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Characterization of hydrophobic interaction and antioxidant properties of the phenothiazine nucleus in mitochondrial and model membranes

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Abstract
The antioxidant properties of the phenothiazine nucleus (PHT) associated with mitochondrial membranes and liposomes were investigated. PHT exhibited hydrophobic interaction with lipid bilayers, as shown by the quenching of excited states of 1-palmitoyl-2[10-pyran-1-yl)]-decanoyl-sn-glycero-3-phophocholine (PPDPC) incorporated in phosphatidylcholine/phosphatidylethanolamine/cardiolipin liposomes, observed even in high ionic strength; and by the spectral changes of PHT following the addition of mitochondrial membranes. Inserted into bilayers, 5 μM PHT was able to protect lipids and cytochrome *c* against pro-oxidant agents and exhibited spectral changes suggestive of oxidative modifications promoted by the trapping of the reactive species. In this regard, PHT exhibited the ability to scavenge DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical. PHT was also able to protect rat liver mitochondria against peroxide- and iron-induced oxidative damage and consequent swelling. At the concentration range in which the antioxidant properties were observed, PHT did not cause alterations in the membrane structure and function. This study contributes to the comprehension of the correlation structure and function of phenothiazines and antioxidant properties.

Keywords: *phenothizine, antioxidant, free radical, mitocondrial membranes, liposomes*

Introduction

The modification of membrane components by nitro-oxidative species has been related to changes in biological functions associated with diseases such as cancer, diabetes, schizophrenia and Parkinson's and Alzheimer's diseases [1–5]. Nitrosative and oxidative species promote chemical modifications of the membrane lipid matrix and membrane-associated proteins, leading to alterations of several key properties of the membranes. In addition to physical factors such as temperature and chemical modifications promoted by oxidative and nitrosative species [6,7], the organization of these membrane complex entities is susceptible to perturbation by membrane-associating molecules [8,9]. Literature data have demonstrated the interaction of some phenothiazine derivatives with biological membranes and hydrophobic domains of proteins. This property may be related to their biological effects. Furthermore, phenothiazine derivatives have photochemical properties [10]. The pro-apoptotic effects of both ground state and excited phenothiazines in tumour cell lines point to the potential use of these

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drugs in cancer treatment [11,12]. The classical phenothiazine derivative trifluoperazine (TFP) affects the activity of membrane proteins by interacting with hydrophobic sites located in the membrane-embedded domains [13]. Also, the presence of antioxidant binding sites in the hydrophobic core of the inner mitochondrial membrane or in the mitochondrial matrix has been previously proposed. These sites may accept a variety of unrelated hydrophobic compounds, modulating ROS-associated mitochondrial processes independently of free radical scavenging activities [14,15]. Previous studies have also demonstrated that thioridazine interacts with the inner mitochondrial membrane, likely close to its surface, acquiring antioxidant activity toward processes with potential implications in apoptosis such as $O_2^{\bullet -}$ accumulation, peroxidation of mitochondrial membrane lipids, mitochondrial permeability transition and the associated release of cytochrome *c* [16].

Phenothiazine derivatives have also been the focus of several biological, chemical, physical-chemical and photochemical studies due to their pharmaceutical properties and applications [17,18]. Particularly, the photochemical behaviour of phenothiazines has gained interest, as the compounds containing a phenothiazine moiety may promote photosensitizing effects in patients under therapy with these drugs [19,20]. It has been demonstrated that the aggregates of three phenothiazine derivates used in the treatment of schizophrenia—thioridazine (TR), trifluoperazine (TFP) and fluphenazine (FP)—form stable cation radicals when photoexcited by UV irradiation [10]. However, in the presence of membranes, phenothiazine cation radicals become reactive in such a way that they promote photodamage in membrane lipids when irradiated [21,22].

Recently, our research group claimed a patent register (PI 0801398-3) for the phenothiazinic nucleous PHT (Figure 1), based on a potential cosmetic-pharmaceutical use of this compound. Parallel studies concerning the biological effects of this drug are crucial for future applications. Furthermore, the present study can contribute to the comprehension of the role played by phenothiazine substituents in the biological effects of these drugs. In the present study, the interaction of PHT with lipid bilayers was characterized and related to the specific antioxidant activity of this compound towards lipids.

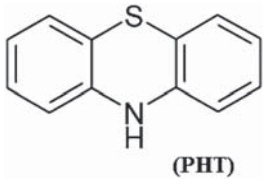


Figure 1. Structure of phenothiazinic nucleous.

Materials and methods

Materials

Phenothiazine (PHT), Cytochrome *c* (horse heart, type III), L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), Cardiolipin (Cl), Sodium phosphate, HEPES, NaCl and herring sperm DNA (fragmented 50 base pair) were obtained from Sigma Chemical Company (St. Louis, MO). PPDPC was obtained from K&V Bioware (Espoo, Finland). All other reagents were of the highest commercially available grade. The drug was solubilized in dimethyl sulphoxide (DMSO) from Merck (Darmstadt, Germany). All stock solutions were prepared using glass-distilled deionized water (a mixed bed of ion exchanger, Millipore) and the pH was measured using a combined glass electrode (Orion Glass pH SURE-FLOW). The reference electrode (ROSS, model 8102) was filled with Orion filling solution (ROSS). The pH meter was calibrated using METREPAK pHydrion standard buffer solutions (Brooklyn, NY). Concentrations of the non-fluorescence phospholipids were determined gravimetrically with a high-precision electrobalance (Cahn Instruments, Cerritos, CA) and the concentration of pyrene containing PPDPC was obtained spectrophotometrically using the molar extinction coefficient $\varepsilon = 42\ 000 M^{-1}$ at 344 nm.

Preparation of liposomes

Preparations of phosphatidylcholine/phosphatidylethanolamine/cardiolipin PCPECl (5:3:2) were first dissolved in chloroform, which was evaporated with N_2 gas. The lipid residue was kept under reduced pressure for at least 2 h and the resulting test-tube film was stored at −20°C until use. The film was hydrated by adding a cold 10 mM sodium phosphate buffer. After the addition of the buffer, the mixture was stirred with a vortex and sonication was performed in Ney Ultrasonik equipment (J. M. Ney Co., Bloomfield, CT) over a 30-min period. The mean diameter of sonicated unilamelar liposomes has been previously determined [23] as 61.1 ± 0.3 nm by dynamic light scattering using a ZetaPlus-ZetaPotential Analyser (Brookhaven Instruments Corporation, Holtsville, NY). When indicated, unilamelar liposomes were also obtained by the extrusion of hydrated lipid dispersions in an Avanti Miniextruder acquired from Avanti Polar Lipids, Inc. (Alabaster, AL). Samples were subjected to 11 passes through two polycarbonate filters (100 nm pore size, Nucleopore, Pleasanton, CA) installed in tandem.

Isolation of rat liver mitochondria

Liver mitochondria were isolated by conventional differential centrifugation [24] from adult rats. The

livers were homogenized in 250 mM sucrose, 1 mM EGTA and 10 mM HEPES buffer (pH 7.4). The mitochondrial suspension was washed twice in the same medium, but containing 0.3 mM EGTA, and the final pellet was resuspended in 250 mM sucrose to a final protein concentration of 80–100 mg/mL.

Steady-state fluorescence measurements

The binding of PHT to PLs was assessed by monitoring the efficiency of the quenching of the fluorescence emission of pyrene containing phospholipid analog PPDPC by the drug [25,26]. The measurements were conducted with a Perkin-Elmer (Foster City, CA) LS50B spectrofluorometer using 5.0 nm band-passes for both the excitation and emission beams 344 and 394 nm, respectively. Two milliliters of 25 μM lipid dispersion were placed into a magnetically stirred four-window quartz cuvette in a holder thermostated at 25°C with a circulating water bath. Subsequently, 2.0 aliquots of 1 mM solution (PHT in DMSO) of the drug were added and the quenching of pyrene fluorescence was observed. Thereafter, proper aliquots of 5 M NaCl, 2 mM DNA or 1 mM PCPECl (5:3:2) LUVs were added to yield the indicated final concentrations. Changes in the pyrene fluorescence between additions were allowed to stabilize for 5–20 min, whereafter the intensity value was recorded and subsequently corrected for sample dilution. The merits as well as limitations of the use of pyrene-labelled lipids in energy transfer measurements have been discussed elsewhere [27].

Electronic absorption spectrometry

Electronic absorption measurements of phospholipids or mitochondrial membrane with the drug and phospholipids with cytochrome *c* were conducted in a photodiode spectrophotometer (Shimadzu Scientific Instruments Inc., Columbia, MD) by using quartz cuvettes of 1.0 or 0.1 cm light path and a slit of 0.5 nm.

Lipid oxidation assays

Oxidative damage of liposomes and mitochondrial membranes was measured by the dosage of lipid hydroperoxide and malonyldialdehyde content. Lipid hydroperoxide (LOOH) measurement was done by the oxidation of Fe^{2+} in the presence of xylenol orange. Sonicated unilamelar liposomes (1 mM) were incubated in the presence or in the absence of 4 μM cytochrome *c* for 30 min at 37°C. Mitochondrial membranes (1 mg/mL) were incubated in the presence or in the absence of $(NH_4)_2Fe(SO)_4$ (50 μM) and 2 mM sodium citrate (used to induce damage). An aliquot of the sample (25 μL) was mixed with methanol (75 μL) and incubated for 30 min at room temperature (25°C) with 900 μL of hydroperoxide reagent, containing 100 μM xylenol orange, 250 μM $(NH_4)_2Fe(SO_4)_2$, 25 mM H_2SO_4 and 4 mM butylated hydroxytoluene in 90% methanol. The oxidation of Fe^{2+} by LOOH generates Fe^{3+} that reacts with xylenol orange to form the coloured compound that absorbs at 560 nm. LOOH concentration was calculated from $\varepsilon_{560nm} = 4.3 \times 10^4\ M^{-1}cm^{-1}$. For the determination of thiobarbituric acid reactive substance (TBARS) content present in liposomes (samples of 1 mM of lipids) or mitochondrial membranes, samples were incubated in the presence or absence of 4 μM cytochrome *c* or 50 μM $(NH_4)_2Fe(SO_4)$ respectively,$_4$ and 2 mM sodium citrate for 30 min at 37°C (final volume 150 μL). To determine TBARS, 150 μL 1% thiobarbituric acid (TBA) prepared in 50 mM NaOH plus 15 μL 10 M NaOH and 75 μL 20 % H_3PO_4 were added to the sample, followed by further incubation for 20 min at 85°C. The MDA-TBA complex was then extracted with 300 μL *n*-butanol and the absorbance measured at 535 nm. TBARs concentration was calculated from $\varepsilon = 1.56 \times 10^5\ M^{-1}$ [28].

Measurements of physical alterations in the mitochondrial membrane

Mitochondria (1 mg protein/mL) were incubated in a medium containing 125 mM sucrose, 65 mM KCl and 10 mM HEPES-KOH, pH 7.4 at 30°C with 75 μM ANS plus 1 μg/mL CCCP. Fluorescence was measured with an F-2500 spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) operating at $\lambda_{excitation}$ and $\lambda_{emission}$ wavelengths of 380 and 485 nm, respectively [29].

Mitochondrial swelling

Mitochondria (0.4 mg of protein) were incubated in 1.5 mL of a medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, pH 7.4, 5 mM potassium succinate (+2.5 μM rotenone) and 10 μM $CaCl_2$ at 30°C. Mitochondrial swelling was estimated from the decrease in absorbance at 540 nm measured by a Hitachi U-2000 Spectrophotometer (Tokyo, Japan).

Determination of protein-thiol content. Mitochondrial membrane thiol groups were measured using 5,5'-dithiobis (2-nitrobenzoic) acid (DTNB, Ellman's reagent) as described in [30]. After 10 min of incubation under swelling conditions, the mitochondrial suspension was submitted to three subsequent freeze–thawing procedures to release matrix proteins and then centrifuged for 15 min at 6000 *g*. The pellet was treated with 0.2 mL of 6% trichloroacetic acid and centrifuged at 6000 *g* for 15 min to precipitate the

mitochondrial membrane proteins. The final pellet was suspended with 1 mL of 0.5 M potassium phosphate buffer, pH 7.6, containing 0.4% SDS. After the addition of 0.1 mM DTNB, absorbance was determined at 412 nm and the number of thiol groups was calculated from $\varepsilon = 13\ 600\ M^{-1}$ [30].

Determination of mitochondrial glutathione content

After 15 min of incubation under swelling conditions, the mitochondria suspension was treated with 0.5 mL of 13% trichloroacetic acid and centrifuged at 900 *g* for 3 min. Aliquots (100 µL) of the supernatant were mixed with 2 mL of 100 mM NaH_2PO_4 buffer, pH 8.0, containing 5 mM EGTA. One hundred microlitres of *o*-phthalaldehyde (1 mg/mL) was added and the fluorescence was measured 15 min later using the 350-/420-nm excitation/emission wavelength pair in a F-2500 fluorescence spectrophotometer (Hitachi, Ltd., Tokyo, Japan) [31].

DPPH photometric assay

PHT and quecertin stock in etanol solutions (1.0 mg/mL) were diluted to final concentrations of 1, 2, 4, 6 and 8 µM in 40 mM sodium acetate buffer pH 5.5. One millilitre of a 0.1 mM DPPH ethanol solution was added to 1.5 mL of sample solutions of different concentrations, and allowed to react at 37°C. After 5 min the absorbance values were measured at 517 nm and converted into the percentage antioxidant activity (AA) using the following formula:

$$AA\% = 100 - \{[(Absorbance_{sample} - Absorbance_{blank}) \times 100]/Absorbance_{control}\}.$$

Ethanol (1.0 mL) plus PHT solution (1.5 mL) were used as a blank. DPPH solution (1.0 mL; 0.3 mM) plus 40 mM sodium acetate buffer pH 5,5 (1.5 mL) was used as a negative control. The positive control was quecertin solution.

Statistical analysis. The experiments were done in triplicate. The results are given as mean and standard deviation (SD). Student's *t*-test was used for comparison between two means. A difference was considered statistically significant when $p = 0.05$.

Results and discussion

PHT partitions into lipid bilayers via hydrophobic interactions without affecting membrane fluidity

PHT promoted the quenching of fluorescence from the pyrene-labelled phospholipid derivate PPDPC incorporated in PCPECL liposomes, revealing the binding of this drug to lipid bilayers (Figure 2A). According to the reversal of fluorescence quenching (RFQ) obtained in the presence of added NaCl, an increase in ionic strength did not lead to dissociation of PHT from PCPECL (Figure 2B). Thus, the fact that essentially no dissociation of PHT from PCPECL was observed even at NaCl concentrations far exceeding the physiological 150 mM (~ 0.75 M NaCl) suggests the occurrence of hydrophobic interactions (Figure 2B). PHT was titrated into PCPECL/PPDPC (5:3:2:0.1) dispersions and, subsequently, unlabelled phospholipids vesicles were added. The reversal of the quenching of the PPDPC residing in the liposome bilayers was analysed during the titration. Consistent with hydrophobic interaction, the addition of PCPECL to 25 µM PCPECL solution led to an increase in fluorescence intensity. In this condition, the increase of PPDPC fluorescence can be attributed to the dissociation of PHT from the PPDPC-marked lipid bilayers, probably caused by competition between PCPECL/PPDPC and PCPECL liposomes (Figure 2C).

The idea of electrostatic interactions being responsible for the binding of PHT to membranes was ruled out by the incapacity of DNA to scavenge PHT from PCPECL liposomes (Figure 2D). DNA molecules contain phosphate groups prone to compete with the phosphate groups of membrane phospholipids when these groups interact with polar and ionic moieties. If a significant amount of electrostatic interaction was present in the interaction of PHT with membrane phospholipids, an increase of PPDPC fluorescence caused by DNA addition would be expected.

The hydrophobic interaction of PHT with PCPECL liposomes pointed out the capacity of this compound to interact with the inner mitochondrial membranes. The interaction of PHT with mitochondrial membranes was characterized by electronic absorption spectroscopy of the drug following the addition of crescent amounts of mitochondrial membranes. The electronic absorption spectra of PHT (15 µM) were determined in the course of the titration using crescent mitochondrial membrane concentrations (mg/mL of protein) and are depicted in Figure 3. Crescent amounts of mitochondrial membrane fragments promoted a decrease and red shift of the absorbance bands at 228, 250 and 315 nm. The observed spectral changes suggest PHT migration from a hydrophilic to a hydrophobic ambient. The insertion of PHT into bilayers could also be accompanied by disaggregation of the drug or eventual aggregation of the domains of membrane mitochondrial lipids or transmembrane proteins.

To probe the effect of PHT on the fluidity of membranes, the drug was assayed with mitochondrial membranes labelled with the fluorescent probe ANS. As shown in Figure 4, PHT did not promote changes in membrane fluidity at a concentration range from 2.5–25 µM since no significant ANS fluorescence decrease was observed. The decrease of ANS fluorescence observed at higher PHT concentrations (above

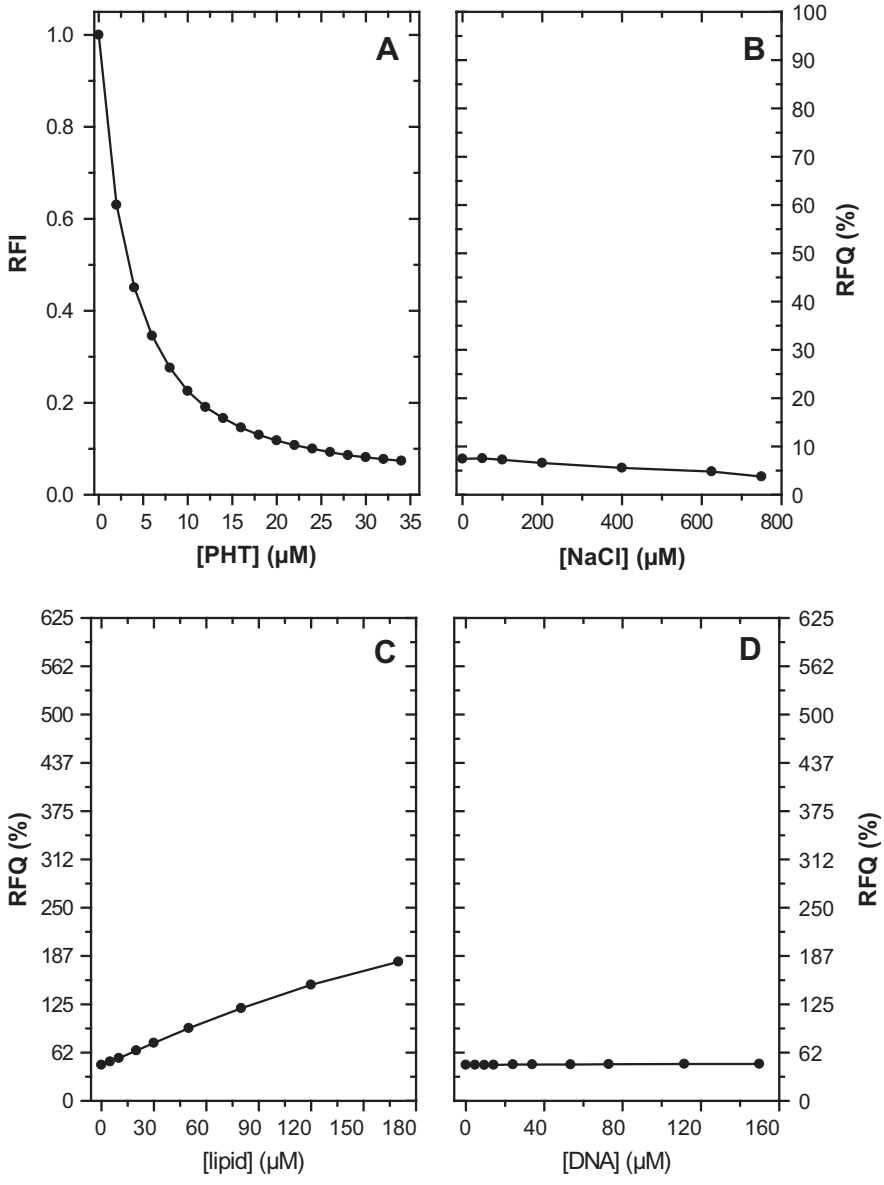


Figure 2. Association of PHT to lipid bilayers. (A) Binding of PHT (•) to PCPECL (5:3:2) as revealed by the decrease in the RFI of PPDPC as a function of [drug]. Subsequent detachment of the bound PHT by added (B) NaCl, (C) PCPECl liposomes or (D) DNA, evident as RFQ compared to the initial value before addition of the indicated solute. Total lipid concentration in 10 mM Sodium phosphate, pH 7.4 was 25 mM. Temperature was maintained at 25°C.

50 μM) was due to a drastic blue shift of the fluorescence (inset of Figure 4) probably caused by the drug's interactions with the fluorescent probe. Considering the absence of substituents in the PHT structure, the above results are according to previous studies by Hendrich et al. [32] showing that phenothiazine compounds substituted with CF_3t position 2 of the ring and acyl chain-connecting ring system with an $NHSO_2CH_3$ group were the most active membrane pertubants.

PHT is a potent antioxidant for lipids and proteins

The interaction of ground state phenothiazine derivatives with biological membranes has been described as an inhibitory action on lipid peroxidation assays in liposomes and mitochondrial membranes [16]. However, the photochemically generated cation radical of phenothiazine derivatives is a pro-oxidant species able to attack lipid membranes [21,22]. The antioxidant effect of ground and excited states of PHT on membranes was investigated by measuring the TBARS content of the membranes assayed with pro-oxidant agents in the presence and in the absence of the drug. The oxidizing compounds used in the mitochondrial mimetic membrane, i.e. PCPECL liposomes and mitochondrial membranes were cytochrome *c* and Fe^{2+}/citrate, respectively. When associated with membranes, cytochrome *c* can react with residual lipid-derived peroxides and aldehydes, leading to the production of free radicals and singlet oxygen. These reactive species promote oxidative damage

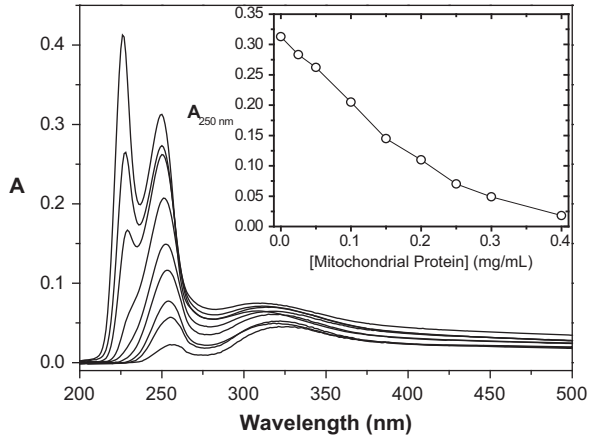


Figure 3. Spectral changes of PHT from the titration of crescent mitochondrial membrane concentrations. The thick line (highest absorbance) corresponds to the PHT spectrum in HEPES buffer at pH 7.4 in the absence of mitochondrial membranes. The thin lines from top to bottom correspond, respectively, to PHT spectra in the presence of crescent mitochondrial membrane concentrations of 0.025 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.15 mg/ml, 0.2 mg/ml, 0.25 mg/ml, 0.3 mg/ml and 0.4 mg/ml. The inset shows the absorbance of PHT at 250 nm as a function of mitochondrial membrane concentration. Concentration of PHT was 15 μM and temperature 25°C.

of the lipid content and the consequent production of TBARS [33].

Figure 5A shows that, in the ground state (dark), 1 μM PHT promoted a 53% decrease in TBARS content in PCPECL liposomes assayed with cytochrome *c*. Under UV irradiation, the same concentration of PHT promoted a 79% decrease of TBARS content. However, this result is not proof of increased antioxidant activity of the PHT triplet state or photo-

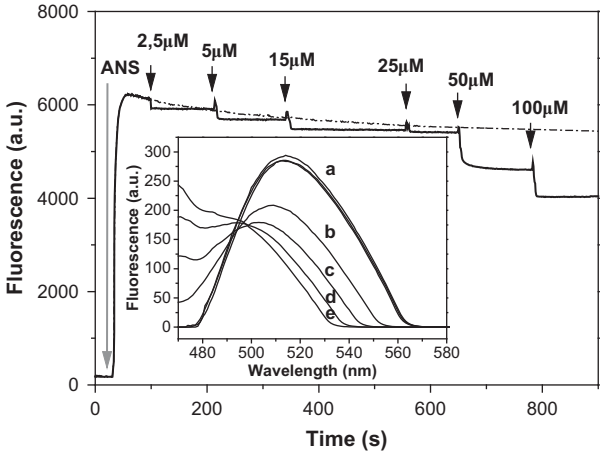


Figure 4. Fluorescence intensity of ANS in the presence of mitochondrial membranes and different PHT concentrations. The dashed line corresponds to the time course of ANS fluorescence in the presence of mitochondrial membranes (1 mg/mL) and the thick line to the effect of the addition of crescent amounts of PHT. The inset shows the electronic absorption spectra of 75 μM ANS in the absence and in the presence of (a) 2.5–25 μM PHT; (b) 75 μM PHT; (c) 125 μM PHT; (d) 175 μM PHT and (e) 225 μM PHT.

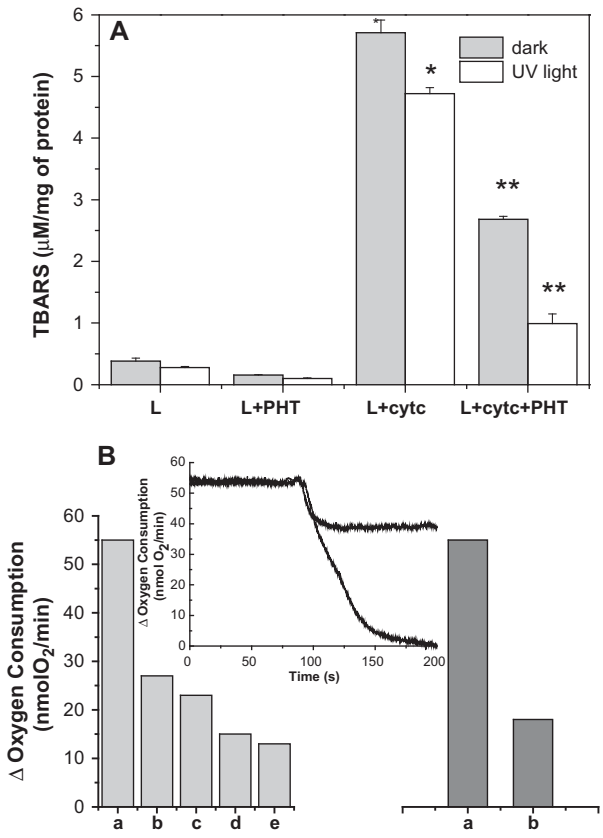


Figure 5. Effect of PHT on lipid oxidation of PCPECL liposomes promoted by Fe^{3+} cytochrome *c* and assayed as TBARS generation and oxygen consumption. (A) TBARS generation of 1 mM PCPECL liposomes incubated under UV irradiation (crossed column) and in the dark (white column) in 5 mM universal buffer, pH 7.4 for 30 min at 37°C under the following conditions: 1 mM PCPECL (control, signed as L), 1 mM PCPECL plus 1 μM PHT (L+PHT), 1 mM PCPECL plus 2 μM cytochrome *c* (L+cyt*c*) and 1 mM PCPECL plus 2 μM cytochrome *c* plus 1 μM PHT (L+cyt*c*+PHT). The symbol *indicates significantly different from the negative control (L) and **indicates significantly different from the positive control (L + cyt*c*). (B) Oxygen consumption of PCPECL liposomes challenged by Fe^{2+} (left panel) in the absence (*a*) and in the presence of 2 (*b*), 10 (*c*), 25 (*d*) and 100 (*e*) μM PHT and cytochrome *c* (right panel) in the absence (*a*) and in the presence (*b*) of 10 μM PHT. The results are the mean of three independent experiments. The inset in (B) is a representative curve obtained in the absence and in the presence of 25 μM PHT. Statistical analysis was done as described in Materials and methods.

generated cation radical. The UV light *per se* led to a decrease of TBARS content, probably due to the degradation of oxidized intermediates. The degradation of TBARS precursors might be enhanced by the reaction with the PHT excited state and the photo-generated cation radical. Figure 5B shows that, in the ground state (dark), PHT promoted a significant decrease of the oxygen consumption that accompanies the oxidation of lipids in PCPECL liposomes promoted by Fe^{2+} and by cytochrome *c*, in a dose-dependent manner.

Figure 6 shows that 1 μM PHT decreased by 92% TBARS content of mitochondrial membranes

challenged by the pro-oxidant system Fe^{2+}/citrate both under irradiation and in the dark. The possibility of an artifact produced by the undesired reaction of PHT with TBA was discarded since PHT did not significantly decrease the TBARS content of mitochondrial membranes when it was added after 30 min incubation with the pro-oxidants.

To evaluate the antioxidant mechanism exhibited by PHT, the capacity of the drug to prevent cytochrome *c* bleaching mediated by free radicals generated in the course of the reaction of the protein with lipid-derived peroxides was probed. Cytochrome *c* spectra were run before and after 30 min incubation at 37°C in a PCPECL solution. Figure 7 shows that in the absence of PHT, extensive cytochrome *c* Soret band bleaching was observed. In the presence of PHT, cytochrome *c* bleaching was almost entirely prevented concomitant with PHT spectral changes, suggestive of drug oxidation. This result is compatible with PHT acting as a trap of pro-oxidant species. Considering that, in addition to free radical production, singlet oxygen is also generated in the course of the reaction of cytochrome *c* with membrane lipids, the assay was repeated in an argon atmosphere (not shown). Identical results were obtained in the argon atmosphere, suggesting that free radicals generated from the reaction of cytochrome *c* with lipid-derived peroxides were responsible for cytochrome *c* bleaching and that PHT acted to trap these reactive species (not shown). PHT exhibited the same spectral changes when incubated in PCPECL liposomes challenged by Fe^{2+}. When present, 10 μM PHT exhibited the same spectral changes observed after incubation with cytochrome *c* and peroxide and compatible with the formation of oxidized PHT derivatives. Furthermore, in a higher PHT concentration (50 μM), concomitant with the spectral changes in the UV region, it was possible to see the appearance of the spectrum of PHT cation radical (not shown). In addition, the antioxidant activity of PHT was evaluated by the ability to scavenge DPPH radicals in comparison with quecertin. DPPH is a free radical, stable at room temperature, which produces a violet solution in ethanol. The solution becomes uncoloured when the free radical is reduced in the presence of an antioxidant molecule [34]. The reducing activity against DPPH was presented as EC_{50}. The EC_{50} values obtained for quecertin and PHT were 1.68 ± 0.39 μM and 3.1 ± 0.43 μM, respectively.

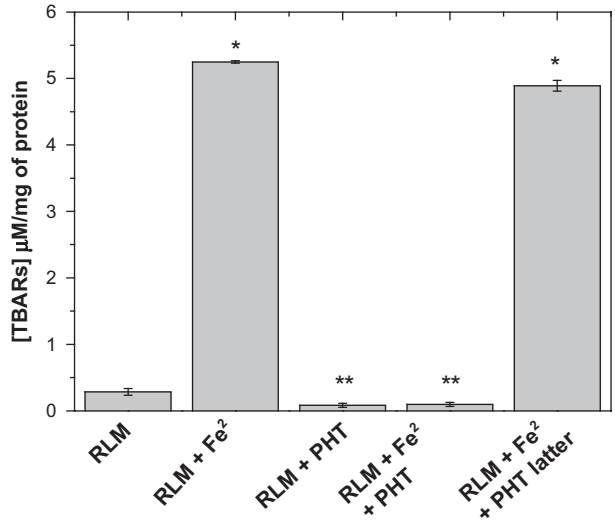


Figure 6. Effect of PHT on the lipid oxidation of mitochondrial membranes assayed as TBARS generation. Mitochondria (1 mg/mL) were incubated in 5 mM universal buffer pH 7.4 for 30 min at 37°C under the following conditions: the control 1 mg protein/mL rat liver mitochondria (control, signed as RLM), 1 mg protein/mL rat liver mitochondria plus 50 μM Fe^{2+} (RLM + Fe^{2+}), 1 mg protein/mL rat liver mitochondria plus 2.5 μM PHT (RLM +PHT), 1 mg protein/mL rat liver mitochondria plus 50 μM Fe^{2+} plus 2.5 μM PHT (RLM + Fe^{2+} + PHT), 1 mg/mL protein of rat liver mitochondria plus 50 μM Fe^{2+} followed by the addition of 2.5 μM PHT at the final time incubation (RLM + Fe^{2+} + PHT latter). The symbol *indicates significantly different from the negative control (RLM) and **indicates significantly different from the positive control (RLM + Fe^{2+}). The results are the mean of three independent experiments. Statistical analysis was done as described in Materials and methods.

PHT protects rat liver mitochondria against MPTP opening promoted by Fe^{2+}/citrate but not by t-BuOOH and teluranas

Considering the efficient antioxidant action of PHT towards cytochrome *c* bleaching (Figure 7) and previous literature data describing the capacity of phenothiazine derivatives to inhibit Ca^{2+}/*t*-BOOH-induced mitochondrial permeability transition [16], the effect of PHT on rat liver mitochondria challenged by *t*-BuOOH and Fe^{2+}/citrate in the presence of Ca^{2+} was probed. In contrast to the derivative thioridazine that is a classical inhibitor of the MPTP (mitochondrial permeability transition pore) induced by Ca^{2+} and *t*-butyl hydroperoxide [16], PHT did not significantly inhibit the mitochondrial swelling induced by *t*-BOOH or the oxidation of mitochondrial thiol groups, glutathione or NADPH (not shown). Similarly, PHT was totally inefficient in protecting rat liver mitochondria against swelling promoted by the organo-teluric (telurana) SH reagent RT-03 (not shown) [35]. However, as evidenced by the mitochondrial swelling and protein thiol group dosage assays, PHT provided significant protection against the mitochondrial damage induced by Fe^{2+}/citrate (Figures 8A and B). Considering that PHT acts as a trap of pro-oxidant species, the above result can be rationalized by the different mechanisms of mitochondrial damage induced by the probed pro-oxidants. *Tert*-butylhydroperoxide promotes oxidative stress because it is decomposed by glutathione peroxidase (GPx) with a concomitant depletion of glutathione. The organic peroxide also generates high valence states of respiratory cytochromes that could

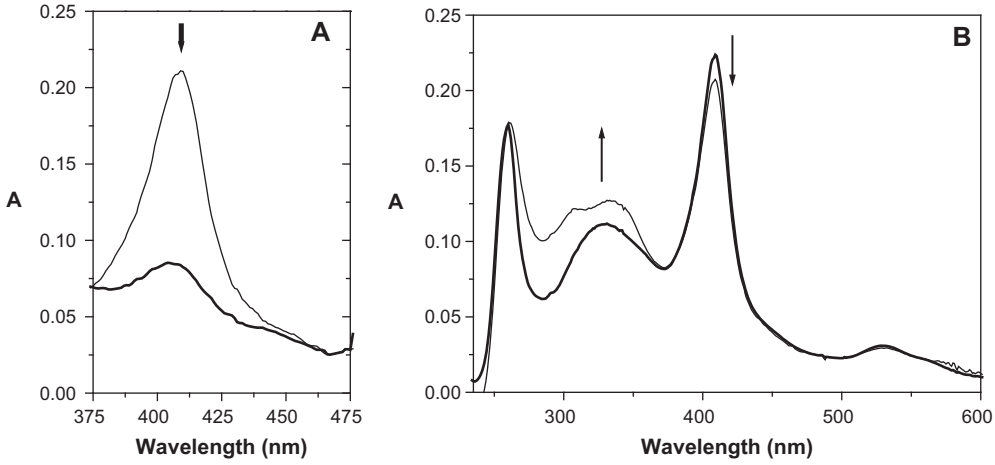


Figure 7. Effect of PHT on the bleaching of cytochrome *c* promoted by lipid-derived free radicals. (A) Bleaching of cytochrome *c* Soret band in the absence of PHT. The thin solid line represents the spectrum obtained immediately after the addition of 2.5 μM cytochrome *c* to 1 mM PCPECL liposomes. The thick solid line represents the spectrum obtained after a 30 min incubation at 37°C. (B) Cytochrome *c* was assayed under the same conditions described in (A), but in the presence of 25 μM PHT. The thin solid line represents the spectrum obtained immediately after the addition of 2.5 μM cytochrome *c* to 1 mM PCPECL liposomes containing 25 μM PHT. The thick solid line represents the spectrum obtained after a 30 min incubation at 37°C. PHT spectral alterations promoted by oxidative attack of free radicals are visible at the 250–350 nm spectral region.

attack both lipid and thiol groups [31]. Therefore, PHT cannot impair the peroxidase activity of either GPx or respiratory cytochromes or the consequent direct oxidation of mitochondrial thiol groups. In contrast, the MPTP opening induced by Fe^{2+}/citrate should be a consequence of the extensive generation of free radicals accessible and rapidly trapped by PHT. Scheme 1 illustrates the above proposal.

Conclusion

According to the presented data, the following conclusions can be outlined:

i) Up to a concentration of 25 μM, PHT interacts with membrane model liposomes and mitochondrial membranes without affecting membrane fluidity. The interaction is evident from the quenching of the fluorescence from the pyrene-labelled phospholipid derivate PPDPC incorporated in PCPECL liposomes and from the drastic spectral changes exhibited by PHT in the presence of rat liver mitochondria.
ii) PHT penetrates into lipid bilayers by hydrophobic interaction, since the drug could not be removed from membranes by high ionic strength or by competitive phosphate groups of DNA.

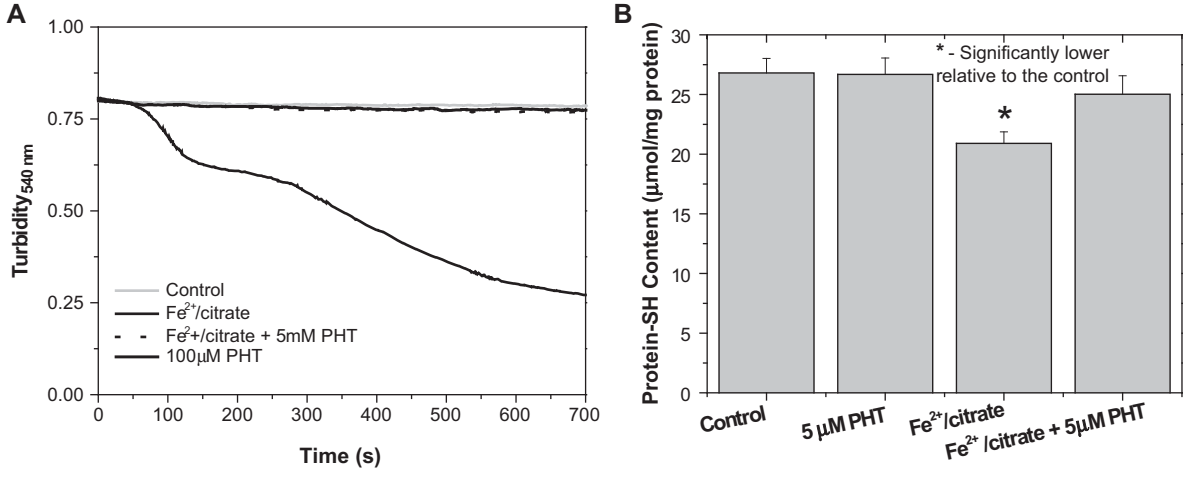
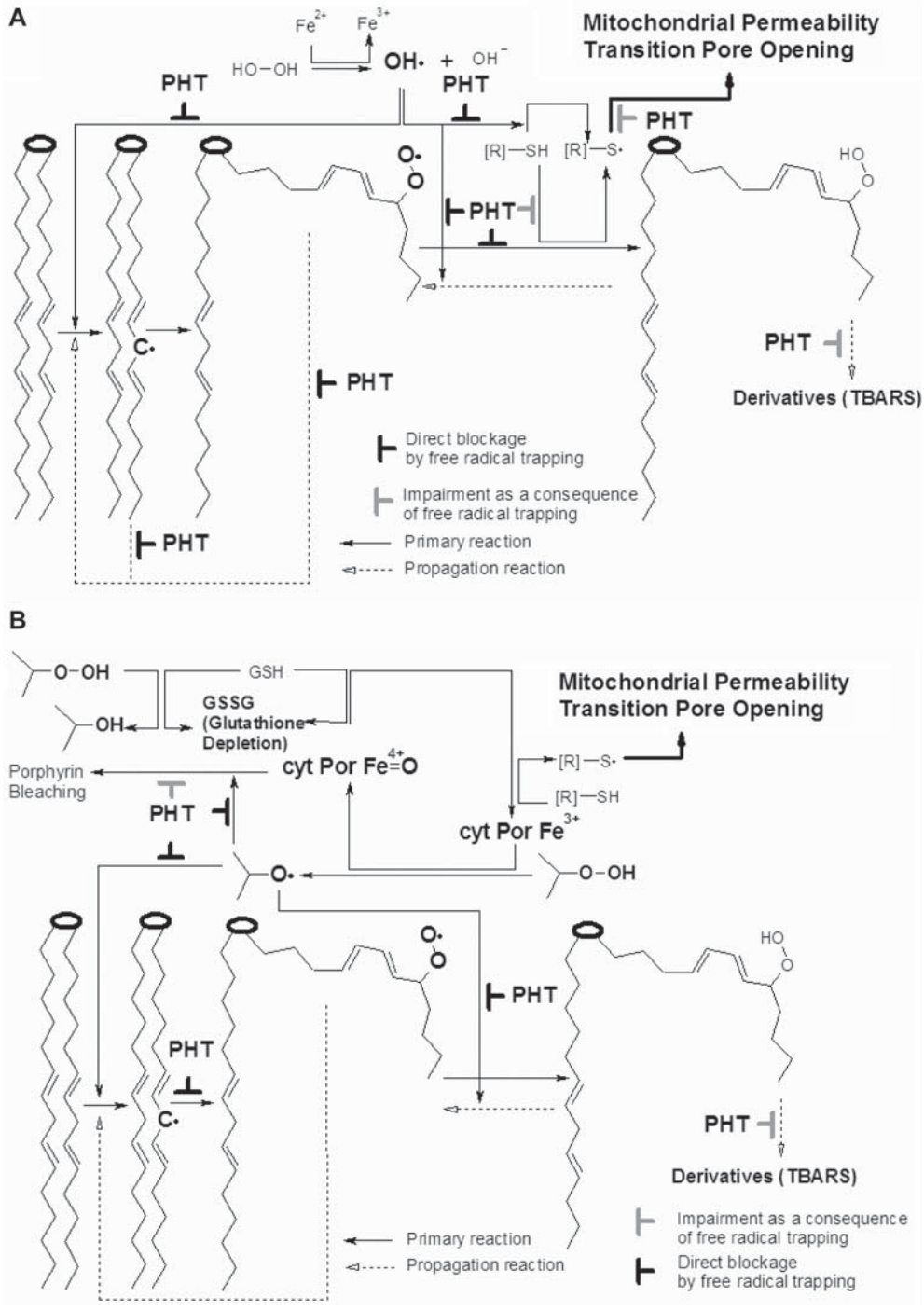


Figure 8. Effect of PHT on the MPTP opening and related oxidation of protein-SH induced by Fe^{2+}/citrate. (A) Thick black solid line represents mitochondrial swelling promoted by Fe^{2+}/citrate; thick gray solid line represents absence of mitochondrial swelling in the absence of Fe^{2+}/citrate and PHT; thin black solid line represents absence of mitochondrial swelling in the absence of Fe^{2+}/citrate but in the presence of 100 μM PHT and thick black dashed line represents absence of mitochondrial swelling in the presence of Fe^{2+}/citrate plus 5 mM PHT. (B) SH content of rat liver mitochondria incubated under the same conditions described above. Mitochondria (0.4 mg protein) were incubated in a standard medium with 5 mM succinate, 2.5 μM rotenone, for 30 min at 37°C. Statistical analysis was done as described in Materials and methods.



Scheme 1. Proposed mechanisms related to the effect of PHT on the MPTP opening induced by Fe^{2+}/citrate (A) and *t*-BuOOH (B).

iii) PHT is a potent scavenger of free radicals and protects lipids and proteins against the attack of these pro-oxidant species. The trapping of free radicals leading to PHT spectral changes that are suggestive of the formation of the drug oxidized derivatives.
iv) Different from derivatives, the photosensitization of PHT does not promote damage on lipids, probably because the photogenerated cation radical of PHT remains stable in the microenvironment of membranes.
v) PHT is unable to revert the oxidation of protein thiol groups. Consequently, this drug does not prevent MPTP openings promoted by RT-03 and by *tert*-butylhydroperoxide.
vi) This study contributes to the comprehension of the role played by phenothiazine substituents on the biological effects of these drugs.

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