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Thioridazine interacts with the membrane of mitochondria acquiring antioxidant activity toward apoptosis – potentially implicated mechanisms

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1 We evaluated the effects of the phenothiazine derivative thioridazine on mechanisms of mitochondria potentially implicated in apoptosis, such as those involving reactive oxygen species (ROS) and cytochrome *c* release, as well as the involvement of drug interaction with mitochondrial membrane in these effects.

2 Within the 0–100 μM range thioridazine did not reduce the free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) nor did it chelate iron.

3 However, at 10 μM thioridazine showed important antioxidant activity on mitochondria, characterized by inhibition of accumulation of mitochondria-generated $O_2^{\bullet-}$, assayed as lucigenin-derived chemiluminescence, inhibition of Fe^{2+}/citrate-mediated lipid peroxidation of the mitochondrial membrane (LPO), assayed as malondialdehyde generation, and inhibition of Ca^{2+}/*t*-butyl hydroperoxide (*t*-BOOH)-induced mitochondrial permeability transition (MPT)/protein-thiol oxidation, assayed as mitochondrial swelling.

4 Thioridazine respectively increased and decreased the fluorescence responses of mitochondria labelled with 1-aniline-8-naphthalene sulfonate (ANS) and 1-(4-trimethylammonium phenyl)-6 phenyl 1,3,5-hexatriene (TMA-DPH).

5 The inhibition of LPO and MPT onset correlated well with the inhibition of cytochrome *c* release from mitochondria.

6 We conclude that thioridazine interacts with the inner membrane of mitochondria, more likely close to its surface, acquiring antioxidant activity toward processes with potential implications in apoptosis such as $O_2^{\bullet-}$ accumulation, as well as LPO, MPT and associated release of cytochrome *c*.
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Keywords: Phenothiazines; thioridazine; trifluoperazine; mitochondrial respiration; lipid peroxidation; mitochondrial permeability transition; permeability transition pore; interaction with membranes; cytochrome *c* release; apoptosis

Abbreviations: ANS, 1-aniline-8-naphthalene sulfonate; BPS, bathophenanthroline-disulphonic acid; CCCP carbonyl cyanide *m*-chlorophenylhydrazone; CsA, cyclosporin A; cyt. *c*, cytochrome *c*; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylene diaminetetraacetic acid; EGTA, ethylene glycol bis (-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulphonic acid); MDA, malondialdehyde; MPT, mitochondrial permeability transition; PTP, permeability transition pore; ROS, reactive oxygen species; TBA, thiobarbituric acid; *t*-BOOH, *tert*-butyl hydroperoxide; TFP, trifluoperazine; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6 phenyl 1,3,5-hexatriene; TR, thioridazine; TRIS, tris(hydroxymethyl)-aminomethane; $\Delta\psi$, electrical transmembrane potential difference

Introduction

Thioridazine is a derivative of the phenothiazines, a class of compounds with antipsychotic activity including trifluoperazine (TFP), a drug widely used in the investigation of several aspects of drug-membrane interactions. Phenothiazines have a three-ring structure in which two benzene rings are linked by a sulphur and a nitrogen atom. TFP has a piperazine ring and a $-CF_3$ group in the side chain, whereas thioridazine has a piperidine ring and a $-SCH_3$ group (Baldessarini, 1995). They are amphiphilic cations whose main feature is the ability to intercalate into biological membranes (Malheiros *et al*., 1998; Pavlov & Glaser, 1998).

A fraction of O_2 available to the respiratory chain in the inner membrane of mitochondria undergoes incomplete reduction generating the superoxide radical ($O_2^{\bullet-}$), which is readily dismutated to H_2O_2 (Gonzalez-Flecha & Boveris, 1995; Boveris & Chance, 1973). Increased $O_2^{\bullet-}$ generation or decreased mitochondrial antioxidant defences in the presence of Fe^{2+} give rise, *via* the Fenton/HaberWeiss reaction, to the highly reactive hydroxyl radical (•OH), which causes

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oxidative damage to biological molecules, namely, peroxidation of the membrane lipids (LPO) (Halliwell & Gutteridge, 1999). The mitochondrial membrane may also undergo a Ca^{2+}-dependent, CsA-sensitive permeability transition process (MPT) mediated by opening of a non-specific channel, referred to as permeability transition pore (PTP), triggered by different agents including pro-oxidants such as *tert*-butyl hydroperoxide (*t*-BOOH) and assessed as mitochondrial swelling (Hunter & Haworth, 1979; Crompton *et al*., 1988; Zoratti & Szabò, 1995; Bernardi, 1999); membrane protein-thiol cross-linkage subsequent to the thiol oxidation by $^{\bullet}OH$ has been proposed as an underlying mechanism (Kowaltowski *et al*., 2001). LPO and mainly MPT onset imply the release of cytochrome *c* from mitochondria into the cytosol, a process now recognized to be closely involved in cell death by apoptotic pathways (Kroemer *et al*., 1998; Skulachev, 2000; Petronilli *et al*., 2001). In this regard, TFP, a classical MPT inhibitor (Pereira *et al*., 1992), was recently reported to inhibit also the HIV-associated apoptosis (Pan *et al*., 1998).

Within this context, in the present study we evaluated the effects of the phenothiazine derivative thioridazine on mechanisms of mitochondria potentially implicated in apoptosis, such as those involving reactive oxygen species (ROS) and cytochrome *c* release, as well as the involvement of drug interaction with the mitochondrial membrane in these effects.

Methods

Chemicals

Thioridazine was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of the highest commercially available grade. The drug was solubilized in water. All stock solutions were prepared using glass-distilled deionized water.

Isolation of rat liver mitochondria

Mitochondria were isolated by standard differential centrifugation (Pedersen *et al*., 1978). Male Wistar rats weighing approximately 200 g were sacrificed by cervical dislocation; livers (10 – 15 g) were immediately removed, sliced in 50 ml of medium containing (mM) sucrose 250 EGTA 1 and HEPES – KOH 10, pH 7.2, and homogenized three times for 15 s at 1 min intervals in a Potter-Elvehjem homogenizer. Homogenates were centrifuged at $770 \times g$ for 5 min and the resulting supernatant was further centrifuged at $9800 \times g$ for 10 min. Pellets were suspended in 10 ml of medium containing (mM) sucrose 250, EGTA 0.3 and HEPES – KOH 10, pH 7.2, and centrifuged at $4500 \times g$ for 15 min. The final mitochondrial pellet was suspended in 1 ml of medium containing (mM) sucrose 250 and HEPES – KOH 10, pH 7.2, and used within 3 h. Mitochondrial protein content was determined by the biuret reaction (Cain & Skilleter, 1987).

Assays of thioridazine in mitochondria-free systems

Reduction of DPPH (100 μM) by thioridazine (0 – 100 μM) was monitored from the change in absorbance at 517 nm, 5 min after the drug was incubated with 40 mM sodium acetate, pH 5.5, and 1 ml ethanol (2.5 ml final volume) (Blois, 1958). Iron chelation was monitored through the formation of the $FeII(BPS)_3$ complex (Bolann & Ulvik, 1987). Thioridazine (0 – 100 μM) was added to the standard medium in the presence of 50 μM Fe^{2+} plus 200 μM BPS and absorbance at 530 nm was measured after 30 min.

Assays with energized mitochondria

The standard incubation medium for all assays was (mM) sucrose 125, KCl 65 and HEPES – KOH 10, pH 7.4. Mitochondria were energized with 5 mM potassium succinate (+ 2.5 μM rotenone) and the respiration medium included also 0.5 mM EGTA and 10 mM K_2HPO_4. Mitochondrial respiration was monitored polarographically with an oxygraph equipped with a Clark-type O_2 electrode (Gilson Medical Electronics, Middleton, WI, U.S.A.). Mitochondria-generated $O_2^{\bullet -}$ was assayed as lucigenin-derived chemiluminescence, monitored with an EG & G Berthold AutoLumat LB 953 apparatus (Bad Wildbad, Germany) (Li *et al*., 1999). Mitochondrial swelling was estimated from the decrease in absorbance at 540 nm using a Model DU-70 spectrophotometer (Beckman, Coulter Inc., Fullerton, CA, U.S.A.). The electrical transmembrane potential difference ($\Delta\psi$) was monitored using 0.4 μM rhodamine 123 as an indicator in a F-4500 spectrofluorometer (Hitachi, Tokyo, Japan) with the 505/535 nm excitation/emission wavelength pair (Emaus *et al*., 1986). The dye rhodamine 123 distributes electrophoretically into the mitochondria in response to $\Delta\psi$ of the inner mitochondrial membrane; the uptake/release of the dye by mitochondria is linearly correlated to the $\Delta\psi$ up to at least 170 mV. The valinomycin-induced K^+ diffusion potential was used to perform a calibration curve. Thus, energized mitochondria were incubated with rhodamine 123 in presence of valinomycin and a titration with K^+ was performed. The $\Delta\psi$ decay due to the electrogenic influx of the cation, determined by the Nerst equation ($\Delta\psi = 59 \log [K^+]_{in}/[K^+]_{out}$; $[K^+]_{in} = 120$ mM), is linearly correlated to the increase in the fluorescence intensity of the dye as it is released from the mitochondria (Åkerman & Wikström, 1976; Emaus *et al*., 1986).

Fe^{2+}/citrate-mediated lipid peroxidation assay

Lipid peroxidation was assayed as MDA generation. One ml of mitochondrial suspension (1 mg protein) was incubated with thioridazine in the standard medium plus 5 mM succinate, 2.5 μM rotenone, 50 μM $(NH_4)_2Fe(SO)_4$ and 2 mM sodium citrate for 30 min, at 37°C (1 ml final volume). For MDA determination, 1 ml of 1% TBA (prepared in 50 mM NaOH), 0.1 ml of 10 M NaOH and 0.5 ml of 20% H_3PO_4 were added, followed by incubation for 20 min at 85°C. The MDA – TBA complex was extracted with 2 ml of n-butanol and absorbance was measured at 535 nm. MDA concentration was calculated from $\varepsilon = 1.56 \times 10^5$ M^{-1} (Kowaltowski *et al*., 1996).

Determination of protein-thiol

After 15 min incubation with thioridazine under the swelling assay conditions, mitochondria (0.4 mg protein) were treated with perchloric acid (5% final concentration) and centrifuged

at 4500 × g for 10 min. The pellet was suspended with 1 ml of medium containing 50 mM EDTA and 100 mM TRIS, pH 8.0. After the addition of 2 mM DTNB, absorbance was determined at 412 nm. The amount of thiol groups was calculated from $\varepsilon = 13\,600\ \mathrm{M}^{-1}$ (Jocelyn, 1987).

Effect of thioridazine on fluorescence responses of ANS and TMA–DPH-labelled mitochondrial membrane

Mitochondria were incubated at 30°C with 75 μM ANS (2 mg protein) or 1.04 μM TMA–DPH (0.5 mg protein) in the standard incubation medium plus 1 μg ml^{-1} CCCP before thioridazine was added (2 ml final volume). Fluorescence was measured with a F-4500 spectrofluorometer (Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 380 and 485 nm, respectively, for ANS and of 362 and 432 nm, respectively, for TMA–DPH (Slavík, 1982; Lee *et al*., 1999).

Determination of cytochrome c

Cytochrome *c* released from mitochondria was determined by an enzyme immunoassay technique using an ELISA kit (Quantikine M., R&D Systems, Abingdon, U.K.). Mitochondria (0.4 mg protein ml^{-1}) were incubated with thioridazine under the conditions of the specific assays and centrifuged at 16,000 × g for 10 min. The supernatant (50 μl) was added to wells and incubated with cytochrome *c* conjugate for 2 h at 25°C. After five washes, substrate solution was added and the plate was incubated for 30 min. The reaction was stopped and optical density was determined using a microplate reader set at 450 nm with correction at 540 nm. Sample concentrations were determined based on a standard curve within a 0.78–25 ng ml^{-1} concentration range (ε = 0,089 ng^{-1} ml).

Results

Effects of thioridazine on respiration and electrical transmembrane potential difference ($\Delta\psi$) of isolated rat liver mitochondria

At relatively low concentrations (10–75 μM) thioridazine slightly increased the rate of succinate-supported state 4 (resting) respiration of mitochondria (Figure 1A), showing that it is a weak uncoupler of oxidative phosphorylation. However, at higher concentrations thioridazine markedly decreased the rate of succinate-supported state 3 (ADP-stimulated) respiration of mitochondria (Figure 1B, IC_{50} = 87.5 μM) in close association with a fall in $\Delta\psi$ (Figure 1C, IC_{50} = 89 μM), showing that thioridazine is also a typical inhibitor of oxidative phosphorylation.

Effect of thioridazine on mitochodria-generated $O_2^{\bullet-}$

A fraction of the O_2 available to mitochondria during respiration is continuously converted to $O_2^{\bullet-}$ which in the presence of its dismutation product H_2O_2 plus Fe^{2+} may produce the $^{\bullet}$OH radical (Boveris & Chance, 1973). As shown in Figure 2, at 10 μM, thioridazine inhibited by about 65% the accumulation of $O_2^{\bullet-}$ generated by respiratory chain during succinate-supported resting respiration of isolated rat liver mitochondria, assayed as lucigenin-derived chemiluminescence.

Effect of thioridazine on Fe^{2+}/citrate-mediated lipid peroxidation of the mitochondrial membrane

The $^{\bullet}$OH radical produced may start a lipid peroxidation process by removing hydrogen atoms from phospholipids and yielding lipid peroxyl radicals (LOO$^{\bullet}$), thus inducing a sequence of propagation reactions (Halliwell & Gutteridge, 1999). Figure 3(A) shows the effect of thioridazine on Fe^{2+}/citrate-mediated lipid peroxidation of the mitochondrial membrane, assayed as MDA generation, and Figure 3(B) and (C) show, respectively, the effects of the drug on the associated swelling of mitochondria and release of cytochrome *c*. Thioridazine, at 10 μM, inhibited by about 80% MDA generation, and therefore peroxidation of the mitochondrial membrane lipids, and also inhibited by about 100% the associated swelling of mitochondria and by about

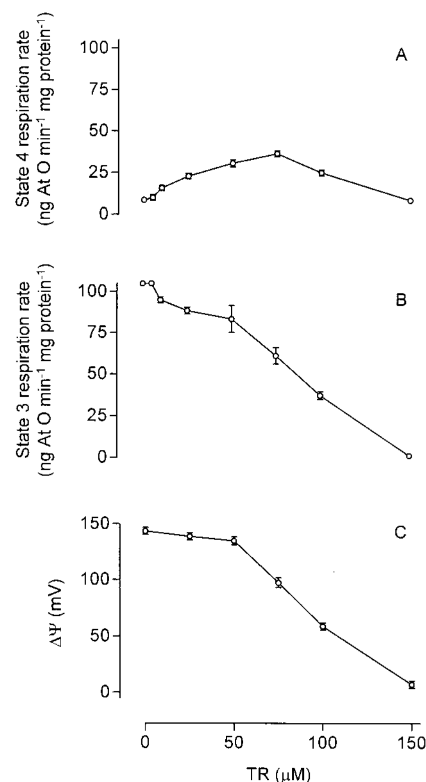

Figure 1 Concentration-response curves for the effects of thioridazine (TR) on state 4 respiration rate (A), state 3 respiration rate (B) and $\Delta\psi$ (C) in isolated rat liver mitochondria. For the respiratory assays, mitochondria (1.5 mg protein) were incubated at 30°C with 5 mM succinate and 2.5 μM rotenone in a standard incubation medium containing 125 mM sucrose, 65 mM KCl and 10 mM HEPES–KOH, pH 7.4, in the presence of 0.5 mM EGTA and 10 mM K_2HPO_4 (respiration medium) in a final volume of 1.5 ml. State 3 respiration was initiated with 0.4 μmol ADP. For the $\Delta\psi$ assays, mitochondria (2 mg protein) incubated in the standard medium plus 2.5 μM rotenone and 0.4 μM rhodamine 123 in a final volume of 2 ml were energized by the addition of 5 mM succinate. The inhibition of the $\Delta\psi$ response of mitochondria was immediate and remained constant for at least 5 min; its extent was calculated 30 s after drug addition by using a calibration curve, as described in Methods. Data are presented as the mean ± s.e.mean of three experiments with different mitochondrial preparations.

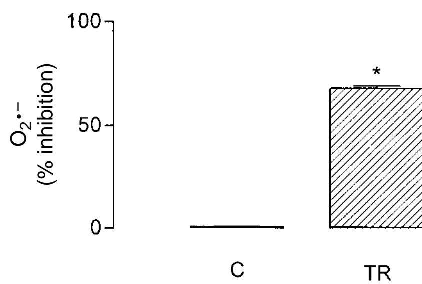

Figure 2 Effects of 10 μM thioridazine (TR) on mitochondria-generated $O_2^{\bullet-}$ assayed as lucigenin-derived chemiluminescence. Mitochondria (0.5 mg protein) were incubated with drugs at 30°C in the respiration medium described in the legend to Figure 1, in a final volume of 2 ml. The lucigenin-derived chemiluminesce response was initiated by adding 5 μM lucigenin; data are per cent inhibition of luminescence (integrated area under the curve) in relation to a control in the absence of the drug. The data are presented as the mean $\pm$ s.e.mean of six experiments with different mitochondrial preparations. Statistical analysis was performed by the Mann–Whitney non-parametric test. *Significantly different from control ($P<0.05$).

60% the release of cytochrome *c* induced in mitochondria under the LPO assay condition.

Effect of thioridazine on Ca^{2+}/t-BOOH-induced mitochondrial permeability transition

One of the lines of evidence with regard to the mechanism of Ca^{2+}/*t*-BOOH-induced MPT is that PTP opening is sensitized by the oxidation of gluthatione and NAD(P)H accumulating ROS such as the $^{\bullet}$OH radical which, in turn, oxidizes thiol groups of membrane proteins followed by cross-linkage formation (Kowaltowski *et al*., 2001). In this regard, the phenothiazine TFP, which is a well established MPT inhibitor (Broekemeier *et al*., 1985), has been proposed to directly interfere with the formation of these membrane protein–thiol cross-linkage (Pereira *et al*., 1992). Thioridazine, at 10 μM, inhibited by about 80% the Ca^{2+}/*t*-BOOH-induced swelling of isolated rat liver mitochondria (Figure 4A), and therefore MPT onset, and also inhibited by about 100% the release of cytochrome *c* induced in mitochondria under the MPT assay condition (Figure 4C). Only about 20% of the mitochondrial membrane protein–thiol groups underwent oxidation under this condition, in agreement with literature data, and thioridazine almost completely protected against it (Figure 4B).

Effects of thioridazine on the fluorescence responses of ANS and TMA–DPH-labelled mitochondria

In order to test the hypothesis that the above effects are mediated by interaction of thioridazine with the mitochondrial membrane, we performed assays with mitochondria labelled with the fluorescent probes ANS and TMA–DPH, which monitor membranes closer to the aqueous interface. ANS is generally assumed to bind to the polar head groups of the phospholipids and to proteins on the membrane surface, with the anionic sulfonate group being the major determinant of binding. The amount of ANS molecules bound to the membranes is highly influenced by the surface charge potential, being inversely proportional to its negative potential (Slavík, 1982) and TMA–DPH is incorporated into

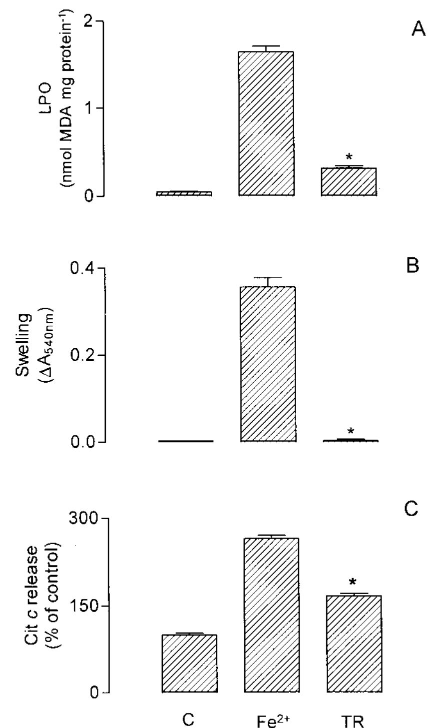

Figure 3 Effects of 10 μM thioridazine (TR) on lipid peroxidation assayed as MDA generation (A) and associated mitochondrial swelling (B) and cytochrome *c* release (C) induced by Fe^{2+}/citrate in isolated rat liver mitochondria (Fe^{2+}). Mitochondria (1 mg protein) were incubated in the standard medium with 5 mM succinate, 2.5 μM rotenone, 50 μM $(NH_4)_2Fe(SO)_4$ and 2 mM sodium citrate for 30 min at 37°C (1 ml final volume). See Methods for MDA, mitochondrial swelling and cytochrome *c* determinations. Data are presented as the mean $\pm$ s.e.mean of nine (A), nine (B) and six (C) experiments with different mitochondrial preparations. Statistical analysis was performed by Kruskal–Wallis non-parametric analysis of variance (ANOVA) followed by Dunn's multiple comparison test. *Significantly different from Fe^{2+} ($P<0.05$ for A and B; $P<0.1$ for C).

the hydrophobic region of membranes oriented parallel to the lipid acyl chain axis, with the cationic trimethylammonium substituent acting as a surface anchor (Lee *et al*., 1999). As shown in Figure 5, thioridazine respectively increased and decreased the fluorescence responses of ANS and TMA–DPH incubated with the isolated rat liver mitochondria, indicating that the drug interacts with the mitochondrial membrane and that interaction is more likely to occur close to its surface.

Effects of trifluoperazine

The effects of TFP on the mitochondrial parameters/responses above were evaluated in parallel, and, except for potency about 10% lower, they were closely similar to those of thioridazine (results not shown).

Discussion

Previous literature data have shown that the classical phenothiazine derivative TFP affects the activity of mem-

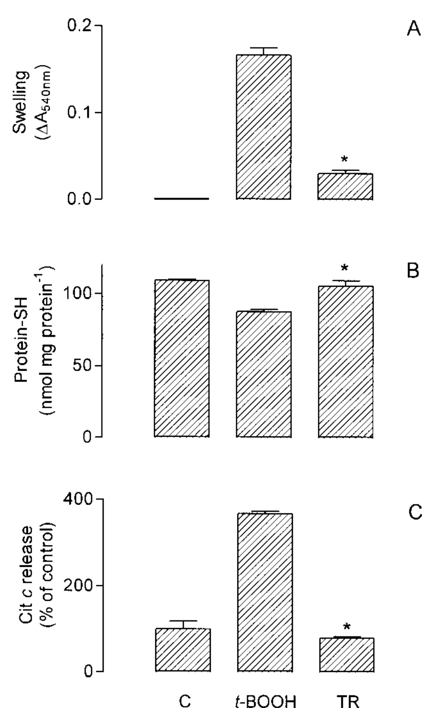

Figure 4 Effects of 10 μM thioridazine (TR) on mitochondrial swelling (A) and associated oxidation of protein-SH (B) and cytochrome *c* release (C) induced by 10 μM $CaCl_2$ + 0.5 mM *t*-BOOH in isolated rat liver mitochondria (*t*-BOOH). Mitochondria (0.4 mg protein) were incubated in the standard medium with 5 mM succinate and 2.5 μM rotenone, at 30°C (1.5 ml final volume). See also Methods for the determinations. Data are presented as the mean $\pm$ s.e.mean of 10 (A), six (B) and six (C) experiments with different mitochondrial preparations. Statistical analysis was performed by Kruskal–Wallis non-parametric analysis of variance (ANOVA) followed by Dunn's multiple comparison test. *Significantly different from *t*-BOOH ($P<0.05$).

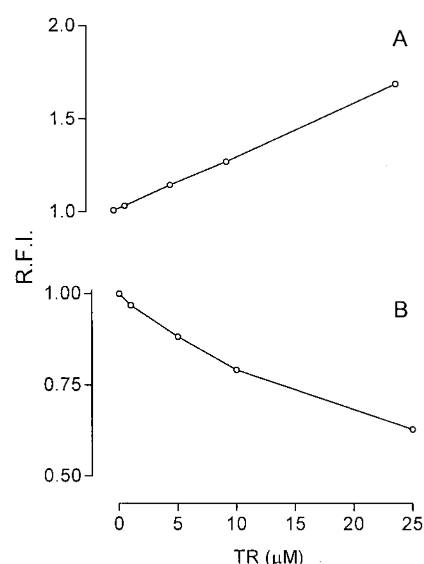

Figure 5 Concentration-response curves for the effects of thioridazine (TR) on membrane-ANS (A) and membrane-TMA–DPH (B) fluorescence response in isolated rat liver mitochondria incubated in the standard medium, as described in Methods. The relative fluorescence intensity (R.F.I.) was measured after drug addition. Data are presented as the mean $\pm$ s.e.mean of three experiments with different mitochondrial preparations.

brane proteins by interacting with hydrophobic sites located in the membrane-embedded domains (Dabbeni-Sala & Palatini, 1990). 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 (Gudz *et al.*, 1997; Elimadi *et al.*, 1998). The structure of phenothiazines *per se* does not suggest free radical scavenging properties. Indeed, within the 0–100 μM concentration range thioridazine did not reduce DPPH (data not shown), a stable free radical potentially reactive with all compounds able to donate a hydrogen atom (Blois, 1958). Nevertheless, in the present study thioridazine showed important antioxidant activity on mitochondria, as characterized by the inhibition of accumulation of the $O_2^{\bullet-}$ continuously generated during resting respiration, and by inhibition of ROS-associated events, such as Fe^{2+}/citrate-mediated peroxidation of the mitochondrial membrane lipids and Ca^{2+}/*t*-BOOH-induced mitochondrial permeability transition.

Inhibition of either respiratory chain or ATP synthase activities is the mechanism accounting for the reduced rate of mitochondrial state 3 respiration. We can therefore expect that thioridazine directly and/or indirectly interacts with mitochondrial proteins that are relevant to the control of state 3 respiration, namely, enzymes comprising segments of the respiratory chain and ATP synthase. However, two lines of evidence argue against participation of these mechanisms in the antioxidant activity of thioridazine on mitochondria: (1) at the concentration at which thioridazine exhibited antioxidant activity (10 μM) the drug had no significant effect on mitochondrial respiration; and (2) in general respiratory chain inhibitors rather stimulate than inhibit $O_2^{\bullet-}$ generation by mitochondria. The coenzyme Q cycle has been reported to be the main pathway for $O_2^{\bullet-}$ generation by the respiratory chain; it accumulates the semiquinone anion in the inner membrane of mitochondria which, in turn, donates electron to O_2 (Kowaltowski *et al.*, 2001). Within this context, we believe that the inhibition of accumulation of mitochondria-generated $O_2^{\bullet-}$ due to thioridazine comes from the interaction of the drug with the inner mitochondrial membrane, thus impairing the electron donation by the semiquinone anion to the O_2.

That thioridazine interacts with the mitochondrial membrane is demonstrated by the increase/decrease in the fluorescence response of mitochondria labelled with ANS/TMA–DPH, respectively. The amount of ANS molecules bound to membranes is inversely proportional to the negative membrane surface potential (Slavík, 1982) and the surface anchor of TMA–DPH is a cationic substituent (Lee *et al.*, 1999). Since thioridazine has a positive charge at the pH of the assays, the increase of the fluorescence response of ANS may reflect a decrease of the negative charge density on the membrane surface by the drug, thereby increasing the binding sites of the probe. On the other hand, the decrease of the fluorescence response of TMA–DPH may reflect a competition between the positive charge of thioridazine and the

probe, thereby reducing its anchoring to the membrane. Therefore, the interaction of thioridazine with the inner mitochondrial membrane is more likely to occur close to its surface and may be responsible, at least in part, for the antioxidant activity of the drug on mitochondria in terms of inhibition of accumulation of mitochondria-generated $O_2^{\bullet -}$, and also of LPO and MPT.

The mechanism of LPO inhibition by thioridazine does not involve chelation of the iron required in the Fenton/HaberWeiss reaction, since the drug did not interfere with the formation of the $FeII(BPS)_3$ complex, as evaluated spectrophotometrically at 530 nm (data not shown). Therefore, we believe that LPO inhibition occurs either at the level of initiation of the process by decreasing the availability of $O_2^{\bullet -}$, and therefore of the $^{\bullet}OH$ radical, as discussed above, or at the level of propagation of processes, by slowing down the LPO free radical reactions as the physical state of the mitochondrial membrane is altered by the drug interaction. Concerning MPT inhibition by thioridazine and considering the proposed mechanism discussed above (Kowaltowski *et al*., 2001), a decrease in the availability of $^{\bullet}OH$ may directly inhibit the protein – thiol oxidation and, if oxidation occurs, a change in the physical state of the mitochondrial membrane would impair the cross-linkage between the oxidized thiols, and therefore the onset of MPT.

The participation of mitochondria in non-apoptotic and apoptotic cell death has been well established. MPT onset can lead to a necrosis-type cell death by ATP depletion and Ca^{2+} homeostasis disruption, without release of cytochrome *c*. However, during apoptosis cytochrome *c* may be released from mitochondria into the cytosol as the mitochondrial membrane becomes permeable due to PTP opening or other processes, and thus acts by triggering the activation of caspases (Susin *et al*., 1998; Bernadi *et al*, 2001). In the present study inhibition of cytochrome *c* release by thioridazine correlated well with inhibition of LPO and MPT onset, indicating that thioridazine may be a useful tool to understand the relationship between mitochondrial mechanisms and induction of apoptosis, playing, in addition, a potential role as a cytoprotective drug in terms of apoptotic cell death.

In general, the present study shows that thioridazine interacts with the inner mitochondrial membrane, more likely close to its surface, acquiring antioxidant activity toward processes with potential implications in apoptosis such as $O_2^{\bullet -}$ accumulation, and also peroxidation of mitochondrial membrane lipids, mitochondrial permeability transition and associated release of cytochrome *c* in mitochondria. Studies on the effects of thioridazine on the mitochondrial mechanisms potentially implicated in apoptosis are now in progress by employing isolated rat liver cells.

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References

ÅKERMAN, K.E.O. & WIKSTRÖM, M.K.F. (1976). Safranine as a probe of the mitochondrial membrane potential. *FEBS Lett.*, **68,** 191 – 197.

BALDESSARINI, R.J. (1995). Drugs and the treatment of psychiatric disorders: psychosis and anxiety. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics* 9th edn ed. Hardman, J.G., Gilman, A.G. & Limbird, L.E., pp. 399 – 430. New York: McGraw-Hill.

BERNARDI, P. (1999). Mitochondrial transport of cations: channels, exchangers, and permeability transition. *Physiol. Rev.*, **79,** 1127 – 1155.

BERNARDI, P., PETRONILLI, V., DI LISA, F. & FORTE, M. (2001). A mitochondrial perspective on cell death. *Trends Biochem. Sci.*, **26,** 112 – 117.

BLOIS, M.S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature,* **181,** 1199 – 1200.

BOLANN, B.J. & ULVIK, R.J. (1987). Release of iron from ferritin by xanthine oxidase. Role of the superoxide radical. *Biochem. J.*, **243,** 55 – 59.

BOVERIS, A. & CHANCE, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem. J.*, **134,** 707 – 716.

BROEKEMEIER, K.M., SCHMID, P.C., SCHMID, H.H. & PFEIFFER, D.R. (1985). Effects of phospholipase A_2 inhibitors on ruthenium red-induced Ca2+ release from mitochondria. *J. Biol. Chem.*, **260,** 105 – 113.

CAIN, K. & SKILLETER, D.N. (1987). Preparation and use of mitochondria in toxicological research. In *Biochemical Toxicology*. ed. Snell, K. & Mullock B. pp. 217 – 254. Oxford: IRL Press.

CROMPTON, M., ELLINGER, H. & COSTI, A. (1988). Inhibition by cyclosporin A of a Ca^{2+}-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. *Biochem. J.*, **255,** 357 – 360.

DABBENI-SALA, F. & PALATINI, P. (1990). Mechanism of local anesthetic effect. Involvement of F0 in the inhibition of mitochondrial ATP synthase by phenothiazines. *Biochim. Biophys. Acta,* **1015,** 248 – 252.

ELIMADI, A., BOUILLOT, L., SAPENA, R., TILLEMENT, J.P. & MORIN, D. (1998). Dose-related inversion of cinnarizine and flunarizine effects on mitochondrial permeability transition. *Eur. J. Pharmacol.*, **348,** 115 – 121.

EMAUS, R.K., GRUNWALD, R. & LEMASTERS, J.J. (1986). Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. *Biochim. Biophys. Acta,* **850,** 436 – 448.

GONZALEZ-FLECHA, B. & BOVERIS, A. (1995). Mitochondrial sites of hydrogen peroxide production in reperfused rat kidney cortex. *Biochm. Biophys. Acta,* **1243,** 361 – 366.

GUDZ, T., ERIKSSON, O., KUSHNAREVA, Y., SARIS, N.E. & NOVGORODOV S. (1997). Effect of butylhydroxytoluene and related compounds on permeability of the inner mitochondrial membrane. *Arch. Biochem. Biophys.*, **342,** 143 – 156.

HALLIWELL, B. & GUTTERIDGE, J.M. (1999). *Free Radical in Biology and Medicine*, pp. 36 – 95. Oxford: University Press.

HUNTER, D.R. & HAWORTH, R.A. (1979). The Ca^{2+}-induced membrane transition in mitochondria. I. The protective mechanisms. *Arch. Biochem. Biophys.*, **195,** 453 – 459.

JOCELYN, P.C. (1987). Spectrophotometric assay of thiols. *Meth. Enzymol.*, **143,** 44 – 67.

KOWALTOWSKI, A.J., CASTILHO, R.F., GRIJALBA, M.T., BECHARA, E.J. & VERCESI, A.E. (1996). Effect of inorganic phosphate concentration on the nature of inner mitochondrial membrane alterations mediated by Ca^{2+} ions. A proposed model for phosphate-stimulated lipid peroxidation. *J. Biol. Chem.*, **271,** 2929 – 2934.

KOWALTOWSKI, A.J., CASTILHO, R.F. & VERCESI, A.E. (2001). Mitochondrial permeability transition and oxidative stress. *FEBS Lett.*, **495,** 12 – 15.

KROEMER, G., DALLAPORTA, B. & RESCHE-RIGON, M. (1998). The mitochondrial death/life regulator in apoptosis and necrosis. *Annu. Rev. Physiol.*, **60,** 610 – 642.

LEE, J., YU, B.P. & HERLIHY, J.T. (1999). Modulation of cardiac mitochondrial membrane fluidity by age and calorie intake. *Free Radic. Biol. Med.*, **26,** 260 – 265.

LI, Y., ZHU, H. & TRUSH, M.A. (1999). Detection of mitochondria-derived reactive oxygen species production by the chemiluminigenic probes lucigenin and luminol. *Biochim. Biophys. Acta,* **1428,** 1–12.

MALHEIROS, S.V., PAULA, E. & MEIRELLES, N.C. (1998). Contribution of trifluoperazine/lipid ratio and drug ionization to hemolysis. *Biochim. Biophys. Acta,* **1373,** 332–340.

PAN, G., ZHOU, T., RADDING, W., SAAG, M.S., MOUNTZ, J.D. & MCDONALD, J.M. (1998). Calmodulin antagonists inhibit apoptosis of CD4+ T-cells from patients with AIDS. *Immunopharmacol.,* **40,** 91–103.

PAVLOV, P.F. & GLASER, E. (1998). Inhibition of protein import into mitochondria by amphiphilic cations: potential targets and mechanism of action. *Biochem. Biophys. Res. Commun.,* **252,** 84–91.

PEDERSEN, P.L., GREENAWALT J.W., REYNAFARJE, B., HULLIHEN, J., DECKER, G.L., SOPER, J.W. & BUSTAMENTE, E. (1978). Preparation and characterization of mitochondria and submitochondrial particles of rat liver and liver-derived tissues. *Meth. Cell. Biol.,* **20,** 411–481.

PEREIRA, R.S., BERTOCCHI, A.P. & VERCESI, A.E. (1992). Protective effect of trifluoperazine on the mitochondrial damage induced by Ca^{2+} plus prooxidants. *Biochem. Pharmacol.,* **44,** 1795–1801.

PETRONILLI, V., PENZO, D., SCORRANO, L., BERNARDI, P. & DI LISA, F. (2001). The mitochondrial permeability transition, release of cytochrome c and cell death. Correlation with the duration of pore openings in situ. *J. Biol. Chem.,* **276,** 12030–12034.

SKULACHEV, V.P. (2000). Mitochondria in the programmed death phenomena; a principle of biology: 'it is better to die than to be wrong'. *IUBMB Life,* **49,** 365–373.

SLAVÍK, J. (1982). Anilinonaphthalene sulfonate as a probe of membrane composition and function. *Biochim. Biophys. Acta,* **694,** 1–25.

SUSIN, S.A., ZAMZAMI, N. & KROEMER, G. (1998). Mitochondria as regulators of apoptosis: doubt no more. *Biochim. Biophys. Acta,* **1366,** 151–165.

ZORATTI, M. & SZABÒ, I. (1995). The mitochondrial permeability transition. *Biochim. Biophys. Acta,* **1241,** 139–176.

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