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Mitochondrial thioredoxin reductase inhibition by gold(I) compounds and concurrent stimulation of permeability transition and release of cytochrome *c*

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Abstract

The effects of auranofin, chloro(triethylphosphine)gold(I) (TEPAu), and aurothiomalate on mitochondrial respiration, pyridine nucleotide redox state, membrane permeability properties, and redox enzymes activities were compared. The three gold(I) derivatives, in the submicromolar range, were extremely potent inhibitors of thioredoxin reductase and stimulators of the mitochondrial membrane permeability transition (MPT). Auranofin appeared as the most effective one. In the micromolar range, it inhibited respiratory chain and glutathione peroxidase activity only slightly if not at all. TEPAu and aurothiomalate exhibited effects similar to auranofin, although TEPAu showed a moderate inhibition on respiration. Aurothiomalate inhibited glutathione peroxidase at concentrations where auranofin and TEPAu were without effect. Under nonswelling conditions, the presence of auranofin and aurothiomalate did not alter the redox properties of the mitochondrial pyridine nucleotides indicating that membrane permeability transition occurred independently of the preliminary oxidation of pyridine nucleotides. Under the same experimental conditions, TEPAu showed a moderate stimulation of pyridine nucleotides oxidation. Mitochondrial total thiol groups, in the presence of the gold(I) derivatives, slightly decreased, indicating the occurrence of an oxidative trend. Concomitantly with MPT, gold(I) compounds determined the release of cytochrome *c* that, however, occurred also in the presence of cyclosporin A and, partially, of EGTA, indicating its independence of MPT. It is concluded that the specific inhibition of thioredoxin reductase by gold(I) compounds may be the determinant of MPT and the release of cytochrome *c*.

Keywords: Auranofin; Aurothiomalate; Chloro(triethylphosphine)gold(I); Mitochondrial permeability transition; Thiol groups; Thioredoxin reductase

1. Introduction

Gold-based compounds are drugs essentially used in the treatment of rheumatoid arthritis [1]. They have also been tested for the treatment of cancer [2], AIDS, bronchial asthma, and malaria [1]. A potential role of mitochondria in the mechanisms of cytotoxicity and antitumor action elicited by gold(I) derivatives has been recently highlighted by McKeage *et al.* [3]. In a previous paper [4], we have observed that auranofin, at submicromolar concentrations and in the presence of calcium ions, was able to induce mitochondrial permeability transition, measured as swelling and membrane potential decrease. This drug adds to a long list of compounds able to act as inducers of mitochondrial membrane permeability transition [5,6]. Thiol reagents and oxidizing substances are particularly active in this process [6]. In the present paper, we have examined and compared the effects of three gold(I) compounds (auranofin, TEPAu, and aurothiomalate) on redox-dependent mitochondrial functions. TEPAu has already been tested on mitochondria [7–9]. In isolated hepatocytes, electron microscopy reveals that, after incubation with 50 μM TEPAu, mitochondria are the first organelles to show morphological alterations [7]. In particular, after relatively long times of incubation mitochondria swell,

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Abbreviations: Auranofin, (*S*-triethylphosphinegold(I)-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside); CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DTNB, 5,5′-dithiobis(2-nitrobenzoic) acid; DTT, dithiothreitol; MPT, mitochondrial permeability transition; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEPAu, chloro(triethylphosphine)gold(I).

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while other organelles do not exhibit significant alterations [7]. At variance with TEPAu, less attention was directed to the interaction between auranofin or aurothiomalate and mitochondria. However, according to Dong *et al.* [10] in some tumor cell lines with altered mitochondria, cisplatin resistance is associated with sensitivity to the killing by several inhibitors of mitochondrial functions including auranofin. In fact, in a cell survival assay [10], very low concentrations of auranofin, comparable to those able to induce permeability transition in isolated mitochondria [4], are sufficient to reduce cell survival. In a particular clone (HeLa-S1a cells), auranofin is the most efficient, and more active than classical inhibitors of the mitochondrial electron flux such as rotenone, thenoyltrifluoroacetone (TTFA), and antimycin [10]. Therefore, mitochondria appear to play a major role in the mechanisms of cytotoxicity and antitumor activity of gold(I)–phosphine complexes [3].

Previously, it was observed that auranofin is a potent inhibitor of both cytosolic [11] and mitochondrial [4] thioredoxin reductase (EC 1.6.4.5) and the action on this enzyme was considered as the major explanation for the effects observed on the other functions such as permeability transition [4]. Here we show that also TEPAu and aurothiomalate are potent inhibitors, at submicromolar level, of thioredoxin reductase although auranofin remains the most efficient. At molecular level, their action is referable to an inhibition of thiol or selenol groups and the present research is mostly aimed at identifying their specific targets. Therefore, in addition to the interaction with the selenoenzyme thioredoxin reductase, their effects on respiration, glutathione, glutathione peroxidase (EC 1.11.1.9), total thiols and pyridine nucleotides redox state were studied. Since an important consequence of mitochondrial membrane alteration is linked to the release of cytochrome *c*, the effect of gold(I) derivatives on the release of the latter was also examined.

2. Materials and methods

Rat liver mitochondria were isolated with differential centrifugation according to Myers and Slater [12], using a medium containing 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 5 mM Hepes at pH 7.0. EDTA was omitted in the washing and in the final resuspension of mitochondria. Mitochondrial matrix was prepared from the mitochondrial suspension (60 mg protein mL^{-1}) by three cycles of freezing and thawing followed by sonication (twice for 30 s each) and centrifugation at 105,000 *g* for 60 min. Pellet was discarded, the supernatant was extensively dialyzed to remove glutathione and, finally, the mitochondrial matrix protein suspension was concentrated in a pressure dialysis system using an Amicon YM10 membrane. Proteins of isolated mitochondria preparations and mitochondrial matrix were estimated with the biuret procedure [13]. Mitochondrial thioredoxin reductase was prepared from isolated rat liver mitochondria and assayed according to Bindoli and Rigobello [14]. Protein content of the thioredoxin reductase preparation was estimated with the procedure of Lowry *et al.* [15]. Glutathione peroxidase activity was assayed according to Little *et al.* [16]. Oxygen uptake was measured polarographically utilizing a Clark-type oxygen electrode [17] inserted in a water-jacketed chamber (25°) with constant stirring and the system was connected to a personal computer [18]. Mitochondrial swelling was followed spectrophotometrically by the decrease in absorbance at 540 nm. Mitochondrial total thiols were measured according to Bindoli and Rigobello [14], while total and oxidized glutathione were estimated as described by Anderson [19]. The redox state of pyridine nucleotides was assessed fluorometrically (λ_{ex}: 350 nm; λ_{em}: 450 nm) in a constantly stirred and termostated (25°) cuvette. Cytochrome *c* release was determined by Western blotting. The various samples were subjected to SDS-PAGE according to Laemmli [20], using 15% (w/v) acrylamide. Gels were then electroblotted, using a "Semi Dry System," to a nitrocellulose membrane (0.2 μm) in 25 mM ethanolamine, 92 mM glycine and 20% (v/v) methanol transfer buffer (pH 9.6), for 90 min at 350 mA. After the saturating step, membranes were treated with the primary monoclonal antibody (clone 7H8.2C12, Biosource International). Membranes were washed and the blots incubated with a rabbit anti-mouse secondary antibody tagged with horseradish peroxidase (DAKO). The detection was performed using the ECL (Amersham, Biosciences).

2.1. Statistical analysis

All the values are the mean ± S.D. of not less than five measurements. Multiple comparisons were made by one-way analysis of variance followed by the Dunnet post-test.

3. Results

Fig. 1 shows oxygen uptake by whole mitochondria in the presence of calcium and increasing concentrations of auranofin. When a pulse of calcium ions is added to respiring mitochondria it is apparent that, after its uptake, low concentrations of auranofin are able to markedly enhance the consumption of oxygen indicating the occurrence of MPT. The concentrations of auranofin used are noticeably lower than those required to inhibit the respiration of submitochondrial particles using NADH or succinate as substrates (not shown). In fact, relatively high concentrations of auranofin, TEPAu, and aurothiomalate are scarcely effective in inhibiting the flux of electrons along the respiratory chain (not shown). Respiration elicited by NADH is inhibited by about 20% by TEPAu higher than 10 μM. The latter is more effective with succinate as substrate since a 50% inhibition of the respiratory chain

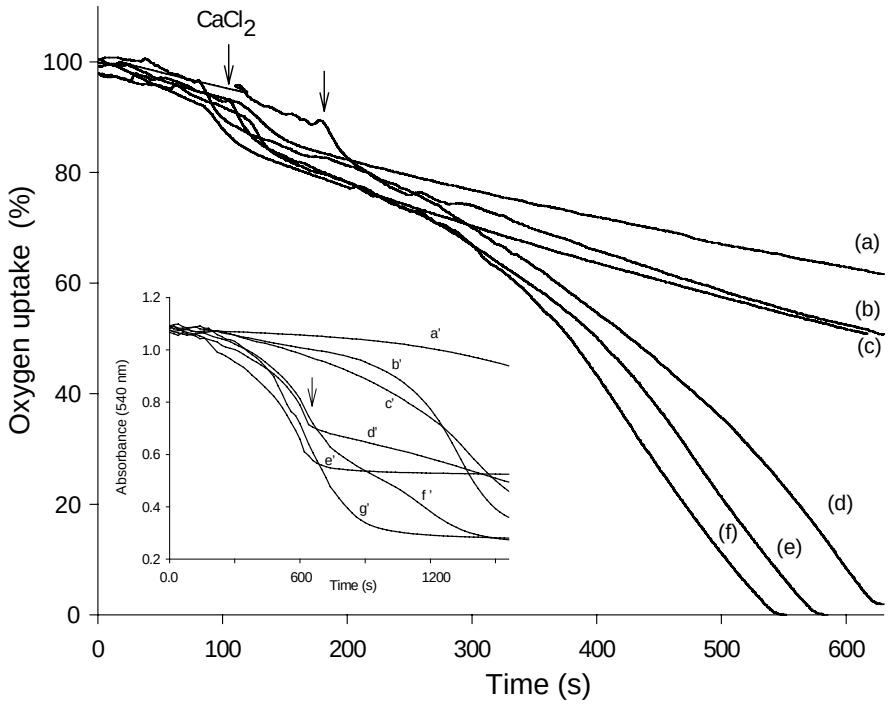

Fig. 1. Mitochondrial permeability transition induced by auranofin observed as oxygen uptake and membrane swelling. Rat liver mitochondria (1 mg mL^{-1}) were preincubated at 25° in 1 mL of 100 mM sucrose, 50 mM KCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4 and 20 mM Hepes-Tris (pH 7.4). DTT (5 mM) and cyclosporin A (5 μM) were also present in b and c, respectively. Respiration was initiated by 5 mM succinate, and, after about 1 min, 100 μM $CaCl_2$ was further added (first arrow). A transient consumption of oxygen is observed due to the energy-dependent calcium uptake. After 1 min (second arrow) were also added: a: none (control); b, c, and d: 0.5 μM auranofin; e: 1 μM auranofin; f: 2 μM auranofin. 100% oxygen corresponds to 278 nmol mL^{-1} of dissolved oxygen. In the inset, the auranofin-induced swelling is reported. Rat liver mitochondria (0.25 mg mL^{-1}) were incubated at 25° in 213 mM mannitol, 71 mM sucrose, 5 mM succinate, 5 μM rotenone, and 3 μM oligomycin. In d′–g′, swelling was triggered by the combined action of 0.25 μM auranofin and 25 μM $CaCl_2$. a′: none (control); b′: 25 μM $CaCl_2$, c′: 0.25 μM auranofin. At the arrow were also added: d′: 0.5 μM cyclosporin; e′: 2 mM DTT; f′: 1 mM EGTA.

occurs at a concentration around 8 μM (not shown). Auranofin is completely ineffective both with NADH and succinate up to concentrations of about 8 μM (not shown). The oxygen uptake stimulated by auranofin in the presence of calcium ions is completely inhibited by cyclosporin A and dithiothreitol (Fig. 1b and c). Results similar to those elicited by auranofin were obtained with TEPAu and aurothiomalate (not shown). MPT is also reported as swelling measured as absorbance decrease at 540 nm (Fig. 1, inset). As apparent, very low concentrations of auranofin in the presence of calcium ions are able to trigger a rapid and extensive mitochondrial membrane permeability transition (g′) while auranofin alone (c′) and calcium alone (b′) are less effective. The addition of cyclosporin A (d′) and EGTA (e′) completely inhibits the swelling induced by the combination of auranofin and calcium, however without reversing it, while DTT (f′) slows down the rate of swelling. Nevertheless, the addition of cyclosporin and DTT before the onset of oxygen uptake, completely inhibit MPT (Fig. 1b and c).

MPT is frequently accompanied by an alteration of the redox state of pyridine nucleotides and most of the inducers determine their oxidation [5,6]. Therefore, the fluorescence changes of pyridine nucleotides in the presence of gold(I) compounds were followed (Fig. 2). The addition of mitochondria to the incubation medium gives rise to a large increase of fluorescence that is further increased and stabilized by the presence of rotenone. The addition of 10 μM auranofin or aurothiomalate does not modify the redox conditions of NAD(P)H. As shown below (Fig. 3), this is in accordance with the inhibitory effect of these substances on thioredoxin reductase and the consequent lack of utilization of NADPH. TEPAu elicits a moderate oxidation of pyridine nucleotides (Fig. 2) as previously observed [9]. At variance with the gold(I) derivatives, both acetoacetate and *tert*-butylhydroperoxide, although with different mechanisms, give rise to an extensive oxidation of the pyridine nucleotides. Acetoacetate and *tert*-butylhydroperoxide oxidize their specific pyridine nucleotides through the action of the enzymes β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and glutathione peroxidase, respectively. Fluorescence measurements have been performed in the absence of substrates. In fact, respiratory substrates, by continuously feeding electrons, decrease the rate and extent of pyridine nucleotides oxidation [21], therefore making the effects of acetoacetate and *tert*-butylhydroperoxide less evident.

We have previously demonstrated that auranofin was able to inhibit thioredoxin reductase either isolated or in its mitochondrial environment [4]. Here, we report a comparative evaluation of the inhibitory properties of the three gold(I) complexes on the isolated enzyme and on the

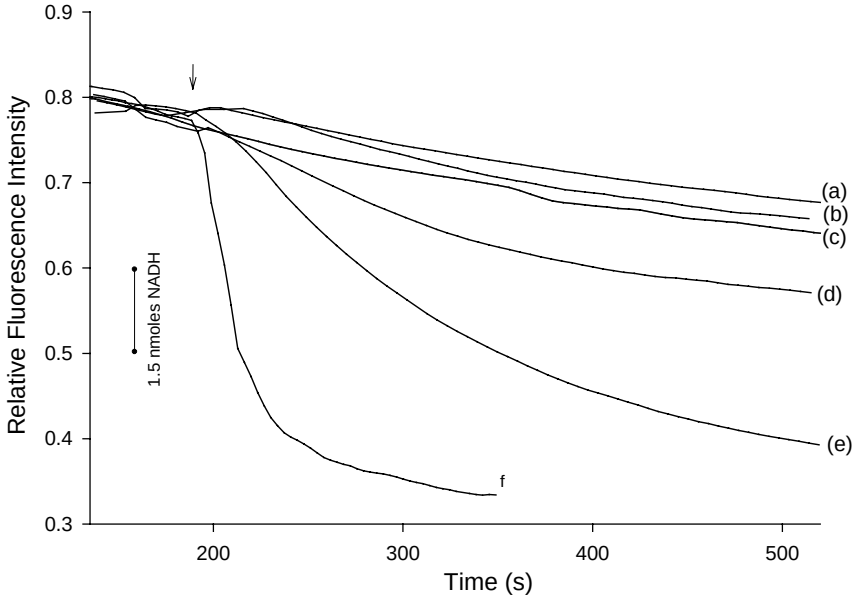

Fig. 2. Estimation of the redox state of mitochondrial pyridine nucleotides in the presence of gold(I) compounds, acetoacetate, and *tert*-butylhydroperoxide. Oxidation of pyridine nucleotides was followed as decrease of fluorescence (λ_{ex}: 350 nm; λ_{em}: 450 nm). Mitochondria (0.6 mg mL^{-1}) were incubated at 25° in 106 mM mannitol, 35 mM sucrose, 62 mM KCl, 1 mM EGTA, 5 μM rotenone, 1.4 mM phosphate, and 15 mM Hepes-Tris (pH 7.4) in a final volume of 2 mL. At the arrow were also added: a: 5 μM aurothiomalate; b: 5 μM auranofin; c: none (control); d: 5 μM TEPAu; e: 50 μM *tert*-butylhydroperoxide; f: 1.2 mM acetoacetate.

dialyzed mitochondrial matrix. As apparent in Fig. 3, all gold(I) compounds are potent inhibitors of the isolated enzyme, again confirming their high specificity towards thioredoxin reductase, possibly interacting with the selenol moiety [1,11]. Auranofin is the most effective in inhibiting thioredoxin reductase since it exhibits an IC_{50} of 2.6 and 36 nM with the purified enzyme and the dialyzed mitochondrial matrix, respectively. TEPAu is slightly less efficient than auranofin displaying IC_{50} of 6 and 59 nM, for the isolated enzyme and the matrix, respectively, while aurothiomalate shows lower inhibitory properties towards thioredoxin reductase with IC_{50} of 25 nM (purified enzyme) and 220 nM (mitochondrial matrix). All these compounds, at submicromolar concentrations, are ineffective towards glutathione peroxidase (Fig. 3). Moreover, in a micromolar range (4–10 μM), aurothiomalate exhibits a marked inhibition towards glutathione peroxidase ($IC_{50} = 1.5$ μM) unlike auranofin and TEPAu that, in the same conditions, are almost completely ineffective. However, it should be considered that thiomalate itself is a good inhibitor of selenium glutathione peroxidase [22].

Considering that, at molecular level, the major target of gold(I) complexes is represented by thiol and/or selenol groups [1,22], their effect on total thiols was examined. Incubation of whole mitochondria with auranofin, TEPAu, and aurothiomalate indicates that these compounds slightly decrease total thiol concentration (Fig. 4). A decrease of the latter comparable to that occurring in the presence of Ca^{2+}-phosphate [21] is apparent. Interestingly, in the presence of the gold(I) compounds, the redox state of mitochondrial glutathione is not modified (not shown).

Bearing in mind the primary role played by mitochondria in the apoptotic process that is essentially mediated by cytochrome *c* release, we have explored the ability of gold(I) complexes to influence the release of this protein and the conditions where the process occurs. As reported in Fig. 5, auranofin, TEPAu, and aurothiomalate are able to stimulate the release of cytochrome *c*. Interestingly, this takes place to a significant extent also in the presence of cyclosporin A and, partially, of EGTA, that is, in conditions where permeability transition is totally inhibited. This is reminiscent of the action of other regents such as retinoic acids [23] and tributyltin [24].

4. Discussion

Auranofin, in the nanomolar range, is an extremely potent inhibitor of thioredoxin reductase (Fig. 3) and is able to elicit the MPT observed as Ca^{2+}-dependent increase of oxygen uptake and swelling of mitochondria (Fig. 1). Moreover, it is almost completely ineffective in inhibiting the enzymes glutathione reductase (EC 1.6.4.2) [4], glutathione peroxidase (Fig. 3) and the respiratory chain. TEPAu and aurothiomalate behave rather similarly to auranofin, although TEPAu shows a more marked inhibitory effect on the respiratory chain (not shown). In fact, the mitochondrial complexes I, III, and IV are scarcely affected by the three gold(I) derivatives, while complex II is inhibited to some extent by TEPAu (not shown). The effect of TEPAu elicited in the presence of succinate is in agreement with the result found by Rush *et al.* [8] where

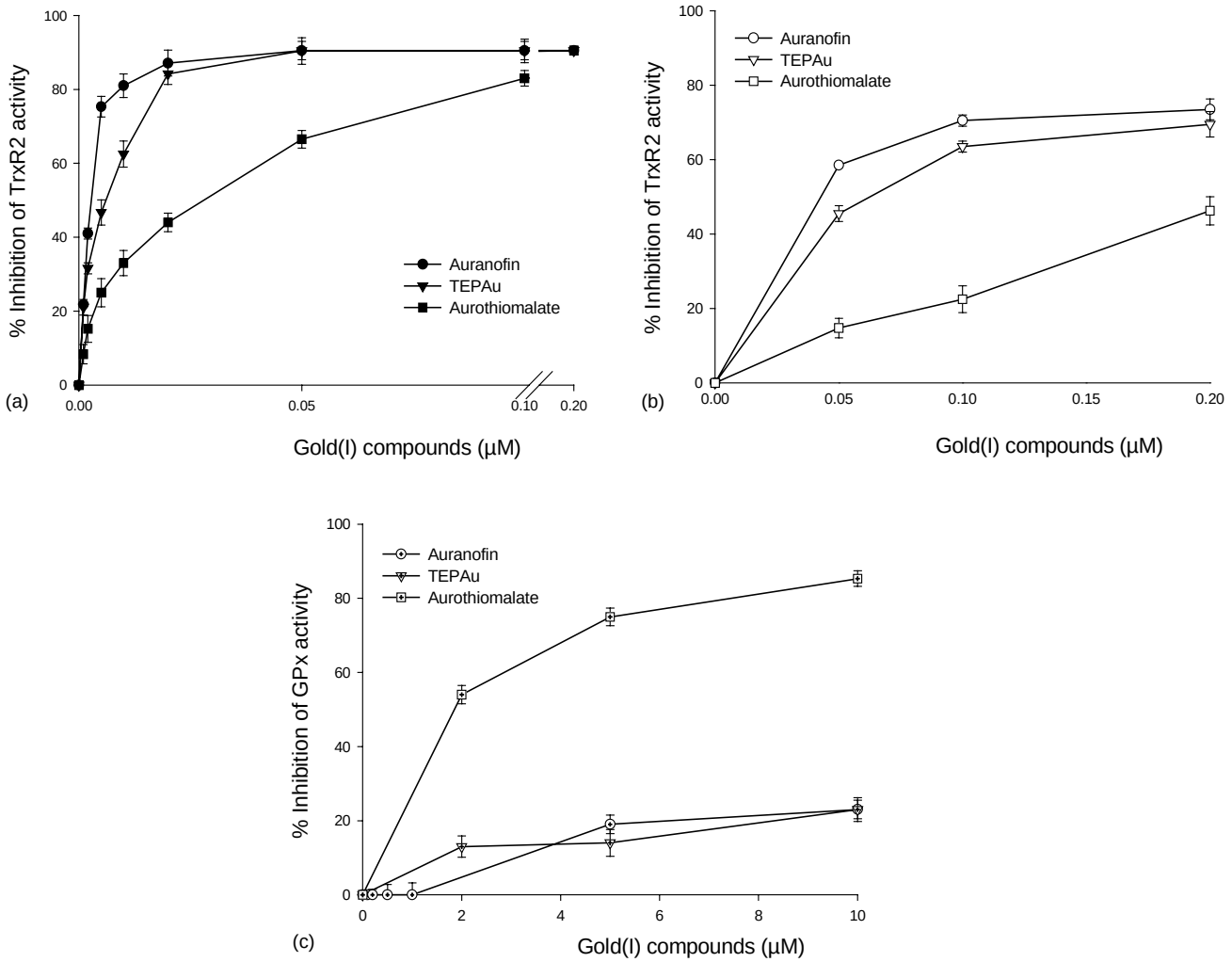

Fig. 3. Inhibitory effects of gold(I) compounds on mitochondrial thioredoxin reductase (TrxR2) and glutathione peroxidase (GPx). Aliquots of purified thioredoxin reductase (a) or mitochondrial matrix proteins (b) at the final concentrations of 0.25 and 0.8 μg mL^{-1}, respectively, were preincubated at 25° for 2 min in 200 mM Na^+K^+-phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.25 mM NADPH. Auranofin, TEPAu, and aurothiomalate were then added at the indicated concentrations and, after 10 min of incubation, the reaction was started by the addition of 3 mM DTNB. The increase of absorbance was followed at 412 nm for about 15 min. Glutathione peroxidase activity (c) was estimated in the mitochondrial matrix (0.3 mg mL^{-1}, final concentration) in 100 mM Tris–HCl buffer (pH 7.0) containing 3 mM EDTA, 0.3 mM NADPH, 0.25 mM GSH, and 0.92 μg mL^{-1} of glutathione reductase at 33°. Reactions were initiated by 0.2 mM *tert*-butylhydroperoxide and the decrease of absorbance was followed at 340 nm for about 10 min. Results are reported as percentage of inhibition with respect to the control and plotted vs. the respective concentrations of gold(I) compounds. Specific activities of the noninhibited reactions were 0.86 μmol min^{-1} mg^{-1} protein for the isolated thioredoxin reductase, 4.3 nmol min^{-1} mg^{-1} protein for the matrix thioredoxin reductase and 75.5 nmol min^{-1} mg^{-1} protein for the matrix glutathione peroxidase.

uncoupled respiration of whole mitochondria shows an IC_{50} of about 5 μM.

The gold(I) compounds used are particularly reactive towards thioredoxin reductase and their effect occurs at the level of the selenol group present at the active site in the C-terminal moiety [1,11]. Both the cytosolic [11] and mitochondrial (Fig. 3) enzymes are inhibited at nanomolar levels of gold(I) compounds. This inhibition is also indirectly confirmed by the high level of reduced pyridine nucleotides observed in whole mitochondria treated with gold(I) compounds (Fig. 2). A correlation between the inhibition of thioredoxin reductase and the alteration of mitochondrial membranes is apparent. In fact, the addition of very low concentrations of gold(I) derivatives to calcium-treated mitochondria in the presence of succinate determines a marked increase of oxygen uptake indicating the occurrence of an ‘‘uncoupling’’ linked to the MPT (Fig. 1) as shown by the concomitant occurrence of swelling (Fig. 1, inset). The effect of the combination of auranofin and calcium ions on oxygen uptake is reminiscent of the effect of paraquat [25], although the latter acts at far higher concentrations as compared to auranofin. Interestingly, in *Escherichia coli*, a NAD(P)H-paraquat diaphorase has been identified as thioredoxin reductase [26]. This reinforces our previous assumption [4] that inhibition or diversion of electrons from thioredoxin reductase influences the permeability status of mitochondrial membranes. Several reagents are in fact both inducers of

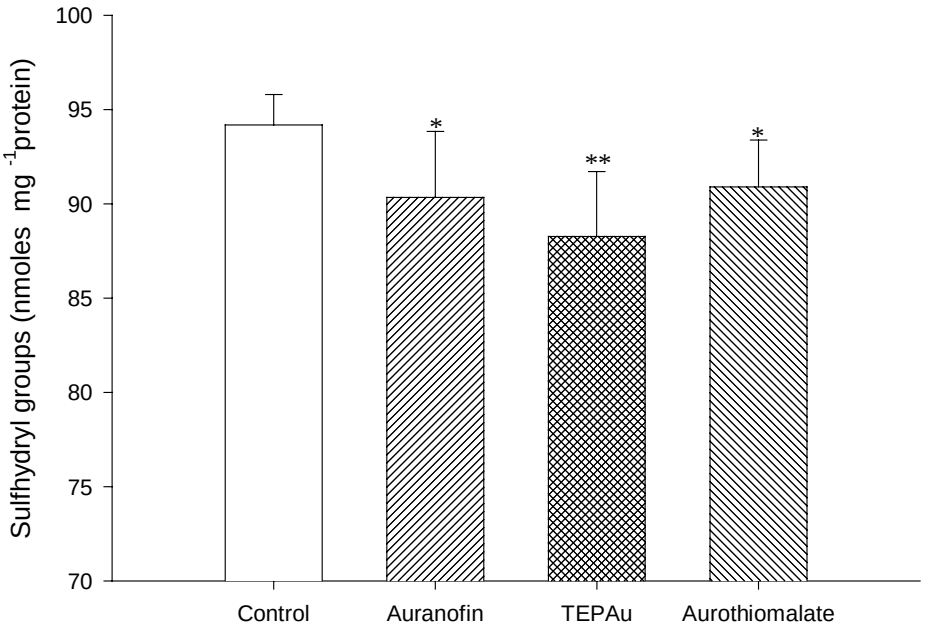

Fig. 4. Effect of gold(I) compounds on total mitochondrial thiols. Mitochondria (final concentration 5 mg protein mL^{-1}) were preincubated at 25° for about 2 min in 106 mM mannitol, 36 mM sucrose, 63 mM KCl, 15 mM Hepes-Tris (pH 7.4), 5 μM rotenone. To 1 mL aliquots of the above indicated suspension, were also added, when indicated, 2 nmol mg^{-1} protein of gold(I) derivatives and the reaction carried out for 30 min, under stirring. Total SH groups were estimated by adding aliquots of 100 μL (0.5 mg mL^{-1} protein) of the mitochondrial suspension to 200 mM Tris–HCl (pH 8.1) containing 10 mM EDTA and 1% SDS (final volume 2.5 mL). Readings were taken at 412 nm before and after the addition of 1.2 mM DTNB. After subtracting the readings measured before the addition of DTNB, total mitochondrial thiol groups were calculated as nanomoles of thiol per milligram of protein. $^{*}P < 0.05$, against control; $^{**}P < 0.01$, against control.

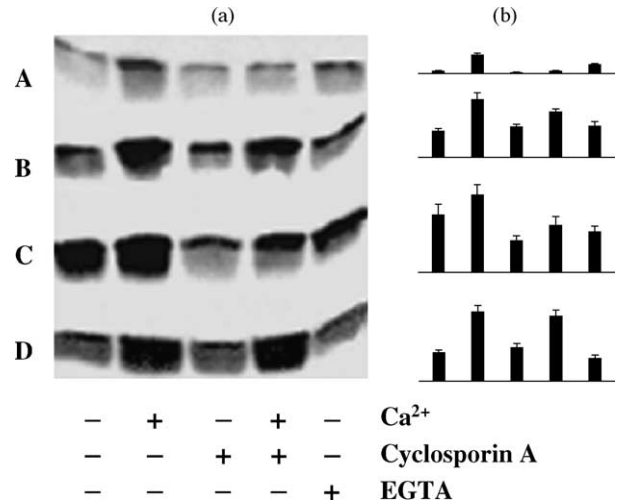

Fig. 5. Release of cytochrome *c* in the presence of gold(I) compounds. Rat liver mitochondria (5 mg protein mL^{-1}) were incubated in 106 mM mannitol, 36 mM sucrose, 63 mM KCl, 15 mM Hepes-Tris (pH 7.4), 5 μM rotenone, 5 mM succinate and in the presence of 10 μM gold(I) compounds: A: none; B: auranofin; C: TEPAu; D: aurothiomalate. When indicated, were also present 50 μM Ca^{2+}, 50 μM cyclosporin A or 40 mM EGTA. For the cytochrome *c* estimation, mitochondria were centrifuged at 11,000 *g* for 2 min and the pellet was discarded. Supernatant was centrifuged again at 15,800 *g* for 10 min and 0.4 mM phenylmethyl sulfonyl fluoride (PMSF) plus 0.4 mM EGTA were added. Aliquots were subjected to Western blotting (a) as indicated under Section 2. Panel b shows the densitometric analysis of the Western blotting performed using Kodak 1D Image Analysis. The columns are arranged in the same order of the spots and their heights are proportional to the areas of the spots.

MPT [6] and inhibitors or substrates of thioredoxin reductase [27]. As apparent by fluorescence measurements (Fig. 2), the redox state of NAD(P)H is not modified by gold(I) derivatives indicating that MPT occurs independently of the oxidation conditions of pyridine nucleotides. This is at variance with many other "pore" inducers (e.g. acetoacetate, *tert*-butylhydroperoxide, diamide) where MPT is concomitant with pyridine nucleotides oxidation [6]. It should be noted that, when mitochondrial glutathione reductase is inhibited by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), oxidation of glutathione with *tert*-butylhydroperoxide was not associated with oxidation of NADPH nor with the efflux of accumulated calcium [28] indicating that an intermediate different than glutathione can be involved in connecting pyridine nucleotides redox state to protein thiol-disulfide transition. The reported results also rationalize the lack of effect of gold(I) derivatives on the glutathione redox state (not shown).

Total thiol groups are slightly decreased (Fig. 4) indicating the occurrence of an oxidative trend involving only very specific thiols and caused by the inhibition of thioredoxin reductase. Probably a more sensitive approach is needed to identify the thiols involved. It is interesting to note that the concentrations of gold(I) compounds used in this study are low if compared to the total mitochondrial thiol groups estimated to be around 90–95 nmol SH groups mg^{-1} protein [14]. In addition, it should also be considered that, in the presence of gold(I) phosphine derivatives, the

release of the triethylphosphine moiety acting as a reducing agent towards disulfides [1] should bring to an overestimation of the total thiol measured. The effect of auranofin is comparable to that of acetoacetate since both compounds act by decreasing total thiol groups, although to a limited extent, without modifying glutathione. Moreover, the action mechanism is different since acetoacetate determines a large oxidation of pyridine nucleotides, at variance with auranofin that acts downstream by inhibiting thioredoxin reductase. In both cases, the flux of electrons towards thioredoxin is interrupted and the latter cannot undergo to its redox cycling any longer.

It has been previously demonstrated that MPT can be modulated by the redox state of pyridines nucleotides and thiols, including glutathione [21,29] therefore indicating that two systems connecting the pyridine nucleotides redox state to thiol redox state are present in mitochondria. The first (''glutathione system'') depends on glutathione and the enzymes glutathione reductase and glutathione peroxidase, while the second (''thioredoxin system'') depends on thioredoxin reductase and thioredoxin peroxidase. According to our results, the thioredoxin system appears particularly involved in the thiol-mediated redox regulation since auranofin and the other gold(I) derivatives do not alter glutathione redox state nor inhibit glutathione reductase and glutathione peroxidase while thioredoxin reductase is highly and specifically inhibited. Consequently, the cyclic reduction of thioredoxin, the natural substrate of thioredoxin reductase is forbidden and hence shifted to a more oxidized conditions.

Exploring the targets of auranofin and similar compounds, it is emerging from our results that, at the concentrations used, these compounds appears to be specifically active on thioredoxin reductase, while total thiols are only moderately interested. On the other hand, we cannot exclude that other targets of gold(I) compounds might be involved such as some critical thiol or selenol groups present in proteins different from thioredoxin reductase. Gold(I) complexes might interact not only with the matrix thioredoxin reductase but also with an intermembrane isoform of thioredoxin reductase that, in turn, might act on the mitochondrial isoform of protein disulfide isomerase, an enzyme belonging to the thioredoxin family and mostly resident in the mitochondrial outer membrane [30].

Mitochondria play a fundamental role in the cell apoptosis by releasing several different apoptogenic proteins present in the intermembrane space, such as AIF [31], Smac/DIABLO [32,33] and cytochrome *c* [34]. The release of the latter can be elicited by MPT, is usually Ca^{2+}-dependent [35] and is due to the large amplitude swelling of mitochondria caused by the increased permeability and enlargement of the inner membrane and the consequent rupture of the outer membrane. However, cytochrome *c* can be released from mitochondria also in the absence of MPT, in a process independent of Ca^{2+} ions [35] and essentially linked to an increase of permeability of the outer membrane due to the presence of factors such as the oligomeric form of Bax that acts as a channel protein [36]. Therefore, the induction of MPT is not strictly required for the release of cytochrome *c* [37–39]. We have previously observed that 13-*cis*-retinoic acid, although able to induce MPT, can release cytochrome *c* also in the absence of the latter process [23]. Similarly, Gogvadze *et al.* [24] showed that tributyltin releases cytochrome *c* in conditions where MPT does not occur. From the reported data, it is apparent that auranofin in conditions of absence of MPT gives rise to a consistent release of cytochrome *c* therefore suggesting a specific molecular basis that involves mitochondria and rationalizes the cytotoxicity and antitumor properties of gold(I)–phosphine complexes [3]. Gold(I) compounds might also help to detach cytochrome *c* from its binding sites in the inner membrane similarly to the action of other agents such as relatively high concentrations of KCl or lipid peroxidation [40].

Thioredoxin was shown to protect the cell against stress-induced apoptosis by suppressing cytochrome *c* release and inhibiting procaspase-9 [41]. In particular, cells overexpressing mitochondrial thioredoxin are resistant to etoposide-induced cytotoxicity [42] and oxidant-induced apoptosis [43]. In addition, these cells are able to increase their mitochondrial membrane potential [42]. On the contrary, mitochondrial thioredoxin deficient cells undergo apoptosis [44]. Moreover, overexpression of mitochondrial thioredoxin reductase is associated with the development hepatocellular carcinomas indicating that alteration of cellular redox status is a critical factor involved in the cell growth [45]. In fact, thioredoxin reductase itself can have direct effects, independent of thioredoxin, on protein dithiol–disulfide balance and peroxide removal. All these results point to a central role for the thioredoxin system in maintaining the efficiency of mitochondrial functions by either removing free radical species or by keeping proteins reduced [44]. Since, thioredoxin reductase plays an essential role in maintaining thoredoxin reduced, its highly specific inhibition exerted by gold(I) compounds, after determining a shift towards a more oxidized condition downstream thioredoxin reductase inhibition, elicits either the permeability transition and/or the release of cytochrome *c*.

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