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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · NOVEMBER 2007

Impact Factor: 3.53 · DOI: 10.1021/tx700092r · Source: PubMed

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Organotellurane-Promoted Mitochondrial Permeability Transition Concomitant with Membrane Lipid Protection against Oxidation

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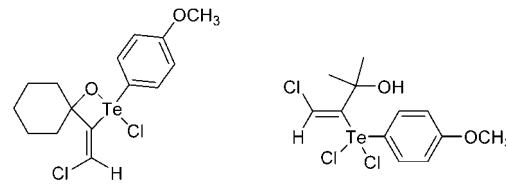
Received March 27, 2007

Organotelluranes exhibit potent antioxidant properties as well as the ability to react with protein thiol groups and, thereby, they are good models to study the mechanism of the mitochondrial permeability transition (MPT). We evaluated the effects of the concentration of organotelluranes, namely RT-03 and RT-04, on rat liver mitochondria. At the concentration range of 0.25–1.0 μM, organotelluranes did not cause any mitochondrial dysfunction. At the concentration range of 5–10 μM, RT-03 and RT-04 caused the Ca^{2+}-dependent opening of the (MPT) pore, regulated by Cyclosporin A. At the concentration range of 15–30 μM the swelling was not inhibited by Cyclosporin A and in the absence of Ca^{2+}, a significant decrease of respiratory control ratio was observed due to concomitant phosphorylation impairment and uncoupling, transmembrane potential disruption, depletion of mitochondrial reduced thiol groups, and alterations in the bilayer fluidity. Above 100 μM, the organotelluranes caused complete inhibition of respiratory chain. Over the whole studied concentration range, RT-03 and RT-04 did not induce mitochondrial oxidative stress assessed by using the reactive oxygen and nitrogen species indicator 2′,7′-dichlorodihydrofluorescein diacetate. Further, the organotelluranes also exhibited protective effect against *t*-butyl hydroperoxide-induced oxidative stress as well as against Fe^{2+}/citrate-induced peroxidation of mitochondrial membranes and PCPECL liposomes. These results point out that MPT pore opening can involve damage exclusively to mitochondrial membrane proteins. The exclusive antioxidant activity observed at nanomolar range is also an interesting new finding described in this work.

Introduction

The mitochondrial permeability transition (MPT) is a well-known event that can precede necrotic and apoptotic cell death (*1–3*). The onset of the MPT is initiated by the opening of the mitochondrial permeability transition pore (MPTP). MPTP is a high conductance pore in the inner mitochondrial membrane that can be opened by many agents such as Ca^{2+}, Pi, alkaline pH, and reactive oxygen species (ROS), and prevented by the imunosuppressive drug cyclosporine A (CsA), Mg^{2+}, acidic pH, and phospholipase inhibitors such as dibucaine, trifluoperazine, and quinacrine. MPTP opening leads to nonselective diffusion of solutes with molecular mass of up to 1500 Da through the inner mitochondrial membrane and promotes mitochondrial depolarization, uncoupling of oxidative phosphorylation and large amplitude swelling (*4*). These events, in turn, lead to ATP depletion and cell death. Although several proteins such as ADP/ATP translocator (ANT), cyclophilin D, and possibly the voltage-dependent anion channel (VDAC) and hexokinase have been assigned as components of MPTP (*1, 5, 6*), the molecular composition of the MPTP remains uncertain. The prevailing model of pore structure does not explain the fact that the MPTP can exist in a regulated and unregulated form. The most incisive evidence against the hypothesis that the MPTP can be formed exclusively by the assembly of specific proteins is the finding that small exogenous pore-forming amphipathic peptides like mitochondrial targeting peptides, alamethicin, and mastoparan also induce onset of a Ca^{2+}-dependent and CsA-inhibitable MPT at low concentrations and a CsA-insensitive MPT at higher concentrations (*7–9*). Considering that many agents that induce MPT such as ROS, oxidants, and thiol reactive agents exhibit the common ability to attack and modify membrane proteins, Lemasters and co-workers (*9*) proposed a model for the MPTP formation in which the chemical attack of mitochondrial membrane proteins leads to the misfolding and aggregation of these proteins to form aqueous channels. Initially, chaperone-like proteins including cyclophilin D regulate the conductance of solutes by the MPTP to confer the properties of regulated PT pores. Binding of chaperones to the misfolded proteins

Chart 1. Molecular Structure of Organotelluranes RT-03 and RT-04



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10.1021/tx700092r CCC: $37.00
Published on Web 09/27/2007

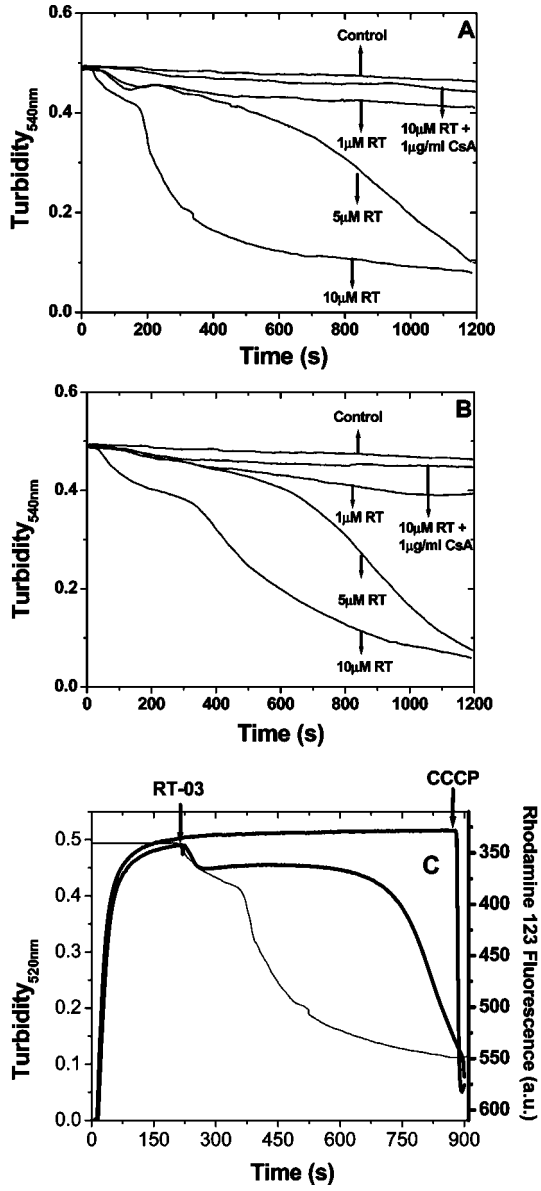


Figure 1. Mitochondrial swelling induced by different concentrations of RT-03 and RT-04. (A) Represents control experiment in the absence of RT-03 and the effect of 1, 5, and 10 μM RT-03 in the absence of CsA, and the effect of 10 μM RT-03 in the presence of 1 μg/mL CsA as indicated in the figure. (B) Represents control experiment in the absence of RT-04 and the effect of 1, 5, and 10 μM RT-04 in the absence of CsA, and the effect of 10 μM RT-04 in the presence of 1 μg/mL CsA as indicated in the figure. (C) Represents the $\Delta\Psi$ of Ca^{2+}-loaded mitochondria in the absence of RT-3 and the loss of $\Delta\Psi$ plus (thick solid line), and the corresponding swelling (thin solid line) after the addition of 10 μM RT-03 in the absence of CsA. Data are presented as the mean of three experiments with different mitochondrial preparations. The reactions were initiated by the addition of mitochondria (0.4 mg protein) 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.

warrants that the pore remains closed until matrix Ca^{2+} raises substantially. The unregulated form of the MPTP occurs when the number of misfolded proteins exceeds the number of chaperones to regulate them. However, according to works of Halestrap (*10*), Ca^{2+} is necessary to cyclophilin D binding and pore opening. In this case, the unregulated pore could occur when the damage in the membrane components are enough to promote protein assembly and pore opening without cyclophilin D binding.

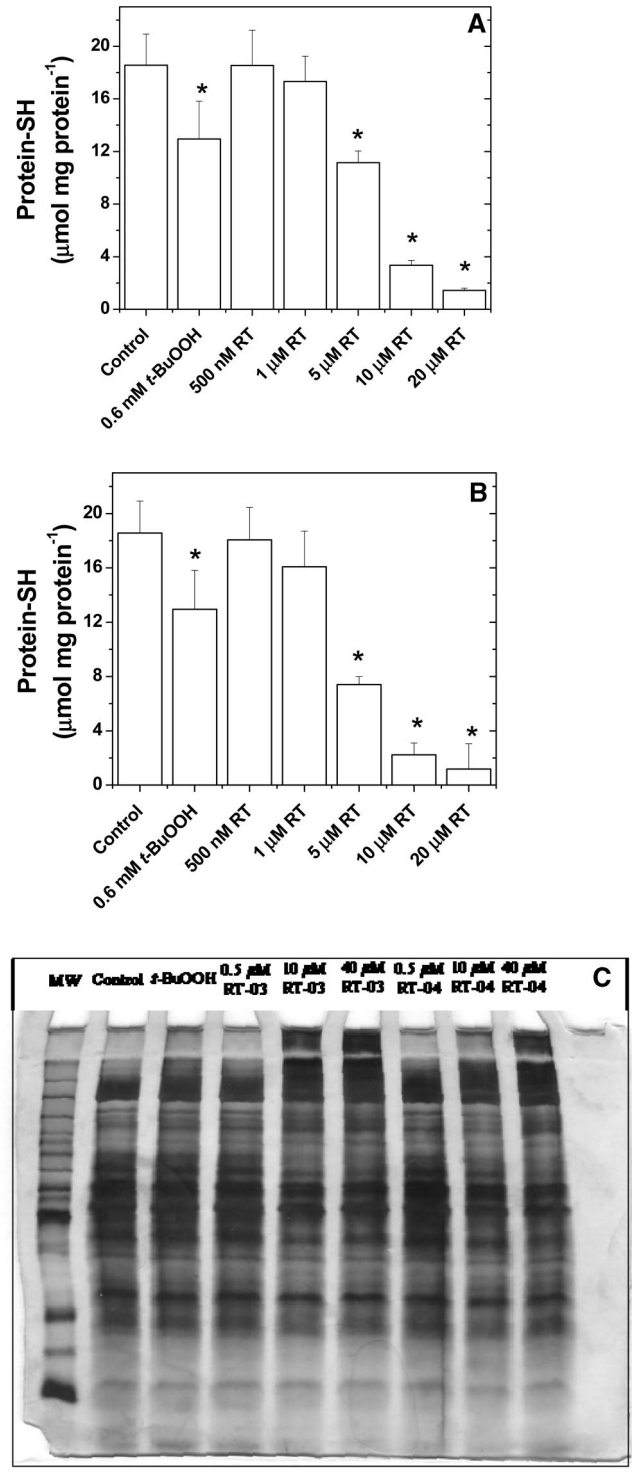


Figure 2. Effect of RT-03 and RT-04 on the total mitochondrial protein thiol group content. (A) Represents control experiment in the absence of RT-03, the effect of 0.6 mM *t*-BuOOH and the effect of 500 nM, 1, 5, 10, and 20 μM RT-03 as indicated in the figure. (B) Represents control experiment in the absence of RT-04, the effect of 0.6 mM *t*-BuOOH, and the effect of 500 nM, 1, 5, 10, and 20 μM RT-04 as indicated in the figure. Mitochondria (0.4 mg protein) were incubated under swelling conditions. See Experimental Procedures for details. Data are presented as the mean +/− SEM of three experiments with different mitochondrial preparations. Asterisk (*) means significant decrease relative to the control. (C) SDS-polyacrylamide slab gel electrophoresis. The lane MW is the Kaleidoscope molecular-weight standard (MW) and the subsequent lanes correspond to mitochondria submitted to the following conditions: absence of drugs (control), 0.6 mM *t*-BuOOH, 0.5, 10, and 40 μM RT-03, and 0.5, 10, and 40 μM RT-04 as indicated in the figure.

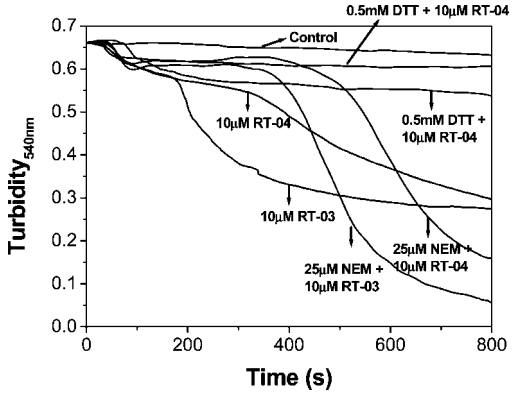


Figure 3. Mitochondrial swelling induced by RT-03 and RT-04. Control experiment in the absence of RT-03 or RT-04, and the effect of 10 μM RT-03 or RT-04 in the absence or presence of 0.5 mM DTT or 25 μM NEM as indicated in the figure. Data are presented as the mean of three experiments with different mitochondrial preparations. The reactions were initiated by the addition of mitochondria (0.4 mg of protein) 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.

Another uncertainty relative to the MPTP is the mechanism responsible for the assembly of the protein components. Literature data have suggested that the oxidative stress could be the link between mitochondrial Ca^{2+} accumulation and MPT (*1*). Despite strong evidence of the relationship between oxidative stress and MPT via direct and indirect changes in the redox state of thiol groups (*1, 11–14*), a lipophylic dithiol reagent is expected to promote cross-linkage of inner mitochondrial proteins and induce the MPTP in conditions in which the oxidative stress is absent. Interestingly, literature data have shown that organotellurium and organoselenium compounds exhibit both reactivity with thiol groups and antioxidant properties, probably because they exhibit glutathione peroxidase-like activity and oxidize thiol groups during the reduction of H_2O_2 (*15*). In addition, these organochalcogens also delay the lipoperoxidation induced by a variety of oxidants. This work, for the first time, clearly demonstrates the occurrence of MPTP in the absence of oxidative stress and further, by a compound with protective effect against lipid oxidation and matrix oxidative stress.

Experimental Procedures

Chemicals. All reagents were commercial products of the highest purity grade available and aqueous solutions were prepared with deionized water (mixed bed of ion exchanger, Millipore) and the pH was measured using a combined glass electrode (Orion Glass pH SURE-FLOW).

Synthesis of the Organotelluranes. The organotelluranes used in this study (RT-03 and RT-04 Chart 1) were synthethized by the electrophilic addition of *p*-methoxyphenyl tellurium trichloride to the corresponding alkynes, namely 1-ethynyl-1-cyclohexanol and 3-methyl-3-hydroxy-butyne, respectively (*16*).

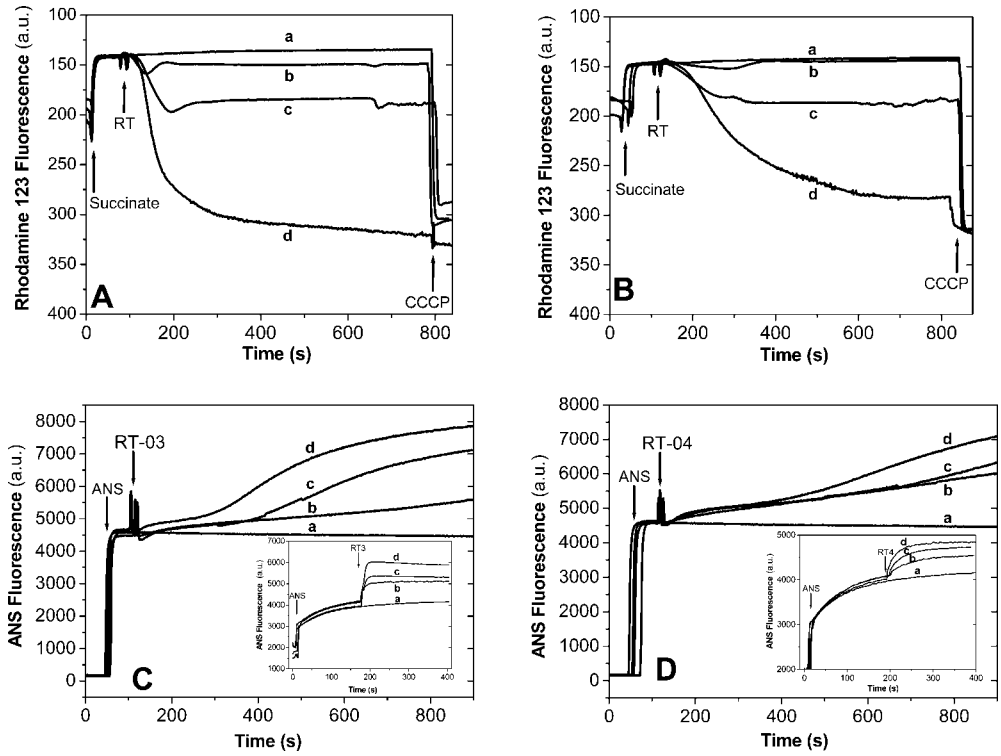


Figure 4. Effect of RT-03 and RT-04 on the mitochondrial transmembrane potential ($\Delta\Psi$) and on the physical properties of mitochondrial membranes and liposome model membranes. (A) Represents the effect of RT-03 on the transmembrane potential in the absence of Ca^{2+}: line a, control; line b, 20 μM RT-03; line c, 25 μM RT-03; and line d, 30 μM RT-03. (B) Represents the effect of RT-04 on the transmembrane potential in the absence of Ca^{2+}: line a, control; line b, 30 μM RT-04; line c, 35 μM RT-04; and line d, 40 μM RT-04. Data are presented as the mean of three experiments with different mitochondrial preparations. (C) Effect of RT-03 on the fluorescence of ANS bound to mitochondrial membrane (see Experimental Procedures for details): line a, control; line b, 10 μM RT-03; line c, 20 μM RT-03; and line d, 40 μM RT-03. The inset shows effect of RT-03 on the fluorescence of ANS bound to PCPECL liposome (see Experimental Procedures for details): line a, control; line b, 10 μM RT-03; line c, 20 μM RT-03; and line d, 40 μM RT-03. (D) Effect of RT-04 on the fluorescence of ANS bound to mitochondrial membrane (see Experimental Procedures for details): line a, control; line b, 10 μM RT-04; line c, 20 μM RT-04; and line d, 40 μM RT-04. The inset shows effect of RT-04 on the fluorescence of ANS bound to PCPECL liposome (see Experimental Procedures for details): line a, control; line b, 10 μM RT-04; line c, 20 μM RT-04; and line d, 40 μM RT-04. The mitochondrial $\Delta\Psi$ measurements were carried out (1 mg/mL) in a medium containing 125 mM sucrose, 65 mM KCl, 0.5 mM EGTA, 10 mM K_2HPO_4, and 10 mM HEPES-KOH, pH 7.4, at 30 °C, plus rhodamine 123 (0.4 μM) was initiated by the addition of 5.0 mM succinate.

Isolation of Rat Liver Mitochondria. Liver mitochondria were isolated by conventional differential centrifugation (*17*) 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 containing 0.1 mM EGTA, and the final pellet was resuspended in 250 mM sucrose to a final protein concentration of 80–100 mg/mL.

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 (*18*) measured by a Hitachi U-2000 Spectrophotometer (Tokyo, Japan).

Determination of Total Thiol Content. After 15 min incubation under swelling conditions, mitochondria were treated with trichloroacetic acid (5% final concentration) and centrifuged at 4500*g* for 10 min. The pellet was suspended with 1 mL of 0.5 M potassium phosphate buffer, pH 7.6, and, after addition of 0.1 mM DTNB, absorbance was determined at 412 nm. The amount of thiol groups was calculated from $\epsilon = 13\,600\ M^{-1}$ (*19*).

Determination of Mitochondrial Glutathione Content. After 15 min incubation under swelling conditions, 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 microliters 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) (*20*).

Analysis of Mitochondrial Reactive Oxygen and Nitrogen Species. Mitochondrial ROS and RNS production was assessed using the oxidative stress indicator 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) as described previously (*21, 22*) The method is based on the intramitochondrial peroxide-dependent oxidation of H_2DCFHDA to form the fluorescent compound, 2′,7′-dichlorofluorescein (DCF). Isolated mitochondria were incubated in a total volume of 2 mL of swelling buffer at 30 °C for 10 min in the presence of 1 μM H_2DCFDA, which was made fresh before use. Further additions are indicated in the figure legends. The relative production of mitochondrial ROS and RNS was measured by using an F-2500 spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) operating at $\lambda_{excitation}$ and $\lambda_{emission}$ wavelengths of 503 and 529 nm, respectively.

SDS-Polyacrylamide Slab Gel Electrophoresis. After 15 min incubation under swelling conditions, samples were incubated in the reaction medium and boiled for 2 min in 250 mM Tris-HCl, pH 7.4, 20% SDS, and 50 mM EDTA according to Liu et al. (*23*). Samples (0.4 μg) were applied to the electrophoresis gel. Electrophoresis was performed by SDS-PAGE in a discontinuous system as described by Laemmli (*24*). The running gel was 10% in acrylamide, the stacking gel was 3.5%, and the voltage was 20 mA. The gels were then stained with silver nitrate as described by Blum et al. (*25*).

Measurements of Mitochondrial Transmembrane Electrical Potential ($\Delta\Psi$). Mitochondrial $\Delta\Psi$ was estimated through fluorescence changes of rodamine 123 (0.4 μM), (*26, 27*) recorded on a model F-2500 spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) operating at $\lambda_{excitation}$ and $\lambda_{emission}$ wavelengths 505 and 525 nm, with a slit width of 5 nm.

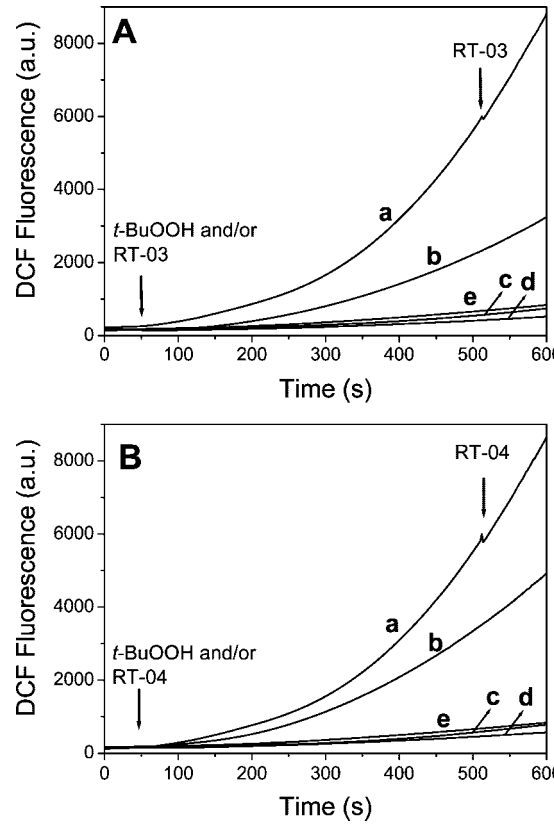


Figure 5. Effect of telluranes on the mitochondrial generation of reactive oxygen and nitrogen species. (A) Effect of RT-03. Rat liver mitochondria (1 mg/mL) were added to a medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, pH 7.2, 5 mM potassium succinate (+ 2.5 μM rotenone), 10 μM $CaCl_2$ at 30 °C, and 0.8 μM DCFDA. *Tert*-butylhydroperoxide alone followed by 100 μM RT-03 addition at 500 s (line a), *Tert*-butylhydroperoxide plus 10 μM RT-03 (line b), or 10 and 40 μM RT-03 (lines c and d, respectively) were added where indicated by the arrows. Line e represents a control experiment without addition of *t*-BuOOH and/or RT-03. (B) Effect of RT-04. Rat liver mitochondria (1 mg/mL) were added to a medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, pH 7.2, 5 mM potassium succinate (+ 2.5 μM rotenone), 10 μM $CaCl_2$ at 30 °C, and 0.8 μM DCFDA. *Tert*-butylhydroperoxide alone followed by 100 μM RT-04 addition at 500 s (line a), *Tert*-butylhydroperoxide plus 10 μM RT-04 (line b), or 10 and 40 μM RT-04 (lines c and d, respectively) were added where indicated by the arrows. Line e represents a control experiment without addition of *t*-BuOOH and/or RT-04.

Measurements of Physical Alterations in the Mitochondrial and Liposome Membrane Lipid Bilayer. Mitochondria (1 mg protein/mL) or 1 mM PCPECL liposomes were incubated in a medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, pH 7.4, at 30 °C with 75 μM ANS plus 1 $\mu g\ mL^{-1}$ CCCP. Fluorescence was measured with a F-2500 spectrofluorometer (Hitachi, Ltd., Tokyo, Japan) operating at $\lambda_{excitation}$ and $\lambda_{emission}$ wavelengths of 380 and 485 nm, respectively (*28, 29*).

Lipid Peroxidation Assay. Lipid peroxidation of mitochondrial membranes was evaluated as MDA generation. Mitochondria (1 mg/mL) were incubated in a medium containing 130 mM KCl, 10 mM HEPES-KOH, pH 7.4, plus 5 mM potassium succinate (+ 2.5 μM rotenone) and 10 μM $CaCl_2$ for 15 min at 30 °C. $(NH_4)_2Fe(SO)_4$ (50 μM) and 2 mM sodium citrate or *t*-BOOH were used to induce damage. 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 further incubation for 20 min at 85 °C. The MDA–TBA complex was extracted with 2 mL of *n*-butanol and the

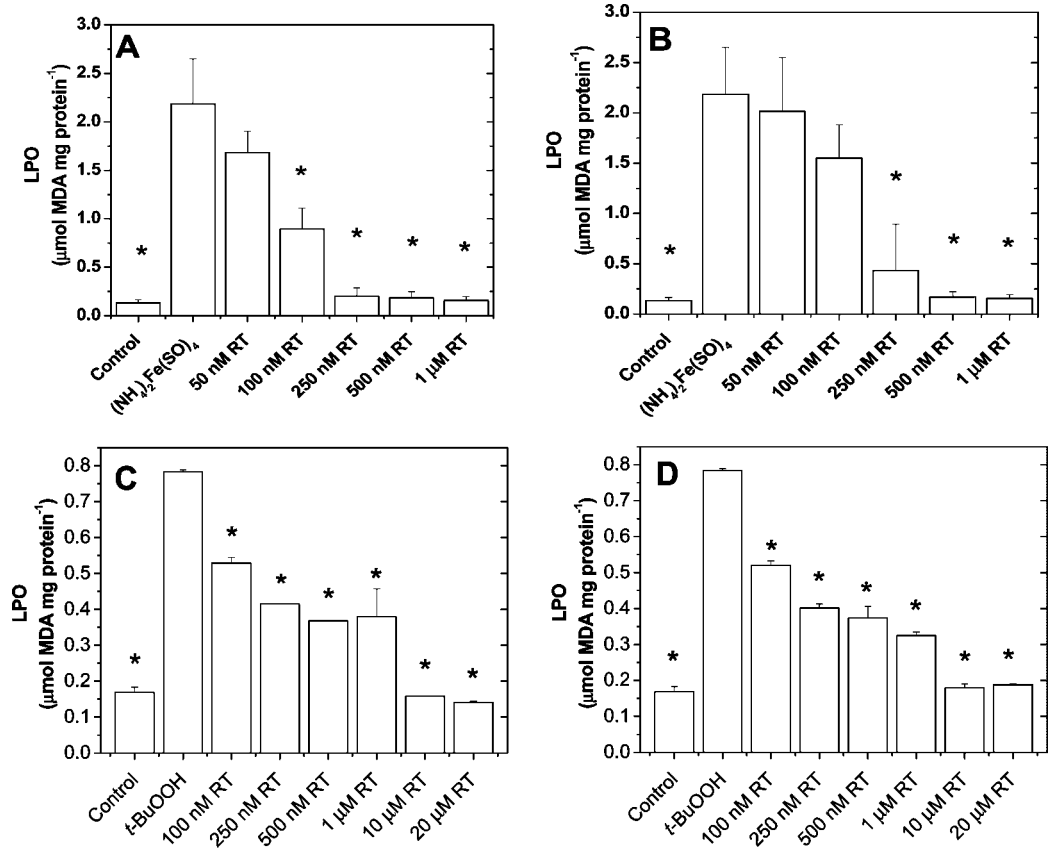


Figure 6. Effects of RT-03 and RT-04 on lipid peroxidation assayed as MDA generation. (A) Control experiment in the absence of RT-03, the effect of 50 μM $(NH_4)_2Fe(SO)_4$ and the effect of 50, 100, 250, 500 nM and 1 μM RT-03 as indicated in the figure. (B) Control experiment in the absence of RT-04, the effect of 50 μM $(NH_4)_2Fe(SO)_4$, and the effect of 50, 100, 250, 500 nM and 1 μM RT-04 as indicated in the figure. (C) Control experiment in the absence of RT-03, the effect of 0.6 mM *t*-BuOOH, and the effect of 100, 250, 500 nM, 1, 10, and 20 μM RT-03 as indicated in the figure. (D) Control experiment in the absence of RT-04, the effect of 0.6 mM *t*-BuOOH, and the effect of 100, 250, 500 nM, 1, 10, and 20 μM RT-04 as indicated in the figure. Mitochondria (1 mg protein/mL) were incubated in a medium containing 130 mM KCl, 10 mM HEPES-KOH, pH 7.4, plus 5 mM potassium succinate (+ 2.5 μM rotenone), for 30 min at 37 °C. $(NH_4)_2Fe(SO)_4$ (50 μM) and 2 mM sodium citrate were used for panel A and B or *t*-BuOOH (0.6 mM) for panel C and D for damage induced. Data are presented as the mean ±SEM of three experiments with different mitochondrial preparations. Asterisk (*) means significant decrease relative to the positive control, $(NH_4)_2Fe(SO)_4$, or *t*-BuOOH-induced lipid peroxidation.

absorbance was measured at 532 nm. MDA concentration was calculated from $\epsilon = 1.56 \times 10^5\ M^{-1}$ (*30*).

Mitochondrial Respiration. Mitochondria (1 mg protein/mL) were incubated in a medium containing 125 mM sucrose, 65 mM KCl, 0.5 mM EGTA, 10 mM K_2HPO_4, and 10 mM HEPES-KOH, pH 7.2, at 30 °C. Mitochondrial respiration was monitored polarographically by an oxygraph equipped with a Clark-type oxygen electrode (Gilson Medical Electronics, Middleton, WI). State 4 respiration was initiated by addition of 5 mM potassium succinate (+ 2.5 μM rotenone) and state 3 respiration was initiated by addition of 400 nmol ADP. Respiratory control ratios (RCR) were determined according to Chance and Willian (*30*).

Statistical Analysis. The statistical analyses for Figure 2A and B and Figure 4A, B, C, and D data were done by GraphPad software available at the following site: http://statpages.org/anova1sm.html. The post-hoc tests (Bonferroni correction) were also done by GraphPad software at http://graphpad.com/quickcalcs/posttest1.cfm.

Results

Figure 1A and B shows that the organotelluranes RT-03 and RT-04 in concentrations above 1 μM induced swelling of Ca^{2+}-loaded mitochondria in a dose-dependent manner. In these conditions, the swelling was accompanied by loss of $\Delta\Psi$ (mitochondrial transmembrane potential) as represented by Figure 1C. CsA was unable to promote complete inhibition of the mitochondrial swelling induced by RT-03 and RT-04 in concentrations above 10 μM (not shown).

The inhibitory effect of CsA on the RT-03- and RT-04-promoted mitochondrial swelling suggested that the organotelluranes were able to mediate the opening of the MPTP, probably via change in the mitochondrial thiol redox state. To confirm the mechanism of MPT induction by RT-03 and RT-04, the effect of organotelluranes on the mitochondrial thiol content (Figure 2A and B) was measured. Figure 2A and B shows that similarly to *t*-BuOOH, RT-03 and RT-04 significantly decreased the mitochondrial total thiol content in the same concentrations in which the CsA-sensitive MPT was observed. Furthermore, the reduction of total thiol group content promoted by RT-03 and RT-04 was accompanied by proportional cross-linkage of mitochondrial membrane proteins, as attested by the SDS-PAGE electrophoresis showed in Figure 2C.

The ability of RT-03 and RT-04 to promote the opening of MPTP by direct or indirect attack to thiol groups of mitochondrial inner membrane proteins was also corroborated by the capacity of DTT to inhibit CsA-sensitive swelling (Figure 3). In this regard, NEM, a well-known monothiol reagent, delayed the CsA-sensitive swelling promoted by the organotelluranes (Figure 3).

Considering the inability of CsA as well as EGTA to inhibit the MPT produced by RT-03 and RT-04 in concentrations above 10 μM, it was necessary to confirm whether loss of $\Delta\Psi$ (mitochondrial transmembrane potential) was involved in the unregulated pore opening. Figure 4A and B, shows that, in the absence of Ca^{2+}, significant RT-03- and RT-04-induced loss of $\Delta\Psi$ was observed only in the organotellurane concentrations able to induce CsA-insensitive swelling.

At this point it was interesting to know whether CsA-insensitive swelling and Ca^{2+}-independent $\Delta\Psi$ loss occurred because the misfolding of proteins was enough to open membrane pores independent of cyclophilin D binding or because the organotelluranes promoted physical damage in the lipid bilayer. Figure 4C and D shows that, in high concentrations, RT-03 and RT-04 promoted physical alterations in the lipid bilayer organization as attested by the increase of ANS fluorescence, both in mitochondrial membranes and PCPECL liposomes (inset of Figure 4C and D, respectively).

The ability of RT-03 and RT-04 to react with thiol groups raised the possibility that oxidative stress could be induced as a consequence of glutathione depletion. In fact, RT-03 and RT-04 promoted significant glutathione depletion in conditions in which the MPT was induced (not shown). To exclude the participation of oxidative stress in tellurane-induced MPT, we measured mitochondrial reactive oxygen and nitrogen species (ROS and RNS, respectively) production (Figure 5A and B) by using the fluorescent probe H_2DCFDA. H_2DCFDA is oxidized mainly by peroxide-derived free radicals and peroxynitrite formed from the reaction of $O_2^{\bullet-}$ with $NO^{\bullet-}$ (*21, 22*) generating DCF, which is highly fluorescent. A fast increase in the rate of DCF production was observed after the addition of *t*-BuOOH (Figure 5A and B, line a), indicating the production of reactive species under this condition. Addition of telluranes after significant H_2DCFDA oxidative cleavage (around 500 s as indicated by arrows on line a of Figure 5A and B) did not decrease the fluorescence which excludes fluorescence quencher artifact. However, when the addition of RT-03 and RT-04 was concomitant with *t*-BuOOH addition (Figure 5A and B, line b), significant decrease in the DCF fluorescence was observed, despite the occurrence of mitochondrial swelling (not shown). DCF fluorescence was not observed in the absence of *t*-BuOOH and telluranes (Figure 5A and B, line c) as well as in the presence of 10 and 40 μM telluranes alone (Figure 5A and B, lines d and e, respectively). The results obtained in the presence of *t*-BuOOH and organotelluranes and in the presence of organotelluranes alone reinforce that MPTP opening can occur in conditions in which oxidative stress was prevented.

Considering that literature data have reported antioxidant capacity by the organotellurium compounds (*14*), we checked the effect of RT-03 and RT-04 on the production of malondialdehyde by mitochondria in the absence and in the presence of Fe-citrate (Figure 6). Figure 6 shows that, despite the capacity to induce CsA-sensitive and CsA-insensitive mitochondria swelling concomitant with glutathione depletion, RT-03 and RT-04 did not induce lipid peroxidation. In contrast, these compounds exhibited efficient antioxidant activity as they were able to protect mitochondria lipids in the nanomolar concentration range. Consistent with the proposal that the MPTP opening could be dependent only on the misfolding of the inner mitochondrial membrane proteins induced by the attack to thiol groups, although the studied organotelluranes have decreased significantly the DCF fluorescence produced by *t*-BuOOH-induced oxidative stress in the matrix (Figure 5A and B, line b), they were not able to prevent the mitochondrial swelling, i.e., MPTP opening, induced by the organic peroxide (not shown).

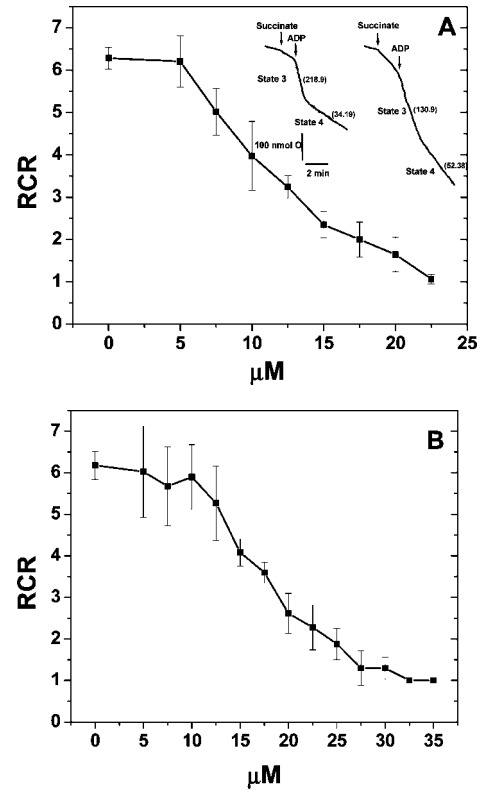
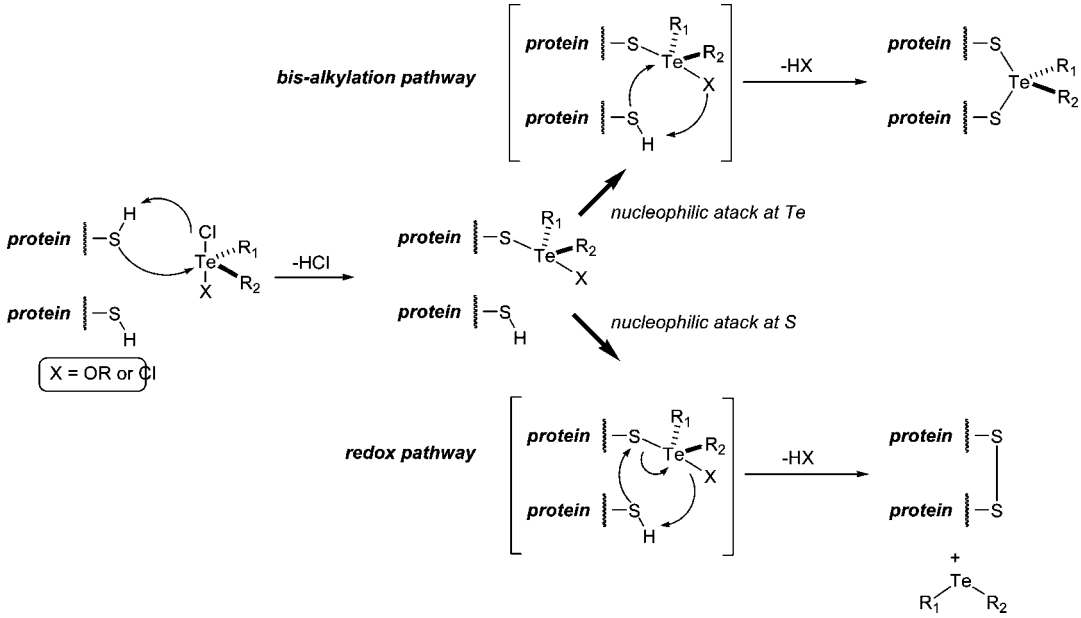


Figure 7. Effects of RT-03 and RT-04 concentration on the RCR of isolated rat liver mitochondria. The experiments were carried out in the following conditions: mitochondria (1 mg protein/mL) was incubated in a medium containing 125 mM sucrose, 65 mM KCl, 0.5 mM EGTA, 10 mM K_2HPO_4, and 10 mM HEPES-KOH, pH 7.2, at 30 °C with addition of 5 mM potassium succinate plus 2.5 μM rotenone. State 3 respiration, by addition of 400 nmol of ADP. Panels A and B show the effect of RT-03 and RT-04, respectively. Data are presented as the mean ±SEM of three experiments with different mitochondrial preparations. The inset shows the original result obtained in the absence (left line) and in the presence (right line) of 15 μM RT-03.

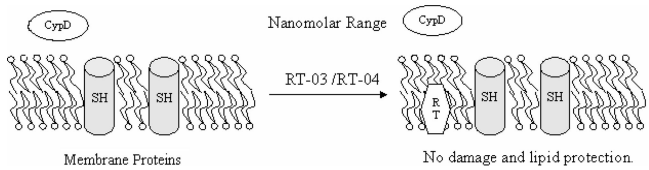
Considering the exceptional antioxidant ability exhibited by RT-03 and RT-04 we tested the effect of these compounds on mitochondrial RCR (respiratory control ratios) in order to establish potential toxicological side effects for these compounds. Figure 7 shows that RT-03 and RT-04 induced a decrease of the RCR in concentrations above 5 and 10 μM, respectively. In this condition, the RCR decrease is resulting from concomitant state 3 respiration rate decrease due to phosphorylation impairment and state 4 respiration rate increase due to uncoupling. These concentrations are around 50-fold the dose necessary to protect the lipid fraction of mitochondria. In this regard, in the nanomolar concentration range, RT-03 and RT-04 did not exhibit any effect on the mitochondria ATP production and glutathione depletion (not shown). The phosphorylation impairment promoted by RT-03 and RT-04 in doses able to induce MPTP opening is expected by considering the ability of these compounds to react with thiol groups. The attack to thiol groups of ANT, for instance, could inhibit ADP phosphorylation by ATP synthase due to the incapacity of mitochondria to exchange ATP for ADP.

Inhibition of the respiratory chain electron transport by RT-03 and RT-04 was observed only in concentrations above 100 μM (not shown). In this condition, damage promoted by RT-03 and RT-04 could be extended to the respiratory chain proteins.

Scheme 1. Proposed Mechanism for the Organotellurane-Mediated Oligomerization of Mitochondrial Proteins Involved in the MPTP Opening



Scheme 2. Effect of Organotelluranes on the Mitochondria at the Nanomolar Range

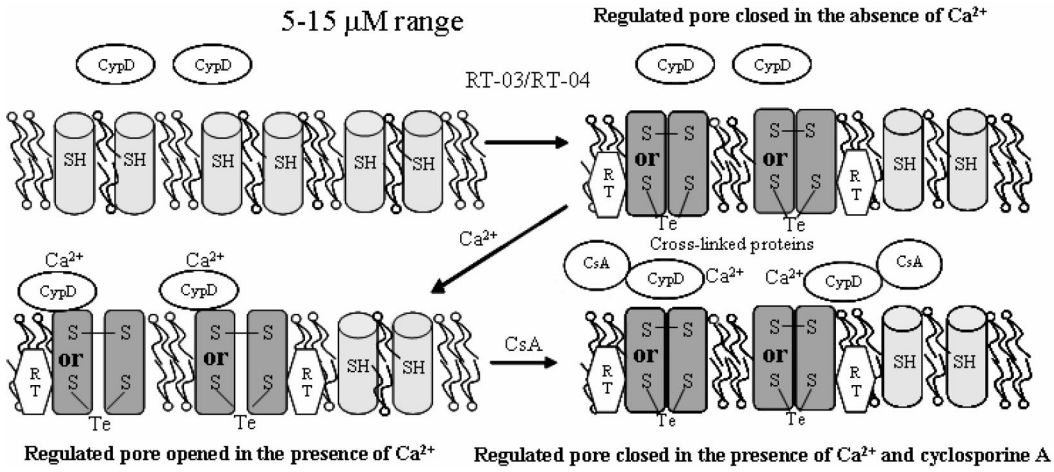


Discussion

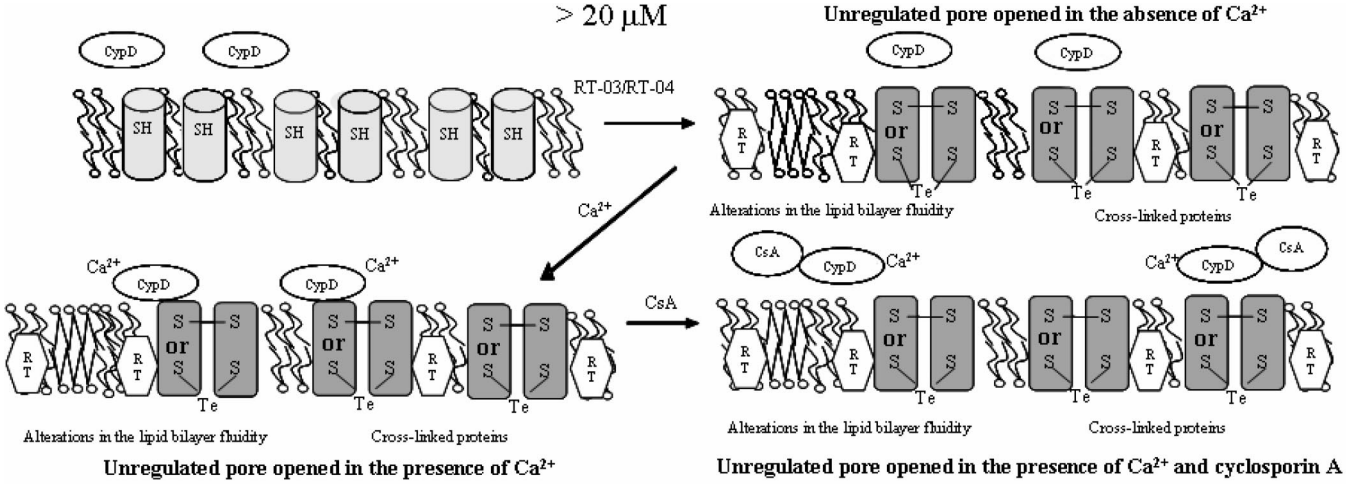
Since MPT was characterized as a key mechanism underlying both necrotic and apoptotic cell death (*9*), this event has been the focus of intense research and controversy. The uncertainty about MPT involves mainly the molecular composition of the pore, but details about the mechanism of MPTP opening also remain a challenge. Particularly concerning the latter uncertainty, the effects of organotelluranes RT-03 and RT-04 on the mitochondria bring light on the requirement of oxidative stress in MPTP opening. RT-03 and RT-04 exhibit structural characteristic to act as dithiol reagents and, this property was further confirmed by the inhibitory action on the cysteine protease cathepsin B (*31*) and by the titration of cysteine thiol groups (*32*). Thus, as expected, RT-03 and RT-04 were able to induce CsA-sensitive and -insensitive MPT in a dose-dependent manner. The regulated and CsA-insensitive MPTP opening triggered by RT-03 and RT-04 was characterized as being associated with the reactivity of these compounds with thiol groups. Thus, the MPTP opening promoted by RT-03 and RT-04 was accompanied by dose-dependent decrease of the thiol group content (Figure 2, A and B), cross-linkage of mitochondrial membrane proteins (Figure 2C), and protective effect by DTT and NEM (Figure 3). At the concentration range in which RT-03 and RT-04 exhibited the ability to promote MPTP opening, they also promoted, in the absence of Ca^{2+}, phosphorylation impairment but not the loss of $\Delta\Psi$ that was observed when Ca^{2+} was present. Therefore, at this concentration range, RT-03 and RT-04 probably reacted with thiol groups of mitochondrial proteins and led them to assemble the pore. When Ca^{2+} was absent the pore remained occluded due to the absence of cyclophilin D binding which prevented the loss of $\Delta\Psi$. However, the attack to thiol groups could affect ANT, a putative component of MPTP (*1, 4, 9*) leading to RCR decrease. However, besides the reactivity with thiol groups, RT-03 and RT-04 also present evident hydrophobicity and, thus, the capacity to affect the inner bilayer structure. The increase of ANS fluorescence induced by RT-03 and RT-04 in mitochondria (Figure 4A and B) at the concentration range coincident with the ability to promote CsA-insensitive MPT as well as in PCPECL liposomes in which proteins are absent (Figure 4C, D, and respective insets) suggests that alterations in the membrane lipid bilayer organization should be involved in this event. Therefore, at high organotellurane concentrations, attack to thiol groups of membrane proteins as well as significant changes in the membrane fluidity (*33*) should be enough to induce misfold of the proteins and open the pore in a cyclophilin D-independent manner. This result suggests that, over the concentration range able to induce CsA-insensitive pore opening, the effect on the membrane organization could have significant influence on mitochondria function.

Literature data have correlated oxidative stress with MPTP opening (*1*). In this regard, there are strong points of evidence for correlation between the NADPH redox status and the occurrence of MPT. On the other hand, Pfeiffer et al. reported the occurrence of CsA-sensitive and -insensitive MPTP opening by the amphypatic peptides mastoparan and MP-14, in a dose-dependent manner (*8*). These peptides exhibited a bimodal mechanism of action to promote MPTP opening. In the submicromolar concentration range, the action of the peptides was Ca^{2+}- and CsA-sensitive, a typical example of CsA-inhibitable MPT. At concentrations above 1 μM, MPT occurred in a nonregulated manner. Although the work of Pfeiffer et al. has characterized that the pores formed in mitochondria in response to both low and high concentrations of mastoparan and MP14 are MPTP, the authors did not investigate the redox status of mitochondrial NADPH and thiol groups and the formation of protein aggregates. Although the peptides can not attack mitochondria thiol groups, the effects on the membrane organization and polarization could trigger oxidative events leading to changes in the redox status of these groups. Anyway, contrary to organotelluranes studied in the present work, these peptides do not exhibit any antioxidant properties and could

Scheme 3. Effect of Organotelluranes on the Mitochondria at the 5-15 μM Concentration Range



Scheme 4. Effect of Organotelluranes on the Mitochondria at the Concentration Range above 20 μM



not provide a condition in which the pore could be formed concomitantly with efficient antioxidant protection.

Literature data have showed that organyl diselenides and ditellurides compounds exhibit potent antioxidant properties due to the inherent reactivity of dichalcogen bonds (*34*). On the other hand, unlike organotellurides, organotelluranes exhibit high reactivity with sulphydryl groups and it is expected that the products of these reactions retain the antioxidant properties since they have mixed dichalcogen bonds.

The fact that the CsA-sensitive MPTP opening was promoted by RT-03 and RT-04 in concentrations unable to promote physical and chemical damages on the lipid bilayer as well as oxidative stress in the mitochondrial matrix, raises, for the first time, evidence that MPTP opening could be promoted exclusively by cross-linkage of the mitochondrial inner membrane proteins. Therefore, the lipid damage observed under conditions in which MPTP opening was promoted by oxidative stress should be only a side effect and was not decisive for the event.

The effect of the organotelluranes RT-03 and RT-04 on the mitochondrial function can be rationalized by the reactivity of these compounds toward nucleophiles. As it is already described, organotelluranes react with thiols (*35–37*) in such way that a sulphydryl group can be "tellurated", and this "tellurated" protein can react further with another thiol group, intra- or intermolecularly in two possible pathways as depicted in Scheme 1. The first pathway, named here as a bis-alkylation pathway, consists of a second substitution at the tellurium atom by the displacement of the second leaving group bonded to tellurium by the second sulphydryl group leading to a tellurium(IV)-bridged protein complex. The "tellurated" protein can also react with another sulphydryl group, by the nucleophilic attack at the sulfur atom, displacing the tellurium moiety that is reduced to a diorganyltelluride as a disulfide bond is formed. This second pathway was named here as a redox pathway, since it involves changes in the redox state of both tellurium and sulfur atoms.

Conclusions

The results described in the present work point out the following conclusions summarized in Schemes 2, 3, and 4: (i) the effects of organotelluranes RT-03 and RT-04 on the mitochondrial function are modulated by the concentration of the drugs; (ii) at the nanomolar concentration range, RT-03 and RT-04 exhibit exceptional antioxidant activity; (iii) the antioxidant activity of RT-03 and RT-04 covered the mitochondrial lipid fraction and matrix but not mitochondrial membrane proteins since, despite of absence of lipid peroxidation and matrix oxidative stress decrease, they were unable to protect the MPTP opening promoted by *t*-BuOOH; (iv) at the concentration range of 5–15 μM, RT-03 and RT-04 induced the Ca^{2+}-dependent MPTP opening regulated by cyclosporine; (v) at concentrations above 20 μM, RT-03 and RT-04 induced the CsA-insensitive MPTP opening, probably due to more extensive protein damages and alterations in the bilayer fluidity; (vi) whatever the type of RT-03- and RT-04-induced mitochondrial pore opening, the mitochondrial lipid fraction was preserved

and matrix oxidative stress was absent despite the depletion of the mitochondrial glutathione content; (vii) for the first time a work shows clear evidence that the MPTP opening can occur in the absence of oxidative stress and concomitant with matrix and membrane lipid protection.

Acknowledgment. This work was supported by the Brazilian research funding agencies Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Fundação de Amparo ao Ensino e Pesquisa da Universidade de Mogi das Cruzes (FAEP-UMC), and Conselho Nacional de Pesquisa (CNPq). We thank Dr. Alicia J. Kowaltowski for the critical reading of the manuscript.

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