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Hemolysis of human erythrocytes induced by tamoxifen is related to disruption of membrane structure

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Abstract

Tamoxifen (TAM), the antiestrogenic drug most widely prescribed in the chemotherapy of breast cancer, induces changes in normal discoid shape of erythrocytes and hemolytic anemia. This work evaluates the effects of TAM on isolated human erythrocytes, attempting to identify the underlying mechanisms on TAM-induced hemolytic anemia and the involvement of biomembranes in its cytostatic action mechanisms. TAM induces hemolysis of erythrocytes as a function of concentration. The extension of hemolysis is variable with erythrocyte samples, but 12.5 μM TAM induces total hemolysis of all tested suspensions. Despite inducing extensive erythrocyte lysis, TAM does not shift the osmotic fragility curves of erythrocytes. The hemolytic effect of TAM is prevented by low concentrations of α-tocopherol (α-T) and α-tocopherol acetate (α-TAc) (inactivated functional hydroxyl) indicating that TAM-induced hemolysis is not related to oxidative membrane damage. This was further evidenced by absence of oxygen consumption and hemoglobin oxidation both determined in parallel with TAM-induced hemolysis. Furthermore, it was observed that TAM inhibits the peroxidation of human erythrocytes induced by AAPH, thus ruling out TAM-induced cell oxidative stress. Hemolysis caused by TAM was not preceded by the leakage of K^+ from the cells, also excluding a colloid-osmotic type mechanism of hemolysis, according to the effects on osmotic fragility curves. However, TAM induces release of peripheral proteins of membrane-cytoskeleton and cytosol proteins essentially bound to band 3. Either α-T or α-TAc increases membrane packing and prevents TAM partition into model membranes. These effects suggest that the protection from hemolysis by tocopherols is related to a decreased TAM incorporation in condensed membranes and the structural damage of the erythrocyte membrane is consequently avoided. Therefore, TAM-induced hemolysis results from a structural perturbation of red cell membrane, leading to changes in the framework of the erythrocyte membrane and its cytoskeleton caused by its high partition in the membrane. These defects explain the abnormal erythrocyte shape and decreased mechanical stability promoted by TAM, resulting in hemolytic anemia. Additionally, since membrane leakage is a final stage of cytotoxicity, the disruption of the structural characteristics of biomembranes by TAM may contribute to the multiple mechanisms of its anticancer action. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Tamoxifen; Human erythrocyte; Hemolysis; Oxidative stress; Partition coefficient; Membrane disruption

Abbreviations: α-T, α-tocopherol; α-TAc, α-tocopherol acetate; AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; DMPC, dimyristoylphosphatidylcholine; DPH-PA, 3-[*p*-(6-phenyl)-1,3,5-hexatrienil]-phenylpropionic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; ER, estrogen receptor; HEPES, 4-(2-hydroxyethyl)-1-piperizine-ethanesulfonic acid; K_p, partition coefficient; PnA, *cis*-parinaric acid; TAM, tamoxifen; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TEMED, *N*,*N*,*N*′,*N*′-tetramethyl-ethylendiamine

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1. Introduction

Tamoxifen (TAM) is a synthetic non-steroidal antiestrogen widely used in the chemotherapy of breast cancer [1]. The established efficiency and low incidence of side effects allowed the use of TAM as a chemopreventive drug [2]. Recently, it was reported that TAM induces multiple cellular adverse effects, including changes in normal discoid shape of erythrocytes with formation of stomatocytes, meaning that the drug may insert in the inner leaflet of the erythrocyte membrane [3]. Additionally, it has been suggested that TAM causes hemolytic anemia [4,5], but the biochemical mechanisms were not identified.

The antiproliferative effects of TAM and other triphenylethylene derivatives in estrogen-dependent breast cancer cells are believed to be mediated by high affinity binding to the estrogen receptor (ER), thereby blocking the growth stimulating action of estradiol [6]. However, TAM also inhibits the growth of ER-negative breast cancer cells and other cell types which lack ER [7–10]. Furthermore, in some ER-positive and ER-negative cell lines, the antiproliferative effect of TAM is not overcome by estrogen addition [8,11]. Moreover, the mechanisms underlying the ER-independent inhibition of tumor cell growth by TAM also remain obscure. Thus, extensive studies have been performed and multiple cellular effects have been described, namely, binding to antiestrogen binding sites [12,13], inhibition of protein kinase C [14,15], inhibition of a nucleoside transporter protein [16], inhibition of cAMP phosphodiesterase [17,18], antagonism of calmodulin by a direct interaction with this protein [19] and induction of apoptosis [20,21].

Biomembranes, where TAM strongly incorporates [22] interacting with lipids [23] and proteins [19,24], have been suggested as a potential target for TAM action [25]. Indeed, TAM induces early modifications in the morphology and structure of breast tumor cell membranes putatively related with its antiproliferative activity [26]. Moreover, TAM has been reported to disrupt unspecifically the structure of model membranes [24,27] and the permeabilization of the mitochondria and the parallel loss of membrane potential induced by TAM has been assigned to a structural disruption of the mitochondrial membrane [28].

At present, effects of TAM on erythrocyte membrane integrity have not been reported. On the other hand, it has been ascertained that free radical-mediated peroxidation of erythrocyte membrane lipids and proteins and changes in cytoskeleton proteins eventually promote hemolysis. Therefore, the reports of hemolytic anemia caused by TAM alone [4] or in combination with mitomycin C, not related to bone marrow toxicity [5], prompted us to search the effects of TAM on isolated human erythrocytes, particularly on the potential oxidative disruption and physical damage of cell membrane and the protection afforded by antioxidants or membrane stabilizers, e.g. α-tocopherol (α-T) and α-tocopherol acetate (α-TAc). Thus, the aim of these studies was to contribute for the elucidation of the underlying mechanisms of TAM-induced hemolytic anemia and to clarify the role of biomembranes in its cytostatic action mechanisms.

2. Materials and methods

2.1. Chemicals

Tamoxifen, 1,6-diphenyl-1,3,5-hexatriene (DPH), HEPES, acrylamide, Coomassie brilliant blue R-250, α-tocopherol acetate (α-TAc) and protein markers for molecular weight were obtained from Sigma (St. Louis, MO, USA). 9,11,13,15-Octadecatetraenoic acid (*cis*-parinaric acid) (PnA) and 3-[*p*-(6-phenyl)-1,3,5-hexatrienil]-phenylpropionic acid (DPH-PA) were from Molecular Probes (Junction City, OR). 2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) was obtained from Polysciences (Warrington, PA). α-Tocopherol (α-T) was purchased from Fluka, Switzerland. Bisacrylamide, TEMED, ammonium persulfate and glycine were from Merck (Darmstadt). All the other chemicals were of research grade. Solutions were prepared in deionized ultra pure water.

2.2. Preparation of erythrocyte suspensions

Freshly collected human blood, obtained from

12 healthy donors (25–50 years old and not submitted to any drug treatment) who are working in our laboratory, was mixed with heparin and centrifuged at 3000 rpm/10 min to separate plasma and erythrocytes. The retrieved erythrocytes were washed three times with 6 vols. of the isotonic phosphate buffered saline (PBS) (150 mM NaCl, 10 mM sodium phosphate, pH 7.4). The buffy coat was carefully removed with each wash. After the last washing, the packed cells were re-suspended in PBS and the hematocrit determined. The erythrocyte suspensions used in experiments were prepared daily.

2.3. Osmotic fragility and hemolysis measurements

The osmotic fragility assays were performed in phosphate buffer solutions (10 mM sodium phosphate, pH 7.4) containing increasing concentrations of NaCl and hematocrit of 0.33%. The hemolysis experiments were carried out in erythrocyte suspensions (4 ml) in PBS with hematocrit of 0.33%. The protective effects of α-T and α-TAc were assessed by preincubating the erythrocyte suspensions with these tocopherols at 37°C/2 h before TAM addition. In a set of samples, the erythrocytes preincubated with α-T and α-TAc were washed three times with 10 ml of PBS before the addition of TAM.

TAM was added from a stock ethanolic solution (2–4 μl) and incubated with erythrocyte suspensions at 37°C for 1 h in a shaking water bath with gentle magnetic stirring. After incubation, the erythrocyte suspensions were centrifuged at 3000 rpm/10 min and hemolysis was estimated from the 540 nm absorbance of hemoglobin released into the supernatant. The results were expressed as % hemolysis. Zero hemolysis was taken as the absorbance of the supernatant of erythrocyte suspensions in isotonic phosphate buffer in the absence of TAM and 100% hemolysis was assigned when buffer was replaced by water, containing an identical volume of ethanol to that used in the experiments with TAM.

The efflux of K^+ from the erythrocyte suspensions was measured with a K^+ ion-selective electrode made in our laboratory, as described elsewhere [29]. The total amount of K^+ was determined by the addition of 0.5% Triton X-100 to the erythrocyte suspensions.

The recordings are typical assays or represent the mean ± S.D. of three independent experiments.

2.4. Evaluation of oxidative stress in erythrocytes

The putative peroxidation occurring during hemolysis induced by 12.5 μM TAM and the peroxidative degradation of the lysate induced by 20 mM AAPH were followed by O_2 consumption and by changes in the hemoglobin absorption spectrum.

The rate of O_2 consumption was monitored using a Clark-type oxygen electrode (YSI model 5331, Yellow Spring Institute) as previously described [30]. The reactions were carried out in a closed glass vessel thermostated at 37°C, provided with magnetic stirring and the hemolysis of erythrocyte suspension (0.33% hematocrit) was induced by addition of 12.5 μM TAM. After 60 min of TAM addition, the azoinitiator AAPH (20 mM) was added and O_2 consumption was followed for another period of 60 min. Oxygen consumption was calculated assuming an O_2 concentration of 177 nmol/ml at 37°C.

During the incubation of erythrocyte suspensions with 12.5 μM TAM and also after addition of 20 mM AAPH, aliquots of the suspension were taken up, centrifuged at 3000 rpm/5 min and the hemoglobin spectrum was analyzed in the supernatant. Withdrawn aliquots were chilled before centrifugation to stop the thermal decomposition reaction of the azoinitiator. Hemoglobin oxidation was followed by the decay of the absorption spectrum at 450–650 nm using a Perkin-Elmer Lambda 6 UV/VIS spectrophotometer (Norwalk, USA).

The oxidation of 1.5 μM PnA incorporated in the membrane of intact erythrocytes (0.05% hematocrit) was induced by 1 mM AAPH at 37°C. TAM (10 μM) was preincubated with the erythrocyte suspension at 37°C/2 min before addition of PnA. After 90 s of recording the basal signal, PnA was added and fluorescence intensity was monitored using a Perkin-Elmer LS-50 spectrofluorimeter provided with thermostated cuvettes and magnetic stirring. The excitation was set at 312 nm and the emission at 450 nm. The excitation and emission slit widths were 5 and 15 nm, respectively. Experiments of spontaneous fluorescence decay of PnA (blank) and in the absence of drug (control) were performed. The experimental

conditions used were previously established to ascertain that the fluorescence intensity of incorporated PnA is linear with its concentration and that the hematocrit is high enough to incorporate most of the probe. The excitation and emission conditions used minimize the fluorescence quenching promoted by hemoglobin.

2.5. Partition coefficient and fluorescence polarization

The partition coefficient (K_p) of TAM was determined by means of second derivative UV spectrophotometry, enabling the quantification of the drug in the lipid phase as previously described [22]. DMPC liposomes and liposomes containing α-T or α-TAc were prepared, after solvent evaporation to dryness, by dispersion in 50 mM NaCl, 10 mM HEPES, pH 7.4. The lipid suspensions were obtained as described elsewhere [22], except that liposomes were vortexed and sonicated during four bursts of 2 min and left to equilibrate overnight at 4°C before use.

The fluorescent probes of membrane fluidity DPH and DPH-PA were incorporated at 37°C/3 h into DMPC liposomes (345 μM in phospholipid) as described elsewhere [23]. Liposomes without or containing α-T and α-TAc were prepared as described above by dispersion in 50 mM KCl and 10 mM Tris-maleate, pH 7 [31]. The lipid/probe molar ratio was about 400. The fluorimetric measurements were carried out in a Perkin-Elmer LS-50 spectrofluorimeter (Beaconsfield, UK) and the excitation was set at 336 nm and the emission at 450 nm, being the band widths 3 and 4 nm, respectively. The degree of fluorescence polarization (P) was determined as reported elsewhere [32]. Adequate control experiments were carried out without added probes to correct for the contribution of light scattering.

TAM was incubated with liposomes at 37°C for 20 min before K_p and P measurements. The values are the mean of three independent experiments.

2.6. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide slab gels were prepared according to the procedure of Laemmli [33]. The slab gel consisted of a 5% polyacrylamide stacking gel and a 10% polyacrylamide separating gel.

Ghost membranes were prepared from erythrocyte suspensions lysed at 37°C/1 h in absence (control) and presence of 12.5 μM TAM and centrifuged at 15 000 rpm/20 min. Hemolysis in control assay was carried out in hypotonic buffer. In either case, the erythrocyte membranes were washed twice with 20 ml of hypotonic phosphate buffer and centrifuged at 19 000 rpm/20 min. The protein of ghosts re-suspended in PBS was estimated by the biuret method [34] in the presence of SDS, using bovine serum albumin as standard and adjusted at 1.5 mg/ml. Slab gels were stained with Coomassie brilliant blue R-250 and destained with a mixture of 25% methanol and 5% acetic acid.

3. Results and discussion

3.1. TAM effects on osmotic fragility and hemolysis of human erythrocytes

The interactions of TAM with biomembranes have been reported to contribute to its multiple cellular effects [23,24,27]. Recently, we suggested that mitochondrial swelling and membrane potential depolarization induced by TAM reflect a direct action of this drug on the inner membrane structure [28]. There-

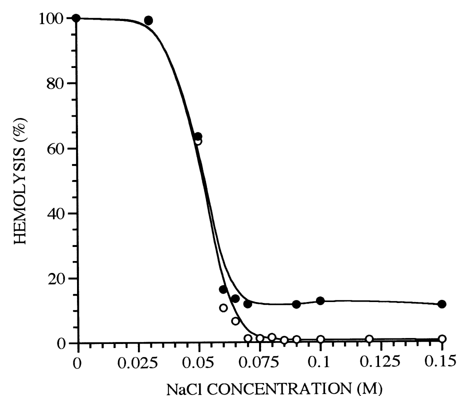

Fig. 1. Effect of TAM on the osmotic fragility of human erythrocytes. Erythrocyte suspensions (0.33% hematocrit) in 10 mM phosphate buffer containing increasing concentrations of NaCl were incubated at 37°C/1 h in the absence (control) (○) and in the presence of 7.5 μM TAM (●). Hemolysis was calculated from the 540 nm absorbance of the supernatant after centrifugation of the erythrocyte suspensions and the results expressed as percentage of total hemolysis.

fore, to confirm the potential relationship between hemolytic anemia and membrane structural defects induced by TAM, the effects of this drug on the osmotic fragility and hemolysis of human erythrocytes were studied.

The effects of TAM on the osmotic fragility, evaluated from the curves of hemolysis as a function of NaCl concentration, are shown in Fig. 1. Incubation of 7.5 μM TAM with the erythrocyte suspensions induces a maximum of 20% hemolysis in the isotonic range. However, TAM does not shift the osmotic fragility curve as compared to erythrocytes incubated in the absence of drug (control), indicating that TAM does not modify the susceptibility of erythrocytes to hypotonic osmotic lysis. Therefore, the hemolytic effect of TAM observed in the isotonic range is not assigned to changes in osmotic membrane permeability, in agreement to K^+ efflux data monitored in parallel to hemoglobin release (Fig. 2). As shown in this figure, the K^+ leakage curve resulting from the incubation of erythrocyte suspensions with 10 μM TAM at 37°C is very close to the hemolysis curve. The similar rates of K^+ and hemoglobin release strongly exclude the lateralization or redistribution of transmembrane proteins into clusters or aggregates induced by TAM, leading to the formation of pores and consequent colloid-osmotic lysis, a mechanism that has been well characterized over the last decade for potent membranolytic agents [35]. Rather, the results support the concept of TAM-induced hemolysis occurring via direct membrane disruption.

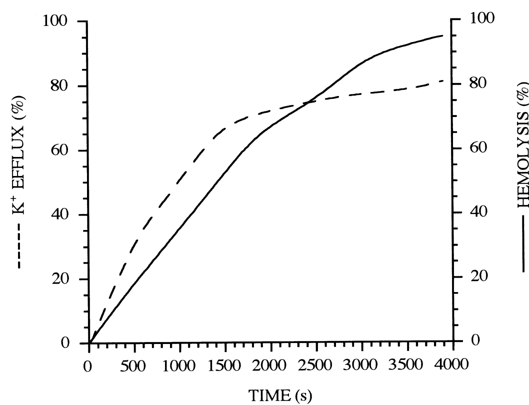

Fig. 2. Time courses of K^+ (- - -) and hemoglobin (—) release from erythrocytes in 150 mM NaCl, 10 mM sodium phosphate, pH 7.4 (0.33% hematocrit), incubated with 10 μM TAM at 37°C. Release of K^+ and hemoglobin were measured with a K^+ ion-selective electrode and by spectrophotometry of the supernatant at 540 nm, respectively. The results are expressed as percentage of total release observed in total hemolysis.

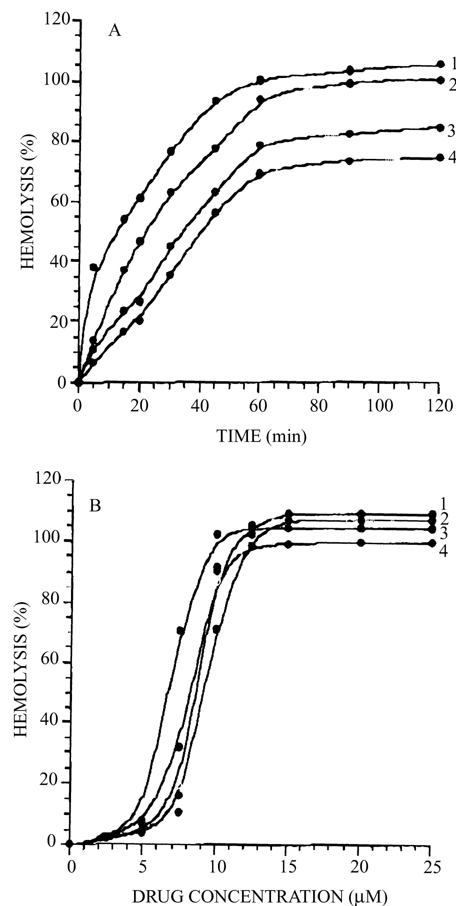

Fig. 3. Hemolytic effect of TAM on human erythrocytes obtained from different donors (lines 1–4) in 150 mM NaCl, 10 mM sodium phosphate, pH 7.4 (0.33% hematocrit). Erythrocyte suspensions were incubated with 10 μM TAM at 37°C as a function of time (A) and at 37°C/1 h as a function of drug concentration (B). Hemolysis was calculated from the 540 nm absorbance of the supernatant after centrifugation of the erythrocyte suspensions and the results expressed as percentage of total hemolysis.

The hemolysis induced by TAM on erythrocytes suspended in isotonic medium (PBS) was studied as a function of incubation time and drug concentration in several erythrocyte suspensions obtained from different donors (Fig. 3, lines 1–4). As shown in Fig. 3A, incubation of red cells with 10 μM TAM at 37°C results in a time-dependent hemolysis and, although the extension of hemolytic effect depends on blood

donor, it reaches a maximum after 1 h of incubation with the drug for all the samples. The hemolysis extension induced by TAM is slower for old subjects (more than 40 years old) (Fig. 3A, lines 3–4), according to other studies pointing that the sensitivity to hemolysis decreases as a function of age while the membrane cholesterol content, that strongly decreases TAM partitioning in biomembranes [22], increases with age. The hemolysis is drug concentration-dependent and complete hemolysis occurs at 12.5 μM TAM incubated at 37°C for 1 h (Fig. 3B), independently of the erythrocyte donor.

It has been reported that TAM partitions strongly in biomembranes [22] and that in humans the levels of TAM are 10–60-fold higher in tissues than in serum (1 μM) [36]. Thus, at high concentrations in vivo, probably the excessive incorporation of TAM may disrupt the erythrocyte membrane and cause extensive hemolysis. Indeed, TAM-induced hemolytic anemia observed in patients under TAM therapy might be consequence of a direct effect on erythrocyte membrane, resulting in the disruption of membrane structure.

To ascertain the potential mechanisms of TAM-induced erythrocyte hemolysis, as putative oxidative disruption and physical perturbation, the effects of antioxidants and membrane stabilizers, e.g. α-T and α-TAc, were studied. The hemolytic effect of TAM is inhibited by low concentrations of α-T previously incorporated into erythrocytes (Fig. 4A). When erythrocytes were washed after preincubation with α-T, the protection against TAM-induced hemolysis is maintained and α-T exhibits nearly the same inhibitory effects as in unwashed erythrocytes. α-T is more effective in suppressing hemolysis than α-TAc since 2 μM α-T inhibits about 80% hemolysis (Fig. 4A) and the same effect is observed only at 15 μM α-TAc (Fig. 4B). After washing of erythrocytes to rule out any interference by free α-TAc, small differences are observed on α-TAc protective effects as compared to unwashed erythrocytes, except for concentrations below 5 μM α-TAc. These observations indicate that α-T, with a selective uptake system in red blood cells [37], and α-TAc incorporate deeply into the erythrocyte membrane and are not significantly released by washing. Therefore, the protective effects of tocopherols against TAM-induced hemolysis probably result from interaction of tocopherols in the membrane core, excluding any surface action. Hence, the hydroxyl group of α-T is not critical for protection of membrane against TAM-induced damage, but rather the chroman ring, according to the effects described elsewhere for inhibition of retinol-induced hemolysis [38].

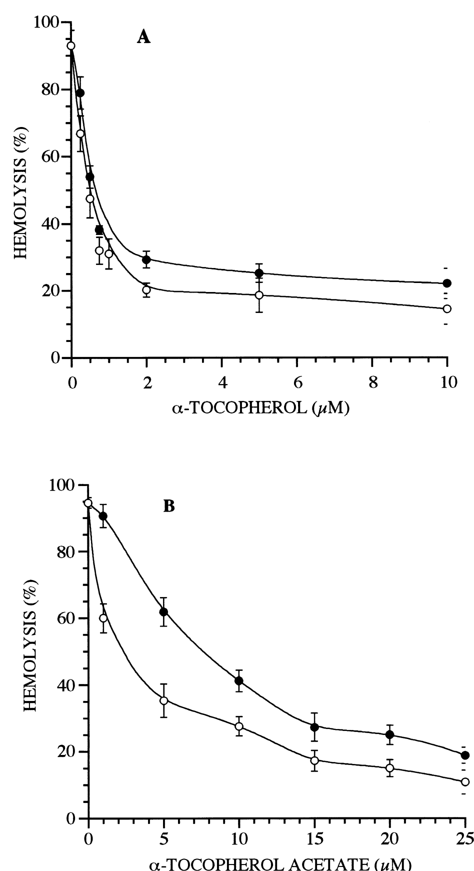

Fig. 4. Inhibition of TAM-induced hemolysis by α-tocopherol (A) and α-tocopherol acetate (B). Erythrocyte suspensions in 150 mM NaCl, 10 mM sodium phosphate, pH 7.4 (0.33% hematocrit) were preincubated with tocopherol compounds at 37°C/2 h and the erythrocytes were washed (●) or not (○) before the addition of 10 μM TAM as indicated in Section 2. The hemolysis extension induced by TAM incubated at 37°C/1 h was measured as described in the legend of Fig. 1. Data represent the mean ± S.D. of three different erythrocyte preparations.

α-T is a widespread naturally occurring compound that acts as the major lipophilic antioxidant in biological systems because of its ability to interrupt the peroxidative process [39]. Moreover, this compound has been shown to inhibit lipid peroxidation in human erythrocyte membranes [40] and to prevent hemolysis induced by oxygen free radicals [41]. Since α-T efficiently inhibits the hemolytic effect of TAM, the

oxidative damage of erythrocytes could in principle be involved in TAM-induced hemolysis. However, TAM is a potent intramembranous inhibitor of lipid peroxidation in model membranes, acting as a peroxyl radical scavenger [30], and an efficient inhibitor of DNA oxidation [42]. Additionally, α-TAc, with the hydroxyl group blocked and hindered of antioxidant activity, also inhibits this hemolytic effect, probably as a consequence of membrane structure stabilization. A major effect of tocopherols is, however, related with withdrawal of TAM from incorporating in the membrane since the partition coefficient is strongly decreased by the tocopherol incorporation occurring at similar putative domains across the membrane structure. Therefore, these observations might suggest that TAM-induced hemolysis is not assigned to an oxidative disruption of the erythrocyte membrane.

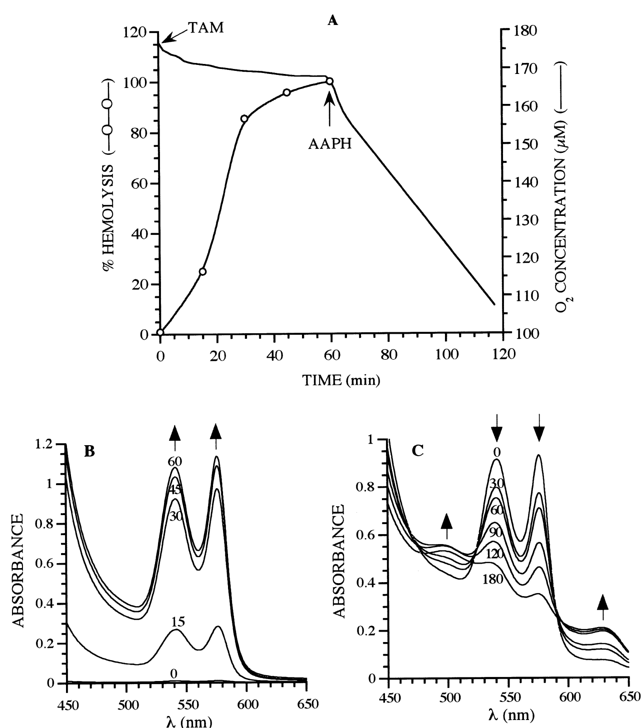

Fig. 5. Rate of oxygen consumption associated with hemolysis of erythrocytes induced by 12.5 μM TAM and by 20 mM AAPH (A) and spectra of hemoglobin after addition of TAM (B) and AAPH (C). Erythrocyte suspensions in 150 mM NaCl, 10 mM sodium phosphate, pH 7.4 (0.33% hematocrit) were incubated with TAM and AAPH at 37°C. O_2 consumption (A) and hemoglobin spectra were monitored simultaneously during incubation of TAM for 1 h (B) and after AAPH addition (C), as described in Section 2. Hemoglobin spectra were recorded at different reaction times indicated by the numbers adjacent to the traces. Upward and downward arrows indicate an increase or decrease in absorbance due to hemoglobin release (B) and hemoglobin oxidation by AAPH added 1 h after TAM (C).

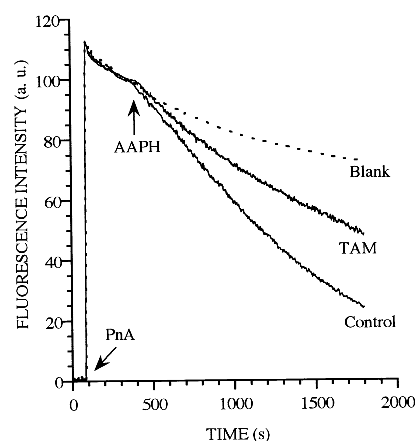

Fig. 6. Effect of 10 μM TAM on the peroxidative degradation of 1.5 μM PnA incorporated into the membrane of intact erythrocytes (0.05% hematocrit). The peroxidative degradation of PnA, indicated by its fluorescence decay, was induced by 1 mM AAPH in the absence (control) and presence of 10 μM TAM (TAM) and kinetically monitored at 450 nm emission setting the excitation at 312 nm with 15 and 5 nm slits, respectively. The dashed line shows the spontaneous fluorescence decay of PnA at 37°C (Blank). The recordings are typical assays of three independent experiments.

3.2. *TAM effects on peroxidative degradation of erythrocytes*

To further clarify the possible involvement of free radicals in the hemolytic effect of TAM, oxygen consumption and hemoglobin oxidation were monitored in parallel during the time course of hemolysis induced by the drug. As observed in Fig. 5A, the hemolysis induced by 12.5 μM TAM is not accompanied by O_2 consumption or changes in the spectra of the hemoglobin released to the supernatant (Fig. 5B). In contrast, when the generator of peroxyl radicals AAPH is added to the incubation medium, an abrupt O_2 consumption is initiated (Fig. 5A) and a gradual conversion of the typical spectra of oxyhemoglobin into that of Met-hemoglobin is observed (Fig. 5C), suggesting an extensive peroxidation of lipids and proteins induced by the radicals generated by AAPH. Clearly, TAM is totally silent regarding oxi-

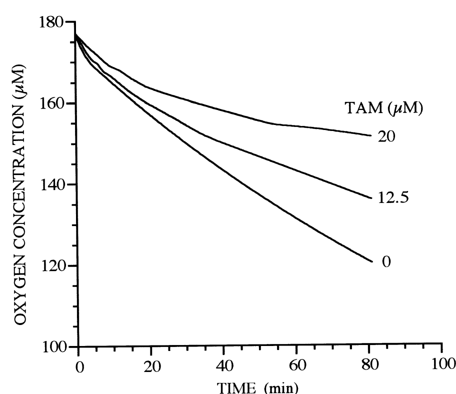

Fig. 7. Effect of TAM on the oxidation of hemolysate induced by 20 mM AAPH at 37°C, as evaluated by O_2 consumption. The hemolysate was prepared from a 0.33% erythrocyte suspension by hypotonic hemolysis. O_2 consumption was monitored by a Clark-type oxygen electrode either in the absence (0 μM) or presence of 12.5 and 20 μM TAM, preincubated at 37°C/20 min before AAPH addition. Results shown are representative of three experiments.

dative challenge of erythrocytes as compared to AAPH.

To emphasize this observation, the effect of TAM on the peroxidative process induced by AAPH in intact erythrocytes was studied by the fluorescence intensity decay of PnA. As can be observed (Fig. 6), the addition of AAPH to erythrocyte suspension in the absence of TAM (control) induces a decrease in PnA fluorescence relative to the spontaneous fluorescence decay of PnA (blank), which is related to the rate of its oxidative degradation [30]. Preincubation of TAM with erythrocytes decreases the rate of fluorescence intensity decay of PnA as compared to the control, indicating an efficient antioxidant activity of TAM in the first stages of erythrocyte membrane lipid peroxidation. Identical antioxidant effect of TAM is also noticed in the hemolysate peroxidation induced by AAPH as evaluated by O_2 consumption (Fig. 7). Exposing the hemolysate to AAPH-derived radicals results in lipid peroxyl radical production due to peroxidation as indicated by an increased rate of O_2 consumption as compared to experiments in the absence of erythrocytes (Fig. 7). The preincubation of TAM with the hemolysate leads to a significant concentration-dependent decrease in the rate of O_2 uptake pointing to a strong scavenger capacity of TAM for lipid peroxyl radicals generated by AAPH as previously described [30]. However, the oxidation of oxyhemoglobin to Methemoglobin in the hemolysate induced by AAPH (Fig. 8A) is not protected by the presence of TAM (Fig. 8B) as detected by the hemoglobin spectra recorded in parallel to O_2 consumption. This inability of TAM to scavenge peroxyl radicals in aqueous phase, occurring identical hemoglobin oxidation induced by AAPH in the absence (Fig. 8A) and presence of TAM (Fig. 8B), strongly corroborates the intramembranous scavenger properties of TAM in model membranes previously described [30].

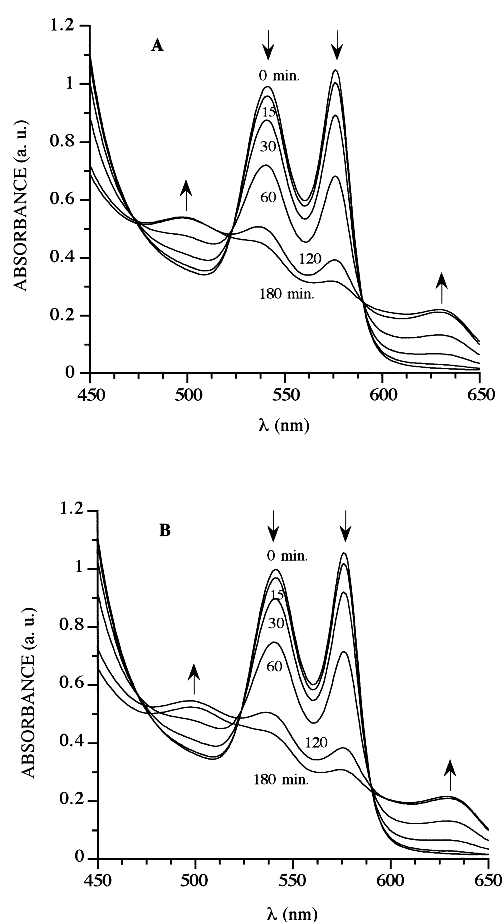

Fig. 8. Effect of TAM on spectral changes of hemoglobin upon reaction of hemolysate with 20 mM AAPH at 37°C. The hemolysate was prepared from a 0.33% erythrocyte suspension by hypotonic hemolysis and AAPH was added in the absence (A) and presence of 20 μM TAM preincubated at 37°C/20 min before oxidant addition (B). Hemoglobin absorption spectra were recorded at different incubation times, indicated by the numbers close to the traces. Downward and upward arrows indicate a decrease or increase in absorbance as the reaction proceeds. Recordings are typical of several experiments.

Therefore, these results indicate that TAM induced-hemolysis is not associated to an increase in erythrocyte membrane lipoperoxidation or hemoglobin oxidative degradation induced by the drug. However, they do not exclude the possibility that TAM-induced hemolysis may result from modifications on erythrocyte membrane proteins and changes on the framework of cytoskeleton and/or plasma membrane proteins.

Previous studies have suggested that membrane proteins are primary targets in cytolysis [43]. Thus, the possibility that TAM-induced hemolysis may occur from interaction with erythrocyte membrane proteins was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This methodology, allowing polypeptide separation as a function of its molecular weight, has been used to study alterations of erythrocyte membrane proteins [44,45], e.g. protein fragmentation and/or polymerization indicated by new bands of lower or higher molecular weight, respectively [40,45].

Fig. 9 shows the electrophoretic profile of the erythrocyte membrane proteins isolated from erythrocyte suspensions hemolysed in hypotonic buffer (control) and after hemolysis with 12.5 μM TAM incubated at 37°C/1 h (TAM). It is clear that TAM did not promote the appearance of new bands of higher or lower molecular weight as compared to the banding pattern of control, suggesting that TAM does not induce polymerization or fragmentation of membrane proteins. Additionally, the relative amounts of spectrin (α and β), bands 3, 4.1, 4.2 and 5 are similar in the absence (control) and presence of 12.5 μM TAM (TAM), indicating minimal changes of these proteins due to hemolysis induced by TAM. However, erythrocytes incubated with the drug display considerable changes in the intensity of some proteins bound to band 3 in the erythrocyte membrane.

The band 3 protein constitutes the anion channel that enables bicarbonate to be changed for chloride and is a binding structure for proteins of the cytoskeleton and cytosol. Recent studies showed that band 4.1 [46], ankyrin, glycolytic enzymes, aldolase and glyceraldehyde-3-phosphate dehydrogenase (band 6), and hemoglobin [47] bind predominantly, if not exclusively, to the band 3 tetramer. TAM decreases the intensity of bands 6, 7 and 16 kDa (hemoglobin monomer), inducing changes on cytoskeleton–membrane connections and lipid bilayer. The release or solubilization of some membrane proteins suggest that TAM changes lipid–protein interactions in the bilayer and the framework of erythrocyte cytoskeleton proteins and plasma membrane. According to the effects in other membrane systems [24], where TAM strongly partitions [22] and induces changes in the bilayer geometry [23], binding unspecifically and interacting with several and distinct membrane proteins [14,19,48], the hemolytic action of TAM putatively results from perturbation of erythrocyte membrane integrity and structure, by impairing lipid–lipid, lipid–protein and protein–protein interactions. Additionally, the release of hemoglobin bound to the membrane could explain the extra absorbance observed in the supernatant of erythrocytes incubated with TAM as compared to the total hemolysis (Fig. 3B), also meaning that TAM disrupts the binding of cytosol and cytoskeleton proteins to

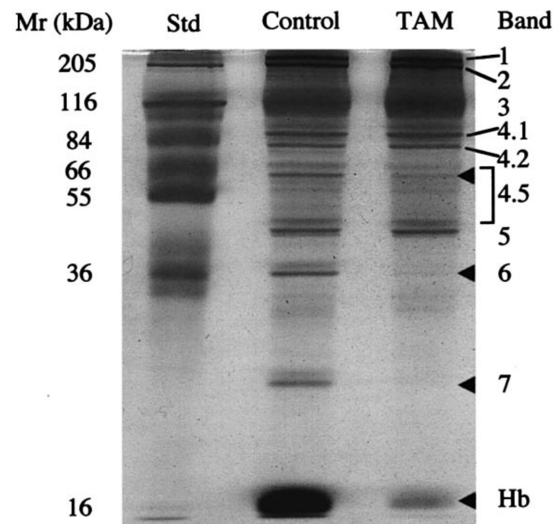

Fig. 9. SDS-PAGE of erythrocyte membrane proteins isolated from 0.33% erythrocyte suspensions preincubated at 37°C/1 h in the absence (Control) and in the presence of 12.5 μM TAM (TAM). Following the preincubation, membranes were separated and washed as described in Section 2. Samples containing 30 μg protein were applied and the peptides were separated by the method of Laemmli [33]. Nomenclature of the bands is given according to the classification of Fairbanks et al. [55] and using the molecular relative weight (Mr) of markers used as standards (Std). The gel shown is representative of three similar separations. Arrowheads indicate the differences in the protein banding pattern between control and TAM.

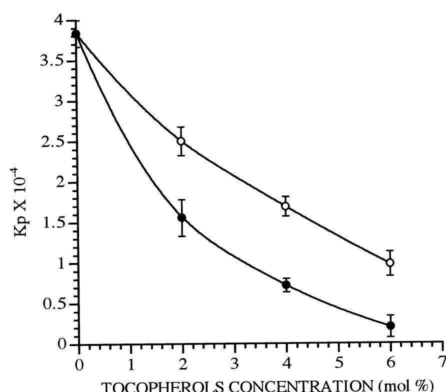

Fig. 10. Partition coefficient (K_p) of TAM into multilamellar DMPC liposomes enriched with α-T (●) and α-TAc (○) at 30°C and evaluated by UV derivative spectroscopy. The K_p of TAM decreases upon α-T and α-TAc incorporation. Each value represents the average of three independent experiments.

band 3, the red cell most abundant intrinsic protein. These defects induced by TAM may contribute to abnormal erythrocyte shape and diminished mechanical stability, resulting in hemolytic anemia.

3.3. *Effect of α-T and α-TAc on K_p of TAM and membrane fluidity*

Owing to its lipophilic character, TAM strongly incorporates in membrane systems and its partition is modulated by several factors, e.g. fluidity degree and membrane composition, being higher in membranes containing protein and low cholesterol concentration [22]. This steroid prevents TAM incorporation, probably due to its known effects on increasing lipid packing and surface density of the bilayer. Furthermore, cholesterol may compete for the microdomains where TAM incorporates, therefore displacing the drug from the membrane [22].

Similarly to cholesterol, α-T and α-TAc, which are membrane stabilizers and condensing agents [49] are expected to affect the partition coefficient (K_p) of TAM, decreasing its hemolytic action. Therefore, the K_p of TAM was estimated as a function of α-T and α-TAc content in membrane model of DMPC multilamellar liposomes, as summarized in Fig. 10. These studies were carried out in liposomes of synthetic lipids to minimize the very strong light scattering of suspensions and interferences in UV spectra when ghosts and erythrocytes are used [22] not allowing reliable collection of spectra data. Partitioning of TAM into DMPC liposomes in the liquid-crystalline phase remarkably decreases with α-T and α-TAc incorporation (Fig. 10), although a stronger effect is exhibited by α-T comparatively to α-TAc.

To further clarify the decrease of TAM K_p upon α-T or α-TAc incorporation in DMPC liposomes, the effects of these compounds on the fluidity of the same membrane model were investigated by fluorescence polarization. To probe the inner and the outer regions of the bilayer two fluorophores were

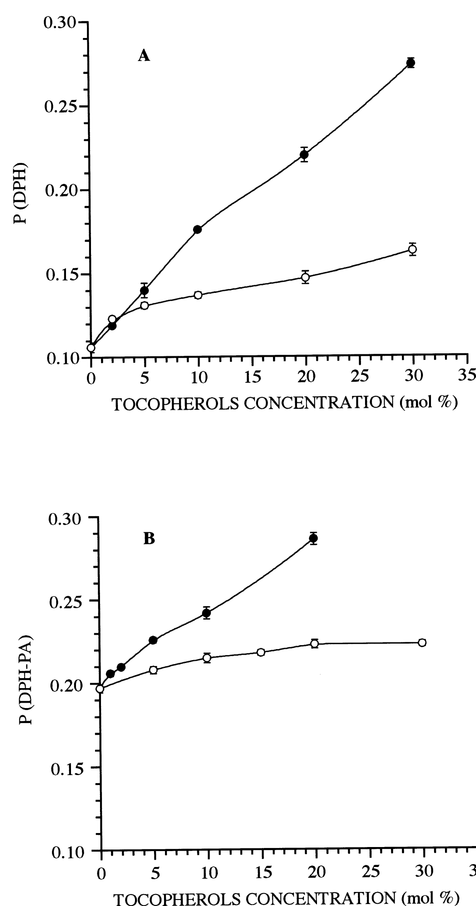

Fig. 11. Fluorescence polarization (P) of DPH (A) and DPH-PA (B) in DMPC multilamellar liposomes (345 μM) enriched with increasing concentrations of α-T (●) and α-TAc (○) at 37°C. The molar ratio of lipid/probe was about 400 and the degree of P was determined at 336 nm excitation and 450 nm emission using 3 nm excitation and 4 nm emission bandwidths. The increase in P is indicative of structural condensation in the regions probed by the fluorophores. Data are means of three different experiments.

used, DPH preferentially located in the hydrophobic core of the membrane and DPH-PA that distributes close to the interfacial region, probing the outer bilayer regions [50,51].

Fig. 11 displays the effects of α-T and α-TAc on the fluidity of DMPC membranes, as evaluated by the fluorescence polarization of DPH (Fig. 11A) and DPH-PA (Fig. 11B). α-T increases the polarization of the fluorophores, meaning an increase in the structural order of lipids across all the thickness of the bilayer [52]. α-TAc slightly increases the polarization of DPH, but not of DPH-PA. This effect of α-TAc is in agreement with its high hydrophobic character and its location predominantly in the central regions of the bilayer [53], apart from the cooperativity region that determines the structural order. A correlation can be established between the extent of α-T and α-TAc effects on TAM K_p and on membrane fluidity, similarly to that described elsewhere for cholesterol [22]. Therefore, the inhibition of TAM-induced hemolysis by α-T and α-TAc may be due to a stabilization of the erythrocyte membrane, decreasing the incorporation of TAM and preventing the physical damage of the erythrocyte membrane resulting from TAM accumulation.

In conclusion, the results described, together with previous data [24,28,54], point to a prominent role of TAM in inducing defects in erythrocyte membrane structure, leading to changes in normal erythrocyte shape and decreased mechanical stability, resulting in hemolytic anemia. Hence, the inhibition of TAM-induced hemolysis by α-T and α-TAc can be regarded as a potential preventive procedure against toxic effects exerted by this anticancer drug in normal tissues. Since cell leakage is a final stage of cytotoxicity, the disruption of the structural characteristics of biomembranes by TAM may also contribute for its multiple mechanisms of anticancer action not related to the estrogen receptors.

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