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Protection of tamoxifen against oxidation of mitochondrial thiols and NAD(P)H underlying the permeability transition induced by prooxidants

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Abstract

The effects of tamoxifen (TAM) were studied on the mitochondrial permeability transition (MPT) induced by the prooxidant *tert*-butyl hydroperoxide (*t*-BuOOH) or the thiol cross-linker phenylarsine oxide (PhAsO), in the presence of Ca^{2+}, in order to clarify the mechanisms involved in the MPT inhibition by this drug. The combination of Ca^{2+} with *t*-BuOOH or PhAsO induces mitochondrial swelling and depolarization of membrane potential ($\Delta\Psi$). These events are inhibited by cyclosporine A (CyA), suggesting the inhibition of the MPT. The pre-incubation of mitochondria with TAM also prevents those events and induces a time-dependent reversal of $\Delta\Psi$ depolarization following MPT induction, similarly to CyA. Moreover, TAM inhibits the Ca^{2+} release and the oxidation of NAD(P)H and protein thiol (–SH) groups promoted by *t*-BuOOH plus Ca^{2+}. On the other hand, the MPT induced by PhAsO plus Ca^{2+} does not induce –SH groups oxidation, supporting the notion that MPT induction by this compound is not mediated by the oxidation of specific membrane proteins groups. However, TAM also inhibits the PhAsO induced MPT, suggesting that this drug may inhibit this phenomenon by inhibiting PhAsO binding to –SH vicinal groups, implicated in the MPT induction. These data indicate that the MPT inhibition by TAM may be related to its antioxidant capacity in preventing the oxidation of NAD(P)H and –SH groups or by blocking these groups, since the oxidation of these groups increases the sensitivity of mitochondria to the MPT induction. Additionally, they suggest an MPT-independent pathway for TAM-induced apoptosis and a potential ER-independent mechanism for the effectiveness of this drug in the cancer therapy and prevention.

Keywords: Mitochondrial permeability transition; NAD(P)H oxidation; Oxidative stress; Tamoxifen; Thiol groups oxidation

1. Introduction

Tamoxifen (TAM) has been successfully used worldwide in the treatment of breast cancer as well as in the prevention of women with high susceptibility to the disease [1–3]. Despite being an anti-estrogenic drug, TAM also inhibits the growth of estrogen receptor (ER)-negative breast cancer cells and other cell types that lack the ER [4–6]. In fact, TAM has been reported to have pro-apoptotic activity in many types of ER-positive [7] and ER-negative tumor cells [8], as well as ability to promote ER-dependent and indepen-

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dent programmed cell death [9,10]. Although many studies have been performed, the mechanisms of antiproliferative effects and pro-apoptotic action of TAM remain controversial and not completely understood.

Mitochondria play a critical role in initiating both apoptotic and necrotic cell death and many compounds are known to induce programmed cell death by pathways that involve mitochondria. A major player is the mitochondrial permeability transition (MPT) [11], characterized by depolarization of mitochondrial membrane potential ($\Delta\Psi$), Ca^{2+} release, mitochondrial swelling and rupture of mitochondrial membrane, leading to the release of cytochrome *c* and other pro-apoptotic molecules, which then activate the caspase cascade that sets apoptosis in motion [12]. TAM is a lipophilic antioxidant with high partitioning into membranes [13] and it has been shown to inhibit the MPT induced in vitro by inorganic phosphate (Pi) and Ca^{2+} [14]. Ca^{2+}-induced MPT can be stimulated by a wide variety of compounds with different chemical characteristics, including Pi, oxidizing agents, dithiol reagents, protonophores and ligands of the adenine nucleotide translocator (ANT) [15]. This phenomenon is accompanied by oxidative stress which in turn induces oxidation of mitochondrial membrane protein thiol (–SH) groups, matrix GSH and depletion of pyridine nucleotides [NAD(P)H] [16–18].

TAM is an efficient inhibitor of lipid peroxidation induced by Fe^{2+}/ascorbate and a strong intramembraneous scavenger of peroxyl radicals [19]. Owing to its antioxidant properties, TAM [19,20] is a potential inhibitor of the MPT stimulated by prooxidants. Therefore, the aim of this study was to evaluate the effects of TAM on the MPT induced by prooxidants such as *tert*-butylhydroperoxide (*t*-BuOOH) [21] and by the thiol cross-linker phenylarsine oxide (PhAsO) [18,22] in the presence of Ca^{2+}, contributing to clarify the molecular mechanisms of TAM-induced MPT inhibition and to better elucidate the signalling pathway for the controversial apoptotic effects of this drug.

2. Materials and methods

2.1. Chemicals

Tamoxifen (TAM), 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (HEPES), bovine serum albumin (BSA), cyclosporine A (CyA), oligomycin, 5,5′-dithiobis(2-nitro-benzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethylether) NNN′N′-tetraacetic acid (EGTA), phenylarsine oxide (PhAsO) and *tert*-butyl hydroperoxide (*t*-BuOOH) were purchased from Sigma Chemical Co. (St. Louis, MO). Tetraphenylphosphonium (TPP^+) and sucrose were from MercK (Darmstadt, Germany). All the chemicals were commercial products of the highest purity grade available.

2.2. Isolation of rat liver mitochondria

Mitochondria were isolated from the liver of fasted overnight rats of either sex (Wistar) by conventional differential centrifugation techniques as described elsewhere [23] with slight modifications. Animals were killed by cervical dislocation and the liver was immediately excised and cooled at 4 °C in isolation medium containing 2250 mannitol, 75 mM sucrose, 10 mM HEPES (pH 7.4), 1 mM EGTA and 0.1% BSA free fatty acid, which was added just prior to use. The cooled liver was then minced with scissors and washed in isolation medium, then diluted and homogenised with same medium and centrifuged at 1200 × *g* for 10 min. The mitochondrial pellet was re-suspended in the washing medium, adjusted to pH 7.2 in the absence of either EGTA and BSA, and mitochondria washed twice were recovered from the supernatant at 10,000 × *g* for 10 min. Mitochondrial protein was measured by the method of biuret [24] with BSA as protein standard.

2.3. Standard incubation procedure

The experiments were carried out at 30 °C in a standard reaction medium containing 200 mM sucrose, 10 mM Tris-Mops (pH 7.4), 1 mM KH_2PO_4 and 10 μM EGTA, supplemented with 2 μM rotenone and 0.5 μg oligomycin/ml as described elsewhere [14]. Mitochondria were pre-incubated with *t*-BuOOH (50 μM) or PhAsO (2 μM) before Ca^{2+} addition. TAM (20 nmol/mg protein) or CyA (0.85 μM) were added from stock ethanolic solutions and pre-incubated with mitochondria for 3 min before succinate addition to start the reaction or after mitochondrial loading with Ca^{2+}, as indicated in the figure legends. The results shown represent typical

recordings from experiments of at least three different mitochondrial preparations.

2.4. Mitochondrial swelling

Changes in mitochondrial osmotic volume were monitored by the decrease in apparent absorbance (light-scattering) at 540 nm, with a Perkin Elmer Lambda 6 UV-Vis spectrophotometer computer controlled (Norwalk, USA), as described previously [25]. Mitochondria (0.5 mg protein/ml) were suspended in 2 ml of standard reaction medium at 30 °C supplemented with *t*-BuOOH (50 μM) or PhAsO (2 μM) and the reaction was started with 5 mM succinate. $CaCl_2$ (50 nmol/mg protein) was added 2 min after energization of mitochondria with succinate.

2.5. Mitochondrial membrane potential measurements

The mitochondrial membrane potential ($\Delta\Psi$) was monitored by evaluating transmembrane distribution of tetraphenylphosphonium (TPP^+) with an ion-selective electrode prepared according to Kamo et al. [26] using a $Ag/AgCl_2$ electrode as reference (model MI 402; Microelectrodes, Inc., Bedford, NH). Reactions were carried out in an open vessel with magnetic stirring in 2 ml of standard reaction medium supplemented with 4 μM TPP^+. The standard reaction medium was supplemented with *t*-BuOOH (50 μM) or PhAsO (2 μM) and the reaction was started with 5 mM succinate and $CaCl_2$ (50 nmol/mg protein) was added after steady-state distribution of TPP^+. $\Delta\Psi$ was estimated from the decrease of TPP^+ concentration in the medium as described elsewhere [26,27].

2.6. Mitochondrial Ca^{2+} fluxes

Mitochondrial Ca^{2+} fluxes were measured by monitoring the changes in Ca^{2+} concentration in the reaction medium using a Ca^{2+}-sensitive electrode, according to previously described procedures [23,28]. Reactions were carried out in an open vessel with magnetic stirring in 2 ml of standard reaction medium supplemented with $CaCl_2$ (50 nmol/mg protein) and started by adding 5 mM succinate to mitochondrial suspensions (0.5 mg protein/ml) pre-incubated with *t*-BuOOH (50 μM).

2.7. Determination of NAD(P)H redox state

The redox state of pyridine nucleotides in mitochondria was followed fluorometrically at 366 nm excitation (slit 3 nm) and 450 nm emission (slit 5 nm) wavelengths in a Perkin Elmer spectrofluorometer LS-50 B [29]. Mitochondria (0.5 mg protein/ml) were suspended in 2 ml of standard reaction medium with *t*-BuOOH (50 μM) and the reaction was started with 5 mM succinate. $CaCl_2$ (50 nmol/mg protein) was added 2 min after energization of mitochondria. At the end of an experiment the auto-fluorescence of the reduced pyridine nucleotides was observed under conditions allowing the reduction of inner mitochondrial pyridine nucleotides. This was achieved by supplementation of reducing respiratory substrates (RS) 5 mM β-hydroxybutyrate and 0.1 mM isocitrate.

2.8. Determination of protein thiol content

Protein thiol groups were measured by the method of Monte et al. [30], with little modification. Mitochondrial suspensions (1 mg protein/2 ml) was added to 0.5 ml of 0.5% (w/v) trichloroacetic acid and the protein sedimented by centrifugation at 3200 × *g* for 12 min (KUBOTA 5400). The supernatant was discarded and the pellet was suspended in 2 ml phosphate buffer (20 mM Na_2HPO_4, 110 mM NaCl and 1 mM EDTA, pH 7.4). To 0.5 ml of this suspension it was added 4.5 ml of phosphate buffer and a 50 μl aliquot of 10 mM 5,5′-dithiobis(2-nitro-benzoic acid) (Ellman's reagent or DTNB) in methanol was then added and mixed well. The absorbance was measured at 412 nm within 15 min.

2.9. Statistical analysis

The percentage of oxidized –SH groups in each experiment was determined relatively to the experiment with Ca^{2+} alone (control) (100%). The results are presented as mean ± S.D. of at least three independent experiments. Comparisons between the different assays were made using the one-way analysis of variance (ANOVA) followed by the Tukey post-test, for multiple comparisons. A P value <0.05 was considered statistically significant.

3. Results

To clarify the mechanisms by which TAM inhibits the MPT, mitochondria were treated with *t*-BuOOH (50 μM) (Fig. 1A) and energized with succinate in the presence of Ca^{2+} (50 nmol/mg protein) (Ca^{2+} + *t*-BuOOH). As observed at 540 nm, mitochondria undergo a rapid and large decrease in the absorbance (Ca^{2+} + *t*-BuOOH), suggesting complete swelling of mitochondria (Fig. 1A). This extensive effect is completely prevented by pre-incubating mitochondria with 20 nmol TAM/mg protein (TAM), as reported previously to OHTAM [23]. Such effects are similar to those observed with cyclosporine A (0.85 μM) (Fig. 1A—CyA), a specific and potent inhibitor of the MPT [31], suggesting that TAM completely protects against the Ca^{2+}–*t*-BuOOH-induced MPT (Fig. 1A—TAM). This concentration of TAM does not significantly affect the $\Delta\Psi$ and phosphorylation efficiency of mitochondria, as previously reported [32]. However, higher concentrations of TAM induce destructive effects in the structural integrity of the mitochondrial membrane [32]. When added alone, neither Ca^{2+} (Fig. 1A—Ca^{2+} alone) nor *t*-BuOOH (Fig. 1A—*t*-BuOOH alone) induce any significant decrease in absorbance, indicating that none of them is able by itself to induce mitochondrial swelling in the used concentrations. PhAsO (2 μM), a thiol cross-linking agent, was also used in combination with Ca^{2+} (50 nmol/mg protein) to induce the MPT (Fig. 1B). Similar to that observed with mitochondria pre-incubated with Ca^{2+} (Fig. 1B—Ca^{2+} alone), PhAsO alone is not able to change the absorbance of mitochondrial suspensions measured at 540 nm. However, mitochondria incubated in the presence of Ca^{2+} plus PhAsO undergo a strong decrease in absorbance at 540 nm, indicative of mitochondrial swelling (Fig. 1B—Ca^{2+} + PhAsO). Similar to CyA (Fig. 1B—CyA), pre-incubation with TAM (20 nmol/mg protein) prevents the occurrence of this effect owing to the inhibition of the MPT induction (Fig. 1B—TAM).

The inhibitory effects of TAM on the induction of the MPT by Ca^{2+} plus *t*-BuOOH are also demonstrated by the fact this drug prevents the Ca^{2+}-induced depolarization of $\Delta\Psi$ associated with MPT induction (Fig. 2A). The addition of Ca^{2+} (50 nmol/mg protein) (Fig. 2A—Ca^{2+} alone) causes a transient depolarization, $\Delta\Psi$ returning to near 205 mV within 2 min. However, in mitochondria treated with *t*-BuOOH, the repolarization of $\Delta\Psi$ after Ca^{2+} accumulation is followed by a dramatic and irreversible depolarization

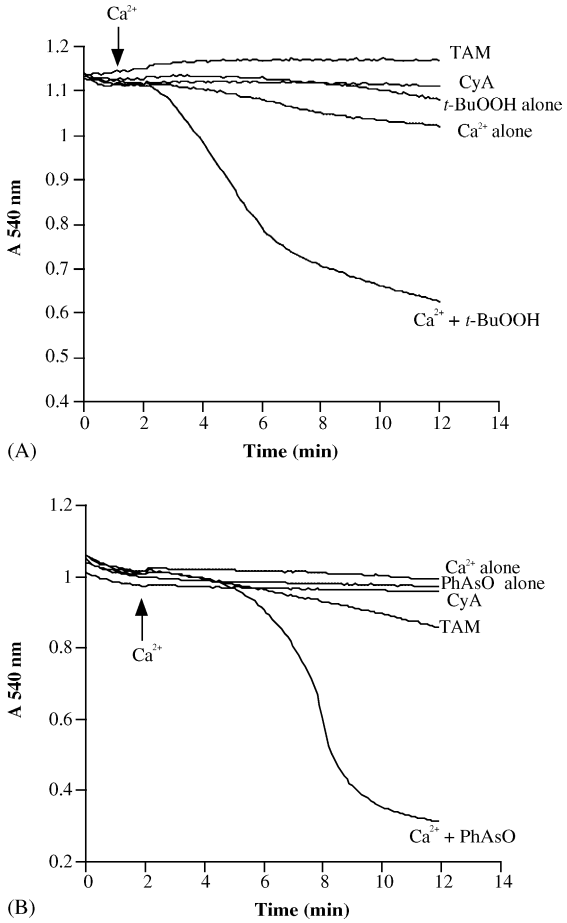


Fig. 1. TAM protects the mitochondrial swelling induced by either *t*-BuOOH (A) or PhAsO (B). Rat liver mitochondria (0.5 mg protein/ml) in 2 ml of standard reaction medium supplemented with 2 μM rotenone and 0.5 μg/ml oligomycin, were energized with 5 mM succinate at 30 °C. Mitochondrial swelling was induced with 50 μM *t*-BuOOH (A) or 2 μM PhAsO (B) pre-incubated with mitochondria in the presence of Ca^{2+} (50 nmol/mg protein) as labelled, respectively, by Ca^{2+} + *t*-BuOOH or Ca^{2+} + PhAsO. Mitochondria were pre-incubated with TAM (20 nmol/mg protein) or CyA (0.85 μM) for 3 min before succinate addition. Mitochondria were also treated with Ca^{2+} alone, with *t*-BuOOH or with PhAsO alone, as indicated in the figures. The mitochondrial swelling was followed continuously at 540 nm and the traces, labelled by the names, are typical of three experiments with different mitochondrial preparations.

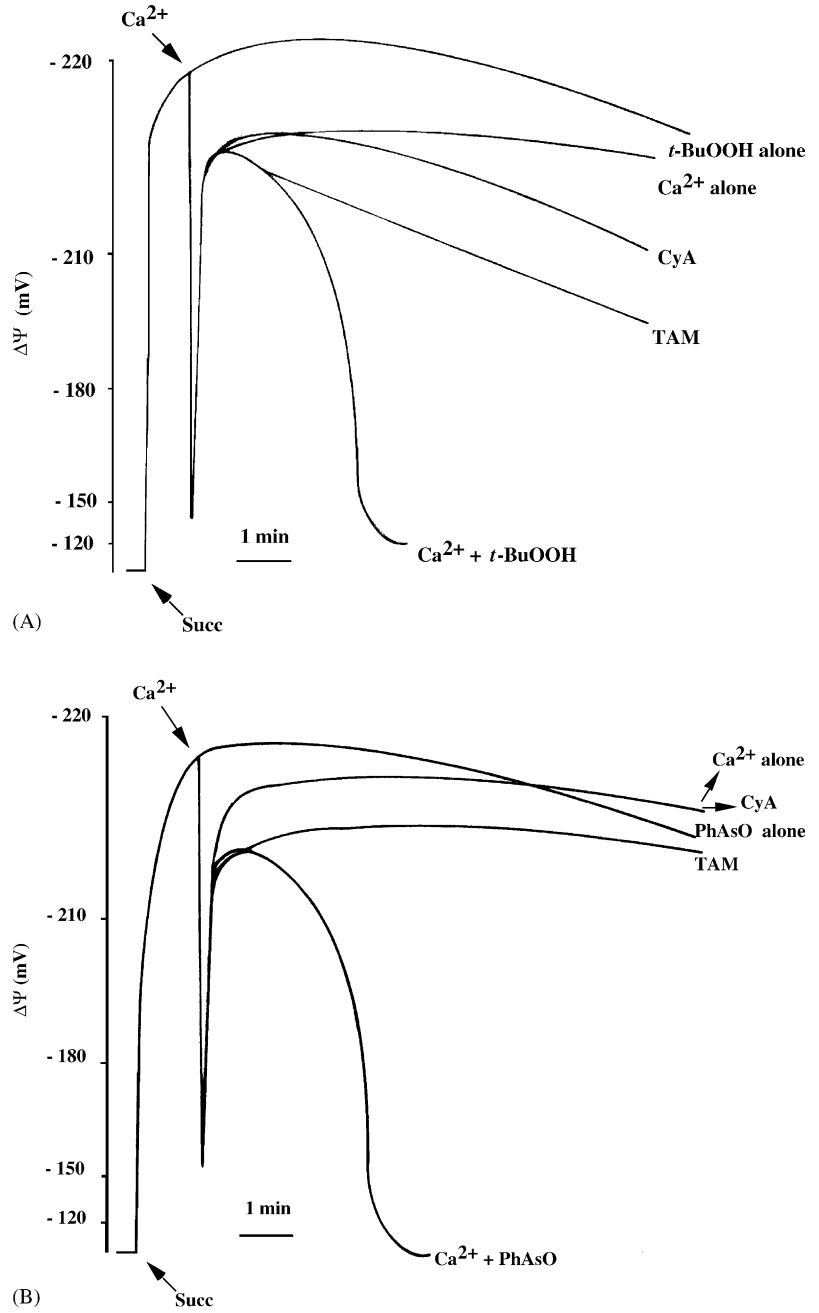


Fig. 2. TAM prevents the mitochondrial depolarization associated with MPT induction. Mitochondria (1 mg) were incubated in the standard reaction medium supplemented with 2 μM rotenone, 0.5 μg/ml oligomycin and 4 μM TPP^+ at 30 °C. The reactions were started with 5 mM succinate and Ca^{2+} (50 nmol/mg protein) was added after steady state distribution of TPP^+. Mitochondria were supplemented with Ca^{2+} after pre-incubation either 50 μM *t*-BuOOH (Ca^{2+} + *t*-BuOOH) (A) or 2 μM PhAsO (Ca^{2+} + PhAsO) (B). TAM (20 nmol/mg protein) or CyA (0.85 μM) were added to mitochondria 3 min prior to adding *t*-BuOOH (A) or PhAsO (B). Either Ca^{2+}, *t*-BuOOH or PhAsO alone were also added to mitochondrial suspensions (traces labelled by the respective names). CyA and Ca^{2+} alone traces are coincident (B). The traces are typical of experiments with three different mitochondrial preparations.

of mitochondria over the course of the next 10 min (Fig. 2A—Ca^{2+} + *t*-BuOOH). Pre-incubation of mitochondria with TAM in the presence of *t*-BuOOH, prior to adding succinate and Ca^{2+}, affords protection against the Ca^{2+}-induced irreversible depolarization of $\Delta\Psi$, similarly to CyA (Fig. 2A—TAM, CyA). Under these conditions, the Ca^{2+} uptake causes a transient depolarization of $\Delta\Psi$ but mitochondria are able to develop and sustain the $\Delta\Psi$. Treating mitochondria with Ca^{2+} in the absence of *t*-BuOOH (Fig. 2A—Ca^{2+} alone) or with *t*-BuOOH alone (Fig. 2A—*t*-BuOOH alone) does not induce mitochondrial depolarization. The effects of TAM were also investigated on the depolarization of $\Delta\Psi$ induced by the combination of Ca^{2+} plus PhAsO (Fig. 2B). As observed with the *t*-BuOOH experiments (Fig. 2A), neither Ca^{2+} alone (Fig. 2B—Ca^{2+} alone) nor PhAsO (Fig. 2B—PhAsO alone) is able to cause $\Delta\Psi$ depolarization, contrarily to that observed with mitochondria treated with PhAsO plus Ca^{2+} (Fig. 2B—Ca^{2+} + PhAsO). Pre-incubation of mitochondrial suspensions with TAM prevents from the PhAsO plus Ca^{2+}-induced depolarization (Fig. 2B—TAM). Moreover, the addition of TAM to mitochondrial suspensions undergoing $\Delta\Psi$ depolarization promoted by either *t*-BuOOH (Fig. 3A) or PhAsO (Fig. 3B) plus Ca^{2+} induces repolarization of $\Delta\Psi$ and the capacity of mitochondria to sustain the $\Delta\Psi$ developed in a similar way to CyA, indicating that this antiestrogen has the ability to prevent and revert the MPT induction promoted by prooxidants and thiol cross-linkers. However, TAM at 20 nmol/mg protein added after Ca^{2+}-induced MPT only partially promotes repolarization of $\Delta\Psi$ as compared to CyA (Fig. 3A and B) since TAM, according to our previous reports [14,32] induces proton leak through the mitochondrial inner membrane.

Further evidence that TAM inhibits the Ca^{2+} plus *t*-BuOOH-induced MPT is provided by the fact that this drug prevents the release of mitochondrial loaded Ca^{2+} (Fig. 4). In fact, mitochondria pre-incubated with *t*-BuOOH and energized with succinate release the accumulated Ca^{2+} within about 15 min (Fig. 4A—Ca^{2+} + *t*-BuOOH). However, TAM affords to mitochondria capacity to accumulate and sustain the added Ca^{2+} similarly to that observed with CyA. Moreover, TAM added during the time course of the Ca^{2+} release, related to MPT promoted by Ca^{2+} plus *t*-BuOOH, induces re-uptake of the released Ca^{2+} by mitochondria (Fig. 4B), in agreement with the repolarization effect referred above (Fig. 3A). However, these effects are also dependent on the time elapsed after Ca^{2+} addition. CyA, even when added late in the reaction, causes total re-uptake of the released Ca^{2+} (Fig. 4B).

The oxidation of mitochondrial pyridine nucleotides [NAD(P)H] also characterizes the induction of the MPT. Therefore, the changes in the oxidized/reduced state of pyridine nucleotides were also followed by monitoring the fluorescence intensity of mitochondrial suspensions treated with *t*-BuOOH in the presence of Ca^{2+} (Fig. 5). The fluorescence of reduced pyridine nucleotides (NADH and NADPH) accounts for most of the cellular auto-fluorescence excited with near UV light, however oxidized pyridine nucleotides (NAD^+ and $NADP^+$) are non-fluorescent. Accordingly, changes in auto-fluorescence reflect changes in the redox state of pyridine nucleotides. During the time course of the MPT induced by Ca^{2+} plus *t*-BuOOH (Fig. 5—Ca^{2+} + *t*-BuOOH) there is hydrolysis and/or oxidation of NAD(P)H after Ca^{2+} addition to *t*-BuOOH pre-incubated mitochondria, as there is a decline in mitochondrial auto-fluorescence. Pre-incubation of mitochondria with TAM prevents the decrease in NAD(P)H content, as it maintains the auto-fluorescence intensity unchanged, similarly to pre-incubation with CyA. Mitochondrial suspensions pre-incubated with Ca^{2+} (Fig. 5—Ca^{2+} alone) or *t*-BuOOH alone (Fig. 5—*t*-BuOOH alone) do not undergo any alteration in auto-fluorescence intensity, which is in accordance with that observed in experiments of mitochondrial swelling (Fig. 1A), $\Delta\Psi$ depolarization (Fig. 2A) and Ca^{2+} release (Fig. 4A).

Considering that the oxidation state of cysteine residues has been proposed as a critical factor regulating the induction of the MPT [33], the thiol groups content of mitochondria treated with Ca^{2+} and *t*-BuOOH (Fig. 6A—Ca^{2+} + *t*-BuOOH) or PhAsO (Fig. 6B—Ca^{2+} + PhAsO) was also determined. The amount of Ca^{2+} used, per se, does not induce the MPT. Therefore, the percentage of oxidized –SH groups in each experiment was determined relatively to the experiment with Ca^{2+} alone (control), that was considered as 100% of –SH in the reduced state. *t*-BuOOH alone is not able to induce significant –SH groups oxidation relatively to the experiment with Ca^{2+} plus *t*-BuOOH. TAM (Fig. 6A—TAM) prevents the oxidation of –SH groups induced by Ca^{2+} plus

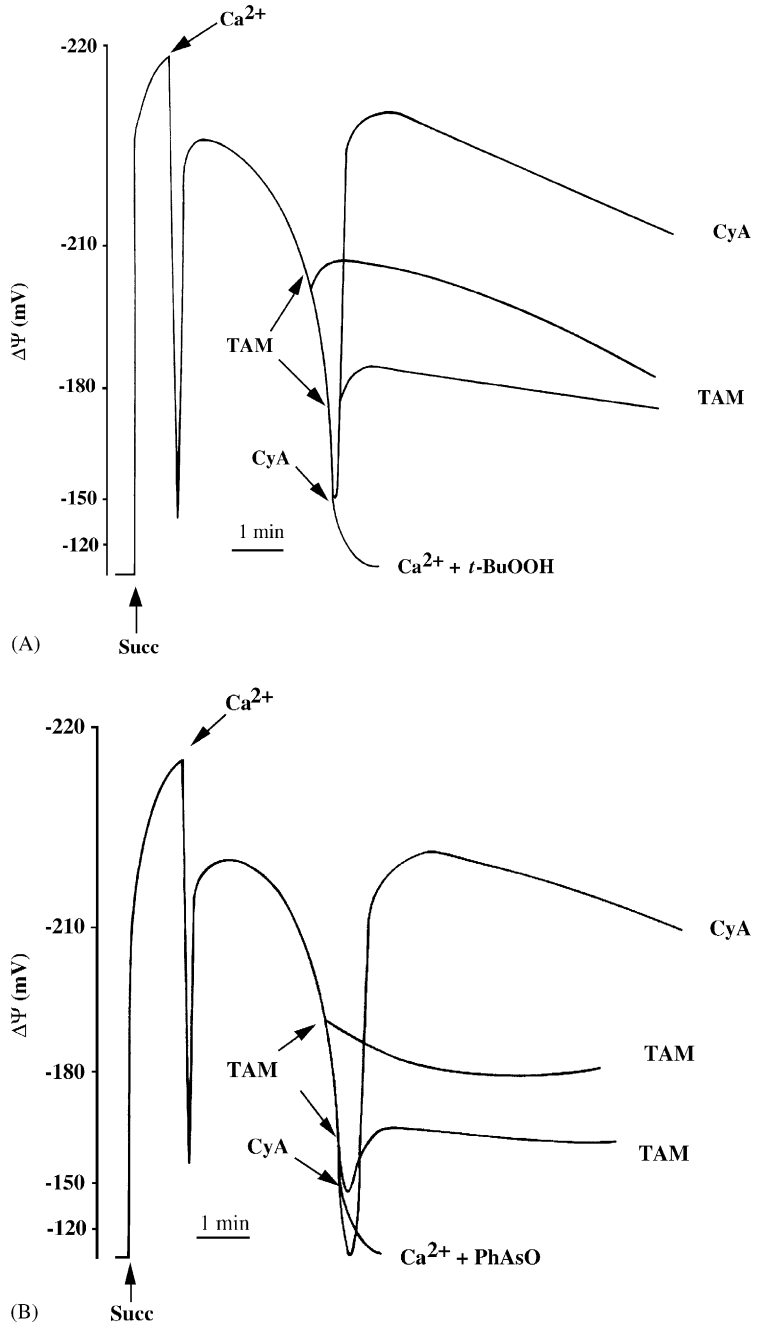
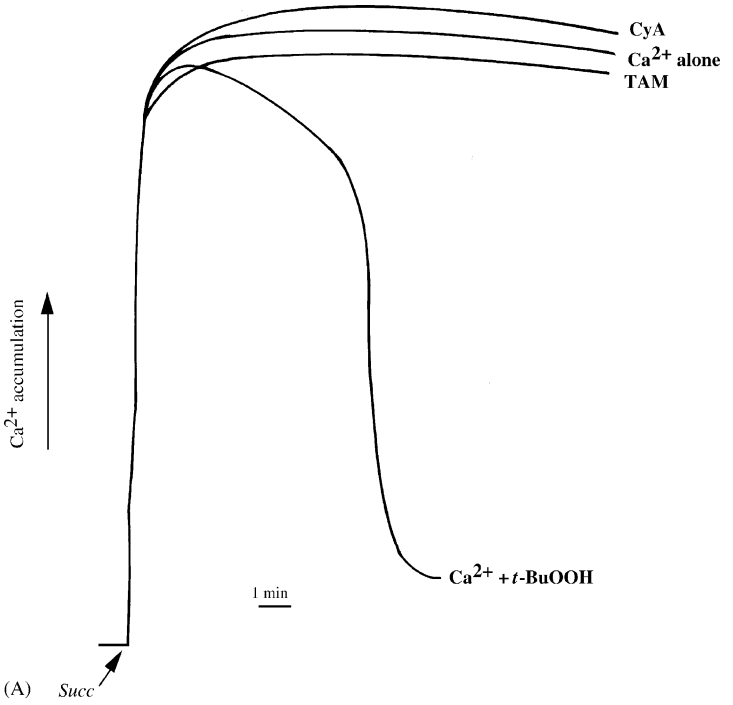
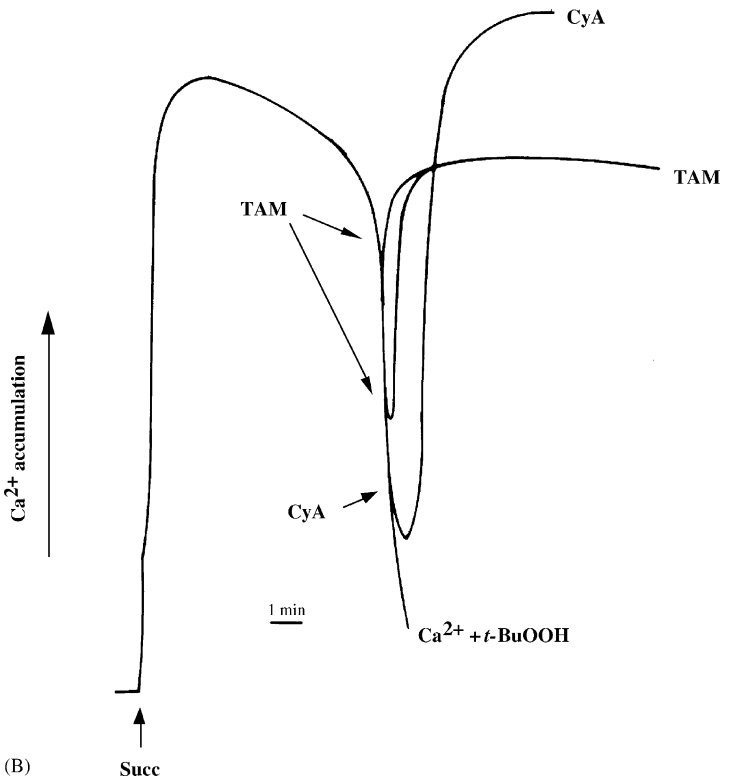


Fig. 3. TAM reverses $\Delta\Psi$ depolarization due to MPT induction by Ca^{2+} (50 nmol/mg protein) plus 50 μM *t*-BuOOH (A) or 2 μM PhAsO (B). The reactions were carried out as described for Fig. 2 except that TAM (20 nmol/mg protein) was added at different times after Ca^{2+}-induced depolarization and CyA added later, as indicated by the corresponding labelled arrows. Mitochondrial depolarization and repolarization were monitored by following $\Delta\Psi$ with a TPP^{+}-selective electrode. All the traces are representative of three separate experiments.


CyA
Ca2+ alone
TAM
Ca2+ accumulation
Ca2+ +t-BuOOH
1 min
(A)
Succ


CyA
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TAM
Ca2+ accumulation
CyA
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Ca2+ +t-BuOOH
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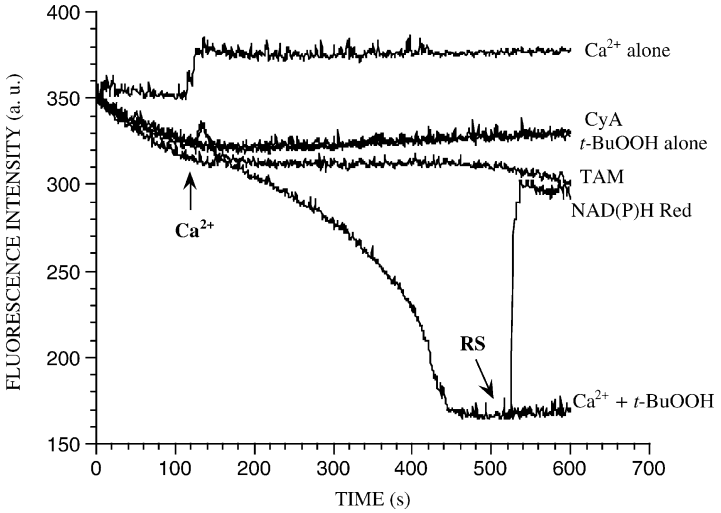

Fig. 5. Effects of TAM on the redox state of pyridine nucleotides during MPT induction as measured fluorometrically (366/450 nm). Rat liver mitochondria (0.5 mg protein/ml) were incubated in 2 ml of standard reaction medium supplemented with 2 μM rotenone, 0.5 μg/ml oligomycin and energized with 5 mM succinate, at 30 °C, before Ca^{2+} (50 nmol/mg protein) addition. The MPT was induced by 50 μM *t*-BuOOH, pre-incubated with mitochondria, in the presence of Ca^{2+} (Ca^{2+} + *t*-BuOOH). Mitochondria were also pre-incubated with TAM (20 nmol/mg protein) or CyA (0.85 μM) for 3 min before succinate addition. Either Ca^{2+} or *t*-BuOOH alone was also added to mitochondrial suspensions, as indicated in the figure. All the traces, labelled by the respective names, are representative of three separate experiments. The supplementation of reducing respiratory substrates (RS) (5 mM β-hydroxybutyrate and 0.1 mM isocitrate) for the re-reduction of pyridine nucleotides [NAD(P)H Red] was as indicated by the arrow.

t-BuOOH. Pre-incubation of mitochondria with CyA also affords protection against –SH groups oxidation (Fig. 6A—CyA), though the effects of TAM are more significant than those of CyA. In the case of induction of the MPT with Ca^{2+} plus PhAsO there is no observable oxidation of –SH groups (Fig. 6B), suggesting that this event is not involved in the MPT induction with this compound. Instead, it reversibly forms a five membered ring with vicinal thiols, inducing thiol cross-linkage and promoting the Ca^{2+}-dependent intramitochondrial NAD^+-hydrolysis [34]. Since CyA inhibits the MPT even when crucial thiols are oxidized, the prevention induced by TAM may be related to its high affinity to bind to membrane proteins, thus blocking the interaction of PhAsO with protein thiol groups components of the MPT.

4. Discussion

In this study an attempt has been made to elucidate the mechanism(s) underlying TAM inhibition of the MPT in isolated rat liver mitochondria. In fact, TAM was able to prevent the mitochondrial swelling promoted by Ca^{2+} plus either *t*-BuOOH (Fig. 1A) or PhAsO (Fig. 1B) and to inhibit the $\Delta\Psi$ depolarization associated with MPT induction by these different inducers (Fig. 2A and B). Moreover, TAM also induces

Fig. 4. The inhibitory effects of TAM on mitochondrial Ca^{2+} release related to the MPT induction. Mitochondria (1 mg) were suspended in 2 ml of standard reaction medium supplemented with 2 μM rotenone and 0.5 μg/ml oligomycin at 30 °C and the reactions were started with 5 mM succinate. Mitochondria were treated with 50 μM *t*-BuOOH in the presence of Ca^{2+} (50 nmol/mg protein) to induce MPT (Ca^{2+} + *t*-BuOOH). TAM (20 nmol/mg protein) or CyA (0.85 μM) were added to mitochondria 3 min prior to adding *t*-BuOOH (A) or at different times during the course of MPT (B). Mitochondria were also treated with Ca^{2+} or *t*-BuOOH alone, as indicated in the figures. The uptake and release of sequestered Ca^{2+} by mitochondria were monitored by using a Ca^{2+}-selective electrode and the traces are typical of three experiments with different mitochondrial preparations.

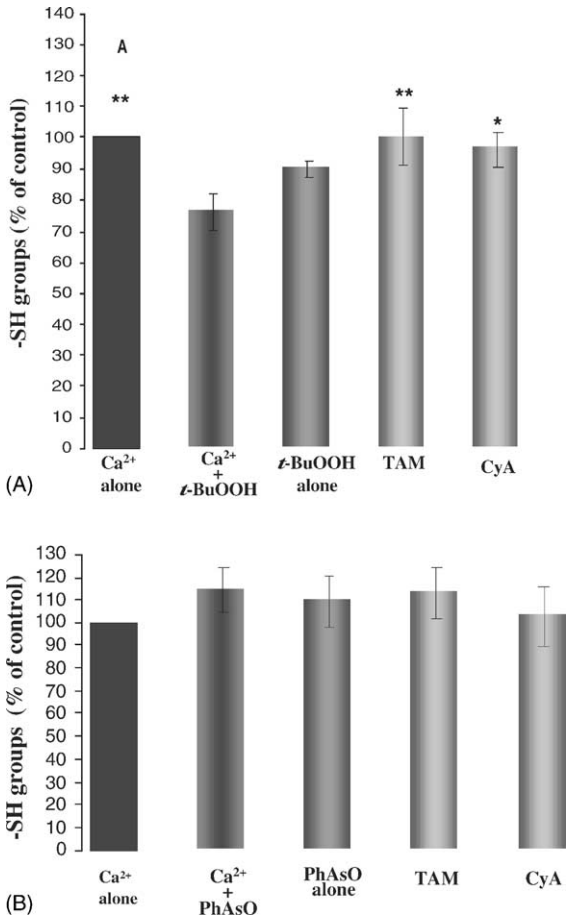

Fig. 6. Effects of TAM on mitochondrial thiol groups oxidation related with MPT induction. Rat liver mitochondria, at 1 mg protein/2 ml of standard reaction medium supplemented with 2 μM rotenone and 0.5 μg/ml oligomycin, were pre-incubated with either 50 μM *t*-BuOOH (A), 2 μM PhAsO (B) after Ca^{2+} (50 nmol/mg protein) uptake and energized with 5 mM succinate at 30 °C. Mitochondria were also pre-incubated with TAM (20 nmol/mg protein) or CyA (0.85 μM) for 3 min prior to starting the reactions and treated with *t*-BuOOH, PhAsO or with Ca^{2+} alone, as indicated in the figures. Thiol groups were evaluated by the Ellman's reagent. Values are expressed as percentage of the thiol groups of mitochondria treated with Ca^{2+} alone (100%) (control) and represent the average of 3–5 experiments ± S.D. Statistical significance: ∗: $P < 0.05$, ∗∗: $P < 0.01$ as compared to the experiment with Ca^{2+} + *t*-BuOOH (A) or with Ca^{2+} + PhAsO (B).

repolarization after MPT induction by the combinations of Ca^{2+} with the prooxidant *t*-BuOOH (Fig. 3A) or with the thiol cross-linker PhAsO (Fig. 3B). TAM also exhibited its inhibitory effects against the mitochondrial Ca^{2+} release related to the MPT induction and induced Ca^{2+} re-uptake by mitochondria after the onset of MPT with Ca^{2+} plus *t*-BuOOH (Fig. 4). As the MPT induction is associated with oxidation of either pyridine nucleotides [NAD(P)H] or critical thiol groups, possibly located in the ANT [18], the effect of TAM on such oxidative reactions was also investigated. The MPT induction with *t*-BuOOH plus Ca^{2+} was associated with NAD(P)H oxidation as demonstrated by the decrease in fluorescence intensity of mitochondria pre-incubated with *t*-BuOOH after Ca^{2+} addition (Fig. 5). A similar behaviour was observed when mitochondria were pre-incubated with PhAsO, instead of *t*-BuOOH, e.g., the addition of Ca^{2+} to mitochondria caused the MPT and resulted in a rapid and extensive pyridine nucleotide oxidation (results not shown). In addition to the oxidation of –SH groups and pyridine nucleotides [NAD(P)H], the generation of ROS within mitochondria (or a decreasing in their detoxification) contributes to the induction of the MPT, as shown by studies with several MPT inducers [35–37]. Different studies from several laboratories have demonstrated that the MPT induced by prooxidants as *t*-BuOOH is triggered by Ca^{2+}-stimulated production of ROS, which accumulate due to exhaustion of the mitochondrial antioxidants GSH and NAD(P)H [38–40]. The attack of ROS to membrane protein thiols produces cross-linkage reactions and opening of membrane pores upon Ca^{2+} binding. In fact, it has already been shown that mitochondrial protein complexes that participate in critical mitochondrial/cellular processes, such as the MPT, contain inner mitochondrial membrane proteins with critical thiols that can be readily oxidized leading to the onset of MPT [37,41,42]. Therefore, concerning to thiol groups oxidation, when the MPT was promoted with Ca^{2+} plus *t*-BuOOH (Fig. 6A) there is oxidation of –SH groups that is inhibited by pre-incubation of mitochondria with TAM. In contrast, when the MPT was promoted with PhAsO plus Ca^{2+} we do not observe –SH groups oxidation (Fig. 6B), probably due to the ability of PhAsO to induce MPT through thiol cross-linkage of vicinal thiols. According to some authors [39,40], the mitochondrial permeabilization induced by thiol cross-linkers, such as PhAsO, is independent of ROS and a consequence of cross-linkage promoted directly by this compound, although Novgorodov et al. [43] claimed to a certain relationship

between the cross-linking of two juxtaposed thiol groups and the free radical process in mitochondria. PhAsO has been shown to exert its effect by attacking vicinal thiols on proteins [44] and seems to be able to attack directly the pore components: it reversibly forms a five membered ring with vicinal thiols and promotes the Ca^{2+} dependent intramitochondrial NAD^+ hydrolysis [34,45].

The reversible permeabilization of the inner mitochondrial membrane induced by Ca^{2+} in the presence of prooxidants is under the regulatory influence of the redox status of both mitochondrial pyridine nucleotides and membrane protein thiols-forming cross-linked protein aggregates [18,46,47], in which Ca^{2+} and prooxidants play a concerted role in ROS accumulation within the mitochondria [36,48]. In fact, the prooxidant and the thiol cross-linker per se were unable to cause mitochondrial swelling (Fig. 1), depolarization of $\Delta\Psi$ (Fig. 2), oxidation of NAD(P)H (Fig. 5) or of thiol groups (Fig. 6), suggesting that Ca^{2+} is essential and must act in additional steps in the sequence of events that lead to mitochondrial permeabilization [38,41]. Therefore, the inhibition of the MPT by TAM seems to be dependent on its antioxidant/scavenging activities [19], protecting against pyridine nucleotides and –SH groups oxidation.

On the other hand, since TAM incorporates to a greater extent into native membranes relatively to the respective lipid dispersions [13], as it binds to proteins in a non specific manner [49], it is probable that in addition the its antioxidant mechanism TAM may bind to the mitochondrial cyclophylin D, a necessary component for the MPT pore, inhibiting the pore assembly, in a similar way to what was described for CyA [15]. Moreover, since TAM also inhibits the MPT induced by PhAsO, this antiestrogen may act by binding to the –SH sites of the ANT, a central component of the MPT pore, protecting thiol cross-linking or oxidation of these critical groups in the MPT induction. Therefore, the MPT inhibition by TAM may depend on different effects of this drug at the mitochondrial level.

Although TAM and its metabolite, OHTAM, inhibit the Ca^{2+}-prooxidant or the Ca^{2+}-Pi-induced MPT [14,23], they exhibit different apoptotic effects [50] in human mammary epithelial cells which may be related to the slightly effects of OHTAM on the mitochondrial bioenergetic functions [51], in contrast with the deleterious effects of TAM as evaluated in rat liver mitochondria [32]. Until recently, the main function of mitochondria was thought to be limited to the production of energy in the form of ATP. However, in a few years a new role for this organelle has emerged. It is now well established that mitochondria play a critical role in the regulation of apoptosis by acting as reservoirs for a multitude of apoptogenic proteins [52]. Most of the mechanisms of necrosis and apoptosis appear to include the MPT that leads to the disruption of the outer membrane and release of apoptotic factors from the intermembrane space [11].

Moreover, this work also demonstrates that TAM inhibits the CyA-sensitive MPT induced by the combination of Ca^{2+} with different inducers and also suggests an MPT-independent pathway for TAM-induced apoptosis and describes an activity distinct from their ER-dependent ability that may lead to necrotic cell death as an additional mechanism for the effectiveness of TAM in cancer therapy and chemoprevention.

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References

[1] V.C. Jordan, Long-term adjuvant tamoxifen therapy for breast cancer, Breast Cancer Res. Treat. 15 (1990) 125–136.
[2] B. Fisher, J.P. Costantino, D.L. Wickerham, C.K. Redmond, M. Kavanah, W.M. Cronin, V. Vogel, A. Robidoux, N. Dimitrov, J. Atkins, M. Daly, S. Wieand, E. Tan-Chiu, L. Ford, N. Wolmark, Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 study, J. Natl. Cancer Inst. 90 (1998) 1371–1388.
[3] I.N. White, Tamoxifen: is it safe? Comparison of activation and detoxication mechanisms in rodents and in humans, Curr. Drug Metab. 4 (2003) 223–239.
[4] W.T. Couldwell, M.H. Weiss, C.M. Degiorgio, L.P. Weimar, D.R. Hinton, G.R. Conti, P.S. Ehresmsan, M.L.J. Appuzo, P. Korblith, N. Detriboler, Clinical and radiographic response in patients with recurrent malignant gliomas treated with high-dose tamoxifen, Neurosurgery 32 (1993) 485–490.
[5] J.D. Croxtall, C. Emmas, J.O. White, Q. Choudhary, R.J. Flower, Tamoxifen inhibits growth of oestrogen receptor-

negative A549 cells, Biochem. Pharmacol. 47 (1994) 197–202.
[6] C. Charlier, A. Chariot, N. Antoine, M. Merville, J. Gielen, V. Castronovo, Tamoxifen and its active metabolite inhibit growth of estrogen receptor-negative MDA-MB-435 cells, Biochem. Pharmacol. 49 (1995) 351–358.
[7] P. Diel, K. Smolnikar, H. Michna, The pure antiestrogen ICI 182780 is more effective in the induction of apoptosis and down regulation of Bcl-2 than tamoxifen in MCF-7 cells, Breast Cancer Res. Treat. 58 (1999) 87–97.
[8] C. Ferlini, G. Scambia, M. Marone, M. Distefano, C. Gaggini, Tamoxifen induces oxidative stress and apoptosis in oestrogen receptor-negative human cancer cell lines, Brit. J. Cancer 79 (1999) 257–263.
[9] S. Mandlekar, V. Hebbar, K. Christov, A.-N.T. Kong, Pharmacodynamics of tamoxifen and its 4-hydroxy and *N*-desmethyl metabolites: activation of caspases and induction of apoptosis in rat mammary tumors and in human breast cancer cell lines, Cancer Res. 60 (2000) 6601–6606.
[10] M. Obrero, D.V. Yu, D.J. Shapiro, Estrogen receptor-dependent and estrogen receptor-independent pathways for tamoxifen and 4-hydroxytamoxifen-induced programmed cell death, J. Biol. Chem. 277 (2002) 45695–45703.
[11] M. Crompton, The mitochondrial permeability transition pore and its role in cell death, Biochem. J. 341 (1999) 233–249.
[12] G. Kroemer, J.C. Reed, Mitochondrial control of cell death, Nat. Med. 6 (2000) 513–519.
[13] J.B.A. Custódio, L.M. Almeida, V.M.C. Madeira, A reliable and rapid procedure to estimate drug partitioning in biomembranes, Biochem. Biophys. Res. Commun. 176 (1991) 1079–1085.
[14] J.B.A. Custódio, A.J.M. Moreno, K.B. Wallace, Tamoxifen inhibits induction of the mitochondrial permeability transition by Ca2+ and inorganic phosphate, Toxicol. Appl. Pharmacol. 152 (1998) 10–17.
[15] M. Zoratti, I. Szabo, The mitochondrial permeability transition, Biochim. Biophys. Acta 1241 (1995) 139–176.
[16] J.L. Scarlett, M.A. Packer, C.M. Porteous, M.P. Murphy, Alterations to glutathione and nicotinamide nucleotides during the mitochondrial permeability transition induced by peroxynitrite, Biochem. Pharmacol. 52 (1996) 1047–1055.
[17] A.J. Kowaltowski, R.F. Castilho, A.E. Vercesi, Mitochondrial permeability transition and oxidative stress, FEBS Lett. 495 (2001) 12–15.
[18] A.P. Halestrap, G.P. McStay, S.J. Clarke, The permeability transition pore complex: another view, Biochimie 84 (2002) 153–166.
[19] J.B.A. Custódio, T.C.P. Dinis, L.M. Almeida, V.M.C. Madeira, Tamoxifen and hydroxytamoxifen as intramembranous inhibitors of lipid peroxidation. Evidence for peroxyl radical scavenging activity, Biochem. Pharmacol. 47 (1994) 1989–1998.
[20] H. Wiseman, M.J. Laughton, H.R.V. Arnstein, M. Cannon, B. Halliwell, The antioxidant action of tamoxifen and its metabolites, FEBS Lett. 263 (1990) 192–194.
[21] A.-L. Nieminen, A.M. Byrne, B. Herman, J. Lemasters, Mitochondrial permeability transition in hepatocytes induced by *t*-BuOOH: NAD(P)H and reactive oxygen species, Am. J. Physiol. 272 (1997) 1286–1294.
[22] E.A. Belyaeva, V.V. Glazunov, S.M. Korotkov, Cyclosporin A-sensitive permeability transition pore is involved in Cd(2+)-induced dysfunction of isolated rat liver mitochondria: doubts no more, Arch. Biochem. Biophys. 405 (2002) 252–264.
[23] C.M.P. Cardoso, L.M. Almeida, J.B.A. Custódio, 4-Hydroxytamoxifen is a potent inhibitor of the mitochondrial permeability transition in vitro, Mitochondrion 1 (2002) 485–495.
[24] A.G. Gornall, C. Bardawill, M.M. David, Determination of serum proteins by means of the biuret reaction, J. Biol. Chem. 177 (1949) 751–756.
[25] J.B.A. Custódio, C.M.P. Cardoso, L.M. Almeida, Thiol protecting agents and antioxidants inhibit the mitochondrial permeability transition promoted by etoposide: implications in the prevention of etoposide-induced apoptosis, Chem. Biol. Interact. 140 (2002) 169–184.
[26] N. Kamo, M. Muratsugu, R. Hongoh, V. Kobatake, Membrane potential of mitochondria measured with an electrode sensitive to tetraphenylphosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state, J. Membr. Biol. 49 (1979) 105–121.
[27] A.J. Moreno, V.M.C. Madeira, Mitochondrial bioenergetics as affected by DDT, Biochim. Biophys. Acta 1060 (1991) 166–174.
[28] V.M.C. Madeira, A rapid and ultrasensitive method to measure Ca2+ movements across biological membranes, Biochem. Biophys. Res. Commun. 64 (1975) 870–876.
[29] R. Catisti, A.E. Vercesi, The participation of pyridine nucleotides redox state and reactive oxygen in the fatty acid-induced permeability transition in rat liver mitochondria, FEBS Lett. 464 (1999) 97–101.
[30] D.D. Monte, D. Ross, G. Bellomo, L. Eklow, S. Orrenius, Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes, Arch. Biochem. Biophys. 235 (1984) 334–342.
[31] K.M. Broekemeier, M.E. Dempsey, D.R. Pfeiffer, Cyclosporine A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria, J. Biol. Chem. 264 (1989) 7826–7830.
[32] C.M.P. Cardoso, J.B.A. Custódio, L.M. Almeida, A.J.M. Moreno, Mechanisms of the deleterious effects of tamoxifen on mitochondrial respiration rate and phosphorylation efficiency, Toxicol. Appl. Pharmacol. 176 (2001) 145–152.
[33] T.E. Gunter, K.K. Gunter, S.S. Sheu, C.E. Gavin, Mitochondria Ca2+ transport: physiological and pathological relevance, Am. J. Physiol. 267 (1994) C313–C339.
[34] C. Richter, M. Schweizer, Oxidative stress in mitochondria, in: J. Scandalios (Ed.), Oxidative Stress and the Molecular Biology of Antioxidant Defences, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1997, pp. 169–200.
[35] R.F. Castilho, A.J. Kowaltowski, A.E. Vercesi, The irreversibility of inner mitochondrial membrane permeabilization by Ca2+ plus prooxidants is determined by the extent of membrane protein thiol cross-linking, J. Bioenerg. Biomembr. 28 (1996) 523–529.

[36] A.-L. Nieminen, A.K. Saylor, S.A. Tesfai, B. Herman, J. Lemasters, Contribution of the mitochondrial permeability transiton to lethal injury after exposure of hepatocytes to *t*-butylhydroperoxide, Biochem. J. 307 (1995) 99–106.
[37] A.J. Kowaltowski, A.E. Vercesi, G. Fiskum, Bcl-2 prevents mitochondrial permeability transition and cytochrome c release via maintenance of reduced pyridine nucleotides, Cell Death Differ. 7 (2000) 903–910.
[38] V.G.R. Valle, M.M. Fagian, L.S. Parentoni, A.R. Meinicke, A.E. Vercesi, The participation of reactive oxygen species and protein thiols in the mechanism of mitochondrial inner membrane permeabilization by calcium plus prooxidants, Arch. Biochem. Biophys. 307 (1993) 1–7.
[39] R.F. Castilho, A.J. Kowaltowski, A.R. Meinicke, E.J. Bechara, A.E. Vercesi, Permeabilization of the inner mitochondrial membrane by Ca2+ ions is stimulated by *t*-butyl hydroperoxide and mediated by reactive oxygen species, Free Radic. Biol. Med. 18 (1995) 479–486.
[40] R.F. Castilho, A.J. Kowaltowski, A.R. Meinicke, E.J. Bechara, A.E. Vercesi, Oxidative damage of mitochondria induced by Fe(II) citrate or *t*-butyl hydroperoxide in the presence of Ca2+ effect of coenzyme Q redox state, Free Radic. Biol. Med. 18 (1995) 55–59.
[41] M.M. Fagian, L. Pereira-da-Silva, I.S. Martins, A. Vercesi, Membrane protein thiol cross-linking associated with the permeabilization of the inner mitochondrial membrane by Ca2+ plus prooxidants, J. Biol. Chem. 265 (1990) 19955–19960.
[42] R. Radi, A. Cassina, R. Hodara, C. Quijano, L. Castro, Peroxynitrite reactions and formation in mitochondria, Free Radic. Biol. Med. 33 (2002) 1451–1464.
[43] S.A. Novgorodov, E.V. Kultayeva, L.S. Yaguzhinsky, V.V. Lemeshko, Ion permeability induction by SH cross-linking reagents in rat liver mitochondria is inhibited by the free radical scavenger, butyl-hydroxytoluene, J. Bioenerg. Biomembr. 19 (1987) 191–202.
[44] W.L. Zahler, W.W. Cleland, A specific and sensitive assay for disulfides, J. Biol. Chem. 243 (1968) 716–719.
[45] V. Petronilli, P. Constantini, L. Scorrano, R. Colonna, S. Passamonti, P. Bernardi, The voltage sensor of the mitochondrial permeability transition pore is tuned by the oxidation-reduction state of vicinal thiols, J. Biol. Chem. 269 (1994) 16638–16642.
[46] M. Schweizer, P. Durrer, C. Richter, Phenylarsine oxide stimulates pyridine nucleotide-linked Ca2+ release from rat liver mitochondria, Biochem. Pharmacol. 48 (1994) 967–973.
[47] A.P. Halestrap, K.-Y. Wodfield, C.P. Connern, Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase, J. Biol. Chem. 272 (1997) 3346–3354.
[48] A.M. Byrne, J.J. Lemasters, A.-L. Nieminen, Contribution of increased mitochondrial free Ca2+ to the mitochondrial permeability transition induced by *tert*-butylhydroperoxide in rat hepatocytes, Hepatology 29 (1999) 1523–1531.
[49] T.R.V. Oosbree, M.R. Olsen, A.C. Tate, V.C. Jordan, G.C. Mueller, An immobilized antiestrogen binds a specific uterine protein in addition to estrogen receptor proteins, Mol. Cell. Endocrinol. 35 (1984) 143–149.
[50] E.C. Dietze, L.E. Caldwell, S.L. Grupin, M. Mancini, V.L. Seewalt, Tamoxifen but not 4-hydroxytamoxifen initiates apoptosis in p53(−) normal human mammary epithelial cells by inducing mitochondrial depolarisation, J. Biol. Chem. 276 (2001) 5384–5394.
[51] C.M.P. Cardoso, A.J.M. Moreno, L.M. Almeida, J.B.A. Custódio, 4-Hydroxytamoxifen induces slight uncoupling of mitochondrial oxidative phosphorylation system in relation to the deleterious effects of tamoxifen, Toxicology 179 (2002) 221–232.
[52] P.A. Parone, D. James, J.C. Martinou, Mitochondria: regulating the inevitable, Biochimie 84 (2002) 105–111.