± 0.01	0.90 ± 0.03
20:4*	10.83 ± 0.13	7.30 ± 0.01	7.75 ± 0.09	8.11 ± 0.06	9.56 ± 0.11	10.82 ± 0.05
20:5*	1.06 ± 0.09	0.36 ± 0.01	0.60 ± 0.03	0.74 ± 0.01	0.91 ± 0.04	0.99 ± 0.06
22:5*	3.97 ± 0.19	2.17 ± 0.12	2.50 ± 0.20	2.57 ± 0.18	3.71 ± 0.06	3.88 ± 0.03
22:6*	1.01 ± 0.12	0.37 ± 0.07	0.45 ± 0.15	0.58 ± 0.05	0.71 ± 0.13	0.81 ± 0.09

Values are means ± SD of triplicate determinations. *Fatty acids that undergo more extensive degradation. Designation *ald* states for dimethylacetal chains of plasmalogenic phospholipid species.

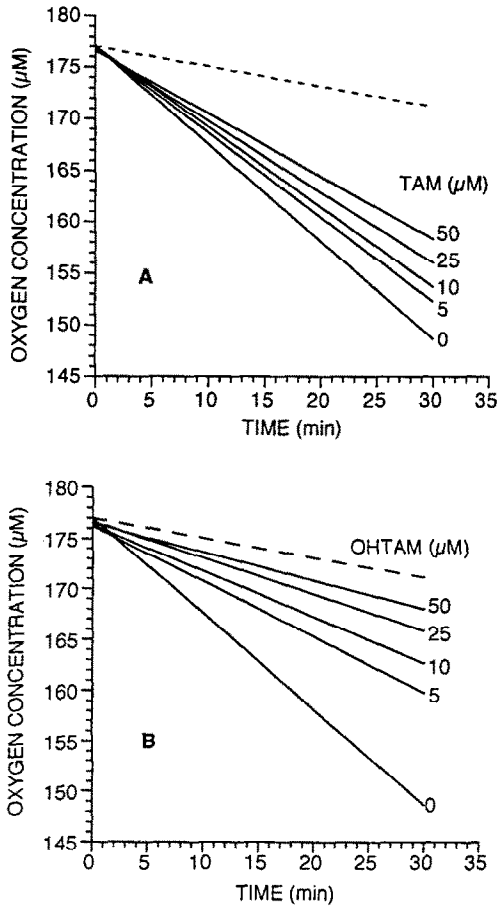


Fig. 4. Rates of oxygen consumption induced by 10 mM AAPH at 37° in the absence (dashed lines) and presence of SR membranes (solid lines), monitored by a Clark-type oxygen electrode and inhibition of oxidation by TAM (A) and OHTAM (B) preincubated with membranes. Negligible effects of drugs on the blanks (dashed lines) are not included. Recordings are typical assays of triplicate experiments.

Interaction of drugs with peroxyl radicals

To evaluate the peroxyl radical scavenging activity of TAM and OHTAM in SR membranes ($ROO^{\cdot}$ from decomposition of azocompounds or from lipid degradation), lipid peroxidation was induced by thermal decomposition of water-soluble (AAPH) and lipid-soluble (AMVN) azo-initiators [28], and the effects were followed by oxygen consumption and PnA fluorescence intensity decrease, as described for other compounds of pharmacological interest [34, 35, 45]. The addition of water-soluble AAPH generates free radicals in the aqueous phase outside SR vesicles, whereas lipid-soluble AMVN incorporated in liposomes generates free radicals within the lipid bilayer [28, 46].

Exposure of SR membranes to AAPH-derived radicals results in lipidic peroxyl radical production from membrane peroxidation as indicated by the increased rate of oxygen consumption (0.94 nmol/mL/min) as compared with blank experiment (0.19 nmol/mL/min) (Fig. 4). When TAM or OHTAM are preincubated with SR membranes, the rate of oxygen uptake is progressively and linearly depressed. The rates of oxygen consumption in the presence of 5 μM and 25 μM OHTAM are about 0.54 and 0.36 nmol/mL/min, respectively, whereas in the presence of identical concentrations of TAM the rates of oxygen uptake are, respectively, 0.81 and 0.69 nmol/mL/min (Fig. 4). Again, OHTAM proved to be a stronger scavenger of peroxyl radicals generated by AAPH than TAM.

The inhibition of lipid oxidation by a chain-breaking antioxidant results in a clear induction (or inhibition) period during which the antioxidant is consumed at a constant rate. When depleted, the inhibition period ends and a fast oxidation proceeds at the same rate as in the absence of the antioxidant [28, 47]. TAM and OHTAM do not induce an initial inhibition period characteristic of typical chain-breaking antioxidants, as indicated by the linear

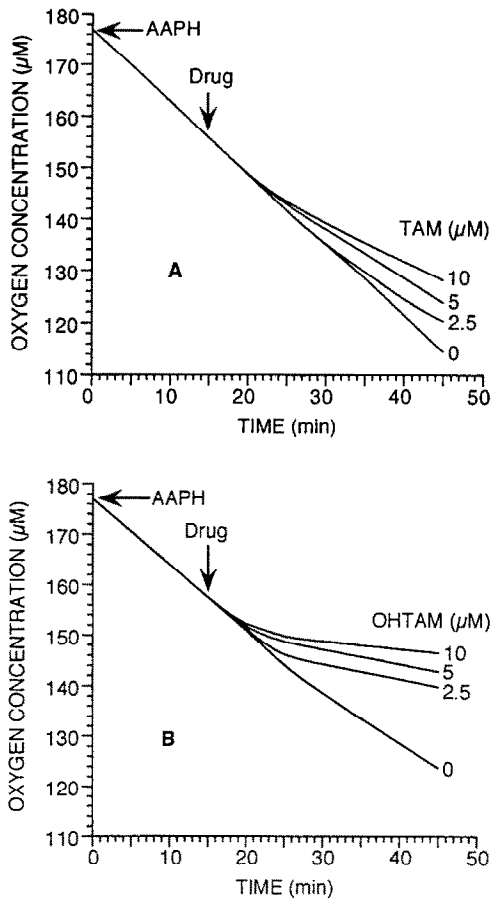


Fig. 5. Inhibitory effects of TAM (A) and OHTAM (B) on the propagation rate of peroxidation in SR membranes induced by 10 mM AAPH at 37°, evaluated by oxygen consumption. TAM and OHTAM were added to the membrane suspensions, from ethanolic solutions at the concentrations indicated by the numbers adjacent to the traces, 15 min after the initiation of peroxidation by thermal decomposition of AAPH, as indicated by the arrows. The recordings are typical results of three assays.

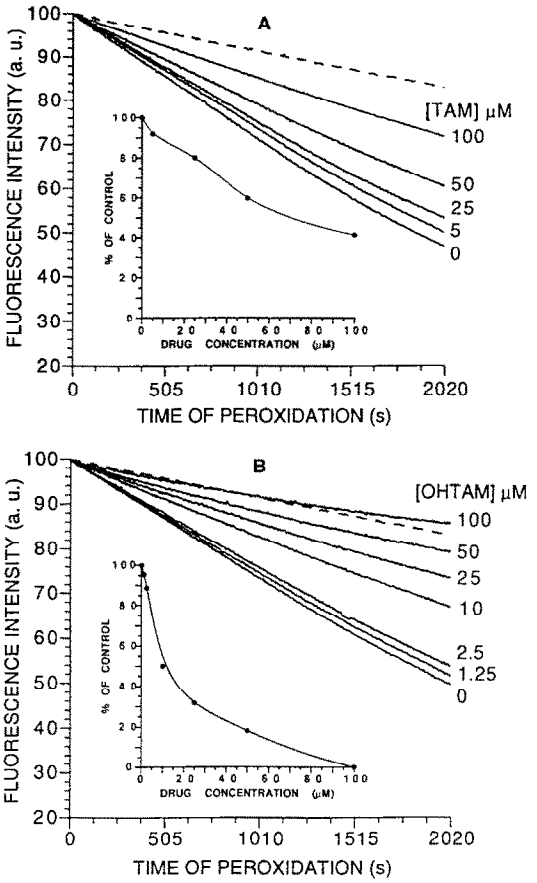


Fig. 6. Inhibition of AMVN-induced PnA fluorescence decay in liposomes of SR lipids by TAM (A) and OHTAM (B). The reaction mixture contained 7.5 μM PnA, 330 μM AMVN and 800 μM SR phospholipid in 20 mM Tris–HCl buffer, pH 7.4, at 50°. Insets depict the inhibition of PnA fluorescence decay promoted by TAM (inset A) and OHTAM (inset B) as a function of concentration, expressed as % in the absence of drugs (control), at 20 min of peroxidation. The dashed lines show the spontaneous fluorescence decay of PnA at 50°. TAM drugs have negligible effects on these blank experiments.

slopes of oxygen uptake (Fig. 4). Therefore, although these drugs are strong scavengers of peroxyl radicals generated in the aqueous phase by AAPH, they do not exhibit typical chain-breaking antioxidant activities.

When TAM or OHTAM is added 15 min after the initiation of peroxidation with AAPH (Fig. 5), there is an inert period followed by partial inhibition indicated by the decreased slope, as a function of drug concentration. The inert period correlates reasonably with the kinetics of drug incorporation since maximal partition takes about 10 min [26]. Therefore, the capacity of drugs to scavenge peroxyl radicals is achieved within the membrane.

The effects of TAM on O_2 consumption are identical when the drug was previously incubated with SR membranes (Fig. 4) or added after 15 min of peroxidation (Fig. 5), i.e., 10 μM TAM inhibits, respectively, 25% and 26% the O_2 consumption relative to the control at 20 min of peroxidation. However, the effects of OHTAM reported in Fig. 5 are significantly higher compared with those of Fig. 4 when the drug was previously incorporated in the membrane phase, i.e., 10 μM OHTAM inhibits, respectively 62% and 45% the O_2 uptake. As shown in Fig. 5, OHTAM added to the aqueous phase is likely to directly scavenge the radicals generated in this phase by thermal decomposition of AAPH since O_2 consumption decreases 2–5 min after OHTAM addition, i.e. a period shorter than the time required for drug incorporation. However, a more efficient scavenger capacity of peroxyl radicals is observed in the lipid phase.

Since no lag phase of oxygen consumption is induced by these drugs during the initiation reaction by AAPH (Fig. 5), it can be concluded that tamoxifen

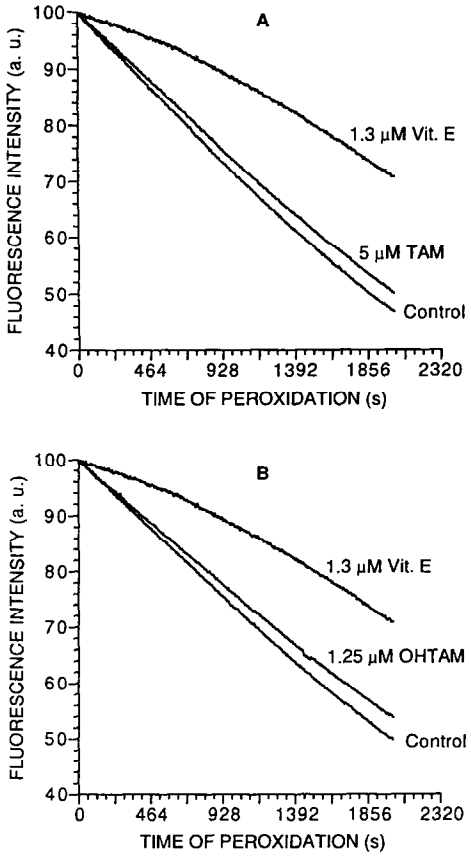


Fig. 7. Typical assays of peroxidation of PnA incorporated into SR liposomes induced by AMVN as indicated for Fig. 6. Inhibition of oxidation by TAM (A) and OHTAM (B) preincubated with lipid suspensions for 20 min before starting the oxidative procedure, and by vitamin E incorporated into the membranes. Whereas vitamin E induces a biphasic response, i.e. an inhibition period of PnA degradation rate followed by a faster rate identical to that observed in the absence of antioxidant, the oxidation reaction in the presence of TAM and OHTAM proceeds smoothly without any induction period.

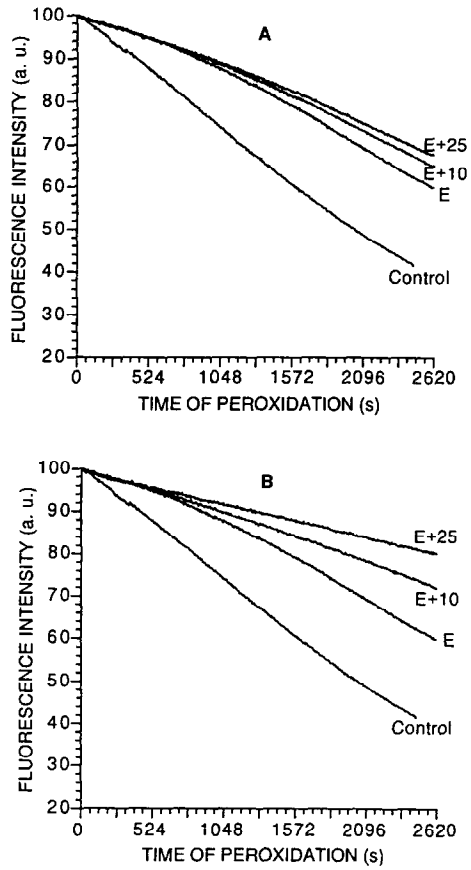


Fig. 8. Inhibition of peroxidative degradation of PnA incorporated into SR liposomes by 1.3 μM vitamin E and its combination with TAM (A) or OHTAM (B). The PnA oxidation was initiated as indicated for Fig. 6. Control represents the PnA fluorescence decay in liposomes without vitamin E or drugs; PnA fluorescence decay inhibition is shown in liposomes containing 1.3 μM vitamin E alone (E) or supplemented with 10 μM (E + 10) and 25 μM (E + 25) of TAM (A) or OHTAM (B). The kinetic traces represent typical experiments of four independent assays.

drugs do not act as a typical chain-breaking inhibitors of lipid peroxidation, as also demonstrated by the above experiments (Fig. 4).

The antioxidant effects of TAM and OHTAM as powerful scavengers of peroxyl radicals into the membranous phase were confirmed by using AMVN as peroxyl radicals generator within the membrane phase of liposomes prepared from SR lipids [28]. The fluorescence of incorporated PnA drops following the addition of AMVN (Fig. 6). Upon incorporation of TAM (Fig. 6A) or OHTAM (Fig. 6B), the AMVN-induced rate of peroxidative degradation decreases as a function of drug concentration, as indicated by a decreased slope of fluorescence quenching. Again, OHTAM proved to be a more efficient intramembraneous scavenger of peroxyl radicals than TAM, as with the results observed when AAPH was used as a radical initiator. The scavenger effect of OHTAM can be described by an IC_{50} value of 10 μM, whereas IC_{50} for TAM is 70 μM, calculated by plotting the % of PnA fluorescence intensity relative to that in the absence of drugs (control), as a function of drug concentration (Fig. 6, insets).

TAM and OHTAM as radical scavengers were compared with the hydrophobic antioxidant vitamin E, as shown in Fig. 7. The incorporation of vitamin E in SR liposomes markedly suppresses the lipid oxidation promoted by AMVN, in agreement with previous studies [28, 47, 48], inducing an inhibition period after which the oxidation proceeds at a similar rate to that without inhibitor, characteristic of a chain-breaking antioxidant. Conversely to the observations with vitamin E, no initial inhibition period was induced by TAM or OHTAM alone (Fig. 7), in agreement with oxygen consumption data (Figs. 4 and 5), suggesting that these antiestrogens do not act as typical chain-breaking inhibitors of lipid peroxidation, contrarily to previous suggestions

[18]. However, the antioxidant activity of the lipid-soluble vitamin E was enhanced by the addition of TAM or OHTAM (Fig. 8) and the rate of oxidation decreased with drug concentration. Therefore, these drugs are strong hydrophobic antioxidants acting as peroxyl radical scavengers generated both in the aqueous and in the membrane phase, thus decreasing the free radical-mediated peroxidation of lipids in membranes.

DISCUSSION

The antiestrogenic drugs TAM and OHTAM are efficient inhibitors of lipid peroxidation, OHTAM being more powerful than TAM, in agreement with the relative membrane partitioning [26] and the potential H donation by OHTAM due to the presence of a hydrogen-donating HO-group in its molecule. Earlier studies proposed that their antioxidant effects could involve alterations in the biophysical properties of membranes in a similar mechanism to the cholesterol stabilizing effect [19, 22, 23]. However, our previous studies indicate that TAM, distributed mainly in the hydrophobic core of the lipid bilayer [24], reduces the membrane fluidity to a greater extent than OHTAM, which preferentially locates in the outer regions of the bilayer [25]. Therefore, the lesser membrane rigidification effect of OHTAM does not explain its more potent antioxidant capacity.

Our data clearly indicate that these anticancer drugs efficiently inhibit lipid peroxidation induced by the Fe^{2+}/ascorbate system, as monitored by the fluorescence quenching of PnA, production of TBARS and degradation of PUFA. Additionally, results with AAPH- and AMVN-derived peroxyl radicals, in the systems where neither transition metals nor reductants were involved, demonstrated that both drugs are strong scavengers of peroxyl radicals, inhibiting the lipid peroxidation in a similar way to that observed with the Fe^{2+}/ascorbate system. Therefore, peroxyl radical scavenging contributes strongly to the overall antioxidant capacity of TAM and OHTAM to inhibit the lipid peroxidation induced by these different systems.

The higher scavenging efficiency of OHTAM, as compared with TAM, was found to occur for peroxyl radicals generated both in the aqueous phase and in the hydrophobic domain of the lipid bilayer, when evaluated by oxygen consumption or by oxidation of the highly hydrophobic substrate PnA. PnA probably locates closer to the polar head group interface of the lipid bilayer with the carboxylate group exposed to the aqueous phase, i.e. regions where OHTAM is also preferentially located [25]. OHTAM, as demonstrated for other phenolic compounds [12], may donate a hydrogen atom of its phenolic HO-group or electrons to quench free radicals and its location in the outer regions of the bilayer enables it to scavenge species capable of abstracting hydrogen atoms, inhibiting the peroxidative degradation of PnA to a greater extent than TAM. This drug preferentially locates in the hydrophobic core of the lipid bilayer and apparently does not contain H donating groups. Its chemical nature, in addition to its distribution in the bilayer core and lower partition coefficient in biomembranes, explains its weaker antioxidant activity as compared with OHTAM, since the relative antioxidant activity will depend on the local concentration, the site of radical production and reactivity towards free radicals [28].

Moreover, the capacity of TAM and OHTAM to inhibit lipid peroxidation may also be due to their ability to chelate transition metals (Fe^{2+}/Fe^{3+}) involved in the peroxidation initiation, and/or to interact with reductants (e.g. ascorbate) necessary for maintaining transition metals in the reduced state. Additionally, the phenolic HO-group of OHTAM may confer capacity to scavenge hydroxyl radicals ($^{\cdot}OH$) generated by Fe^{2+}/ascorbate [49] or peroxyl radicals resulting from lipid peroxidation.

Our findings also indicate that both cytostatics are efficient intramembranous scavengers of peroxyl radicals generated in water or lipid phases, without a typical chain-breaking antioxidant activity, since in the presence of these drugs the oxidation proceeds smoothly without any inhibition period (Figs. 4, 5 and 7). The antioxidant effectiveness of these antiestrogens enhances the antioxidant capacity of vitamin E, presumably by regeneration through its radical reduction or by preventing attack from reactive oxygen species. However, further studies on the reactivity and production of the intermediates, or molecular products by interactions with oxidizing species, are required to better elucidate these mechanisms. The association of these drugs with vitamin E suggests that the therapeutic antioxidant potential of these cytostatics may be increased by association with other antioxidants.

The free radicals and other oxygen reactive species may oxidize DNA bases, proteins, cell antioxidant defences and cell membranes through a chain reaction, a characteristic of cancer process initiation by oxyradicals and other carcinogens [50]. Therefore, an apparent relationship between lipid peroxidation and potential risk of later-developing breast cancer is possible [6]. Consequently, TAM may prevent initiation and promotion of breast cancer by scavenging mechanisms of free radicals and the stronger scavenger antioxidant capacity of its more active metabolite, OHTAM, may contribute to the anticancer and chemopreventive action of TAM on development of breast cancer.

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