1. Introduction

Cisplatin combination chemotherapy is the cornerstone of treatment of many cancers. Cisplatin and the follow-on drugs carboplatin and oxaliplatin are used to treat 40–80% of cancer patients [1]. The high effectiveness of cisplatin in the treatment of several types of tumors is severely hindered by some clinical problems related to its use in curative therapy, such as normal tissue toxicity and the frequent occurrence of initial and acquired resistance to the treatment [1–4]. The subsequent successful development of antitumor platinum drugs has paved the way for studying other metal-based chemotherapeutic compounds [3]. During the last few years there has been a renewed interest in the application of gold(I) and gold(III) compounds in cancer chemotherapy [5,6]. Attention was directed towards gold compounds for two reasons: (1) gold(III) centers are isoelectronic to Pt(II) compounds and adopt square-planar configurations similar to that of cisplatin, and (2) gold(I) compounds are well known pharmaceuticals, some of which are currently being used to treat rheumatoid arthritis. Gold compounds (Fig. 1) have displayed strong tumor cell growth inhibition effects by a non-cisplatin-like mode of action. Antiarthritic linear gold(I)-phosphine auranofoin and related derivatives A [7–13] and gold(I)-N-heterocyclic carbens B [14] have been reported to induce apoptosis via selective inhibition of the mitochondrial isoform of thioredoxin reductase via induction of ROS formation. Antitumor effects of tetrahedral gold(I) complexes ([Au(dppe)2]4+ and related compounds c are ascribed to their delocalized lipophilic cation properties [14–16].

Gold(III) complexes currently hold great potential to enter clinical trials since a few of them are highly cytotoxic to solid cancer tumors in vitro and in vivo while causing minimal systemic toxicity [17–21]. Most gold(III) compounds display reduced affinity for DNA and it seems reasonable that DNA is neither the primary nor the exclusive target for most gold(III) complexes. Recent studies have proposed different modes of action for these compounds. Gold(III)-porphyrin complexes D, known gold-based DNA intercalators, induce intracellular oxidation and apoptosis through both caspase-dependent and -independent mitochondrial pathways [21–23]. Gold(III) complexes with dithiocarbamate ligands E inhibit thioredoxin reductase activity, generate free radicals, increase ERK1/2 phosphorylation, affecting mitochondrial functions [18,24]. These compounds have also been described to cause a strong inhibition of the proteasome system, via both redox-dependent and -independent processes [25]. Gold(III) derivatives with polydentate N,N ligands F, G, H are also potent inhibitors of thioredoxin reductase [8,26]. Recently, histone deacetylases [27], mTOR, and cyclic-dependent kinases have been proposed as possible biochemical targets for some of the gold(III) complexes [27]. Moreover, a recent proteomic study of dinuclear oxo gold(III)
compound H showed that its mode of action is strictly related to that of auranofin (gold(I)) that they induced changes in protein expression that are limited and selective, that both compounds trigger caspase 3 activation and apoptosis, and that a few affected proteins are primarily involved in cell redox homeostasis [28]. While some gold(III) compounds are reduced easily to gold(I) derivatives in vitro (and have therefore a similar mode of action), for gold(III) complexes with dithiocarbamate E or porphyrin ligands D the “activation by reduction” mechanism has been discarded [28].

One of us have recently reported on the synthesis of apoptotic organogold(III) complexes containing iminophosphorane ligands whose stability in solution and oxidation state can be easily followed by $^{31}$P NMR spectroscopy (selected compounds for the present study in Fig. 2) [29]. The choice of secondary ligands like dithiocarbamate is related to their known chemoprotective effects. Other secondary ligands like water soluble phosphines (in 3) were used to increase the solubility of the cycloaurated compounds in water. Cationic compounds (like 2) containing dithiocarbamate ligands, are the most
cytotoxic in vitro against HeLa human cervical carcinoma and Jurkat-T acute lymphoblastic leukemia cells [29]. Compounds 1 and 3 are mainly apoptotic but in the case of the more cytotoxic compound 2, cell death is activated due to both apoptosis and necrosis. We confirmed by 31P NMR spectroscopy that especially 1 and 2 do not get reduced to Au(I) derivatives in solution. Also, a possible interaction of these compounds with DNA has been discarded. Compound 2 manifests a high reactivity toward cytochrome c and thioredoxin reductase (as confirmed by spectroscopic methods) [29].

We report here on the cytotoxic effect of 3 of these iminophosphorane–organogold(III) complexes with different ligands (Fig. 2) and we describe cell death pathways activated by these compounds, focusing on the role of Bcl-2 proteins, caspases and ROS. Our present results indicate that these compounds induce intracellular oxidative stress that subsequently provokes mitochondrial dysfunction. Mitochondrial alterations induced by compounds 1 and 2 depend partially on Bax/Bak activation. Caspases make a limited contribution to the toxicity of compounds 1–3. ROS production at mitochondria seems to be the key event for the toxicity of these compounds, as antioxidants can fully revert their killing activity and Jurkat rho0 cells are highly resistant to the toxicity of 1–3.

2. Experimental

2.1. Drugs and chemicals

\[ \text{[Au}([\text{r}^2-C,\text{N}-\text{C}_6\text{H}_4(\text{PPH}_2 = \text{N}([\text{C}_6\text{H}_5]-2)](\text{S}_2\text{CN-Me}_2)\text{PF}_6 (1), [29,30] \text{[Au}([\text{r}^2-C,\text{N}-\text{C}_6\text{H}_4(\text{PPH}_2 = \text{N}([\text{C}_6\text{H}_5]-2)](\text{Cp}(m-\text{C}_6\text{H}_4-SO_3\text{Na}_2))_3\text{Cl})\text{PF}_6 (3) [29] \text{were prepared as described previously. The general caspase inhibitor z-VAD-fmk was from Bachem (Geneve, Switzerland). Any other chemicals were purchased from Sigma-Aldrich (Madrid, Spain).}

2.2. Cell culture

The human T-cell leukemia Jurkat (clone E6.1) was from the ATCC collection. Jurkat cells lacking Bak were generated using RNA interference techniques, as described previously [31]. In all cases, cell lines containing the empty vectors were used as suitable controls. Jurkat-p0 cell line (Dr. Ignacio Aguiló, University of Zaragoza, Spain), lacking normal mitochondrial DNA, was generated by the long-term culture in the presence of low concentrations (50 ng/ml) of ethidium bromide. Cells were routinely cultured at 37 °C in RPMI 1640 medium supplemented with 5% or 10% (Jurkat p0) fetal calf serum (FCS), L-glutamine and penicillin/streptomycin (hereafter, complete medium). Jurkat-p0 cultures were also supplemented with glucose (4.5 mg/ml), sodium pyruvate (0.1 mg/ml) and uridine (50 pg/ml).

Blood samples from healthy donors were obtained from the ‘Banco de Sangre y Tejidos de Aragón’. Leukemic blood samples from patients with the clinical diagnosis of B-cell chronic lymphocytic leukemia (CLL) were obtained from the ‘Hospital Clínico Universitario Lozano Blesa’. All subjects gave written informed consent and the Ethical Committee of Aragón approved the study. PBMC were freshly isolated by Ficoll–Paque density centrifugation, as previously described [32]. After isolation, normal PBMC and leukemic CLL cells were kept in complete medium. CLL cells were cultured in the presence of 20 ng/ml of IL-4 (Preprotech).

2.3. Cytotoxicity studies

To evaluate the toxicity of compounds 1–3, Jurkat cells (3 × 10^5 cells/ml) or PBMC from healthy donors or CLL patients (3 × 10^6 cells/ml) were cultured in 96-well flat-bottom plates with different concentrations of each compound. The cytotoxic effect of 1–3 was assessed after 24 h by measuring simultaneously the mitochondrial membrane potential and the exposure of phosphatidilserine, as described below. For caspase inhibition assays the cells were preincubated with 100 μM Z-VAD-fmk for 1 h before the addition of compounds 1–3. DMSO was added to controls in the experiments with compounds 1 and 2.

2.4. Cell death analysis

Quantification of cell death was performed by the simultaneous analysis of phosphatidilserine (PS) exposure and mitochondrial membrane potential (ΔΨm) in a flow cytometer (FACScan, BD Bioscience, Spain). In brief, 2.5 × 10^5 cells in 200 μl were incubated in ABB (140 mM NaCl, 2.5 mM CaCl_2, 10 mM Hepes/NaOH, pH 7.4), with either 5 mM DiOC6(3) or 60 mM tetramethylrhodamine ethyl ester (TMRE) (both from Molecular Probes) at 37 °C for 10 min. AnnexinV-PE or AnnexinV-FITC (Invitrogen) at a concentration of 0.5 μg/ml was added to samples and incubated at 37 °C for further 15 min. In all cases, cells were diluted to 1 ml with ABB to be analyzed by flow cytometry.

To additionally assess cell viability after treatment with the compounds, 2.5 × 10^5 cells were harvested and incubated in 200 μl of PBS containing 50 ng/μl of 7-Amino-Acetoinomycin D (7-AAD, Immunoestop). When analyzed simultaneously to either PS exposure or ΔΨm, 7-AAD was added to samples in ABB.

2.5. Nuclear morphology

Morphology of nuclei after the treatment with the different compounds was analyzed by staining cell cultures with Hoechst 33342 (Molecular Probes) at 25 ng/ml. Cells were visualized in a fluorescence microscope (Nikon Eclipse 50i) and ACT Software was used for the acquisition of the images.

2.6. Intracellular ROS quantification

Oxidative stress was analyzed by intracellular staining with the fluorescent probe 2-hydroxyethidium (2-HE, Molecular Probes). After 16 h of culture in the presence of compounds 1–3, cells were incubated with 2 μM 2-HE at 37 °C for 15 min. Red fluorescence produced by reduction of 2-HE to ethidium was quantified in a flow cytometer.

3. Results

3.1. Leukemia cells are more sensitive to iminophosphorane–organogold (III) compounds 1–3 than normal PBMC

The sensitivity to compounds 1–3 of T-cell leukemia Jurkat cells was compared to that of normal lymphocytes (PBMC). As shown in Fig. 3, Jurkat and CLL cells were more sensitive to the cytotoxic effect of compounds 1–3 than PBMC. The IC50 of compound 1 was 2.57 μM for Jurkat cells and 6.0 μM for PBMC (Fig. 3A). Compound 2 at 0.5 μM induced cell death in around 100% of Jurkat cells but only in 17% of PBMC (Fig. 3B). Finally, compound 3 induced killing of 96% of Jurkat cells at a concentration of 25 μM (IC50 = 16.1 μM) whereas only 33.5% of PBMC were dead at the same concentration (Fig. 3C). The IC50 of the compounds for CLL cells ranged from 0.48 to 1.2 μM for 1, 0.06 μM to 0.1 μM for 2 and from 2.7 μM to 14.2 μM for compound 3.

3.2. Iminophosphorane–organogold (III) compounds 1–3 induce mitochondrial depolarization and apoptosis or necrosis

We have analyzed cell and nuclear morphology in Jurkat cells after 16 h in the presence of compounds 1–3. Nuclear staining showed that only a fraction of cells exhibited chromatin condensation and fragmentation, typical features of apoptosis (Fig. 4). However, a high percentage of cells, around 50%, had lost...
mitochondrial transmembrane potential ($\Delta\Psi_m$), in the same cultures. Since collapse of mitochondrial transmembrane potential is a feature of cell death in most models [33], we concluded that a significant fraction of cells were dead without exhibiting apoptotic morphology. This discrepancy was more evident in the case of compound 2. These results suggest that compounds 1–3 induced mitochondrial depolarization and later cell death that can proceed both through apoptosis and necrosis.

### 3.3. Implication of Bax/Bak in the toxicity of iminophosphorane–organogold (III) compounds 1–3

The toxicity of compounds 1–3 was analyzed in Jurkat-shBak cells, lacking both Bax and Bak. Control Jurkat cells were highly sensitive to these compounds and around 50% (1 and 3) or 80% (compound 2) of cells bound annexin V-PE after 24 h, with doses of 10 $\mu$M for compounds 1 and 3 and 0.5 $\mu$M for compound 2 (Fig. 5A and B). Analysis of $\Delta\Psi_m$ loss indicated that the three compounds induced mitochondrial depolarization in a similar percentage of cells (Fig. 5B). Cisplatin at 25 $\mu$M caused death of around 60% of Jurkat control (CN) cells. Jurkat-shBak cells were completely resistant to cisplatin but still displayed moderate sensitivity (40–50%) to compound 2. Also, compound 1 exerted some toxicity on Jurkat-shBak cells, with around 20% annexinV-PE+ and $\Delta\Psi_m$low cells (Fig. 5A and B). Cell death produced by compound 3 seemed to be dependent on the presence of Bax and Bak, since Jurkat-shBak cells were almost completely resistant to the toxic effect of this compound (Fig. 5A and B). These results indicate that, whereas compound 3-induced cell death is totally dependent on the presence of multidomain proteins of the intrinsic/mitochondrial route, some Au(III)-iminophosphorane compounds, such as 1 and 2, activate both Bax/Bak-dependent and -independent cell death mechanisms.

We further analyzed the mechanism of cell death induced by Au(III)-iminophosphorane compounds in Bax/Bak-deficient Jurkat cells. As shown in Fig. 5C, compounds 2 and 3 induced necrosis and apoptosis at similar levels in control Jurkat cells. Around a third of death cells analyzed after treatment with compound 1 were necrotic (Fig. 5C). In contrast, cell death induced by compounds 1–3 in Jurkat-shBak cells was mainly necrotic (Fig. 5C). In the case of compound 3, both apoptotic and necrotic cell death were prevented in Jurkat-shBak cells. These results suggest that necrosis observed in Jurkat control cells after treatment with compound 3 could be a secondary event to apoptosis. However, necrosis induced by compounds 1 and 2 could be induced by a primary mechanism independent of Bax/Bak.

### 3.4. Caspase inhibition do not prevent cell death induced by iminophosphorane–organogold (III) compounds

The general caspase inhibitor z-VAD-fmk did not significantly reduce the loss of mitochondrial transmembrane potential and plasmatic membrane integrity (7-AAD+ cells) induced by compounds 1–3 (Fig. 6A). A limited protection was observed for compound 3, probably indicating the implication of caspases in an amplification loop that accelerates the execution of apoptosis [31]. In the same way, cell death induced by compounds 1–3 in Jurkat-shBak cells was mainly caspase-independent, as z-VAD-fmk did not significantly mainly

![Fig. 3](image_url) Leukemia cells are more sensitive to 1–3 than normal PBMC Jurkat cells (3 $\times$ 10^5 cells/ml) and PBMC from normal donors or CLL patients (2 $\times$ 10^5 cells/ml) were cultured for 24 h in the presence of the indicated doses of each compound (1–3). Cell death was evaluated by annexinV-PE labeling and quantification of fluorescent cells by flow cytometry. Results are mean±SD of three independent experiments.

![Fig. 4](image_url) Compounds 1–3 induce apoptosis and necrosis Jurkat cells were cultured for 24 h in the presence of compounds 1–3 or left untreated. Nuclei were stained with Hoechst 33342 (10 $\mu$g/ml) and cells were photographed under UV light (left panels). Bright-field pictures are shown (right panels). Loss of transmembrane potential was assessed in a portion of each sample by flow cytometry after staining with DiOC6(3) as indicated in the Experimental section. Figures in left panels indicate the percentage of $\Delta\Psi_m$low cells.
caspase-independent, as z-VAD-fmk did not significantly reduce the percentage of annexin V-PE+ or 7-AAD+ cells (Fig. 6B). These results indicate that these iminophosphorane–organogold(III) compounds activate caspase-independent pathways that lead to cell death.

3.5. Iminophosphorane–organogold (III) compounds induce cell death through ROS production

Gold compounds have been described to induce intracellular oxidative stress [22]. ROS production in Jurkat cells treated with compounds 1–3 was analyzed by flow cytometry after staining with 2-hydroxyethidium. As shown in Fig. 7, the three compounds induced ROS production in Jurkat cells (Fig. 7A). To evaluate the real contribution of ROS to the toxicity of these compounds, we treated Jurkat cells with 1–3 in the presence of the anion superoxide scavenger MnTBAP, the antioxidant glutathione (GSH) or its precursor N-acetylcysteine (NAC). MnTBAP almost completely protected Jurkat cells from the toxicity of compound 3 and it also offered a partial protection against compound 1-induced cell death (Fig. 7B). The general antioxidants glutathione and NAC effectively inhibited cell death induced by 1–3 (Fig. 7B). Taken together, these results indicate a role of ROS in the killing activity of the Au(III) compounds 1–3, and particularly the implication of superoxide anion in the toxicity of 3 and 1.

Mitochondria are in most cases the main source of intracellular ROS, due to electron flow in the respiratory chain. Rho0 cells, lacking mitochondrial DNA, show a very limited capacity to generate ROS at the mitochondrial level [34]. When we tested the sensitivity of Jurkat-rho0 cells to compounds 1–3, we observed that these cells were completely resistant to them (Fig. 7C), even at a concentration twice the IC50 for Jurkat parental cells (Fig. 3).

In order to determine whether ROS were critical in the commitment of cells to death, we studied the growing potential of Jurkat cells transferred to fresh medium after 24 h treatment with 1–3 in the presence of NAC. Total viable cells in cultures were analyzed 24 h and 48 h after removal of compounds 1–3 and NAC (Fig. 7D). Cells that had been treated with 1–3 in the presence of NAC fully recovered their growing potential, as compared with control cells, doubling their number from 24 to 48 h. Cells cultured only with 1 and 2 did not multiply. A fraction of cells treated with 3 alone were able to divide after being transferred to fresh medium.

4. Discussion

In the last years several gold compounds are being investigated as potential antitumor agents [5,6]. We have studied the mechanisms of the
The antitumor effect of three organogold(III) compounds (1–3, Fig. 2) that we have previously reported to induce cell death due to both apoptosis and necrosis [29]. Necrosis has often been regarded as an unregulated process but new data indicate that a programmed necrotic death can be activated in some models [35]. Our more recent studies presented here, have first shown that these complexes are more sensitive to Jurkat-T acute lymphoblastic leukemia and B-CLL leukemia cells than to normal lymphocytes (PBMC), consistent to what some of us have found for those (1–3) and related iminophosphorane organogold(III) complexes in oral epithelial cells [36]. Mitochondrial depolarization preceded cell apoptotic or necrotic cell death induced by compounds 1–3, suggesting that mitochondria could be the cellular target of these compounds. Similar results have been found for compound 3 in oral human cancer cells MDA686LN [36]. In this sense, it is accepted that most gold compounds do not interact with DNA and their mechanism of action could involve either direct mitochondrial damage or proteasome inhibition or modulation of specific kinases [6]. For instance, gold(I) N-heterocyclic carbenes accumulate selectively in the mitochondria of tumorigenic cells and cause loss of ΔΨm, depletion of the ATP pool and apoptosis [15,16]. Mitochondrial permeabilization during cell death is regulated by proteins of the Bcl-2 family. Antiapoptotic members of this family, such as Bcl-2, Bcl-XL or Mcl-1, block mitochondrial permeabilization and the release of apoptogenic proteins (cytochrome c, AIF, Smac/Diablo and others). Proapoptotic proteins of the Bcl-2 family classify in two groups, the “BH3-only” and the “multidomain” subfamilies. Multidomain proapoptotic proteins Bax and Bak are essential for the onset of mitochondrial permeabilization and apoptosis through the intrinsic pathway [37]. Gold (III) dithiocarbamate complexes (like E, Fig. 1) are known to down-regulate the antiapoptotic molecule Bcl-2, upregulate the proapoptotic molecule Bax and induce apoptosis on prostate cells and xenografts [20] and on human acute myeloid leukemia cells [38]. The implication of Bax/Bak in cell death induced by 1–3 was studied using Bax- and Bak-deficient Jurkat cells. Differences were found in the behavior of the compounds regarding the implication of Bax/Bak. The neutral compound 1, containing two chloride ligands [Au(μ2-CN-C6H4(PPh2=N(C6H5)-2)Cl2], and the cationic compound 2, with a dithiocarbamate ligand [Au(μ2-CN-C6H4(PPh2=N(C6H5)-2)(S2CN-Me2)]PF6, activate both Bax/Bak-dependent and -independent cell death mechanisms. However, the behavior
of 3, a cationic compound with a water-soluble phosphine and a chloride ligand \([\text{Au}(\text{m-C}_6\text{H}_4\text{SO_3Na})_2]\) [28]. In our case the behavior of iminophosphorane only dithiocarbamate ligands of the type \((\text{III})\) compounds [29] and it also offered a partial protection against compound completely protected Jurkat cells from the toxicity of compound [29]. When we tested the sensitivity of Jurkat-rho0 cells to compounds 1–3, we observed that these cells were completely resistant to them, even at a concentration twice the IC50 for Jurkat parental cells. These results point to mitochondria as the main target of compounds 1–3, causing ROS generation (all three compounds) and/or Bax/Bak activation (compound 3 and partially for compound 1).

In summary, we have demonstrated here that iminophosphorane–organogold(III) compounds are highly cytotoxic to Jurkat T-cell acute lymphoblastic leukemia cells and B-CLL cells, showing a higher toxicity against these cells than to normal T-lymphocytes. We have found significant differences in the mechanisms activated by 1–2 and 3. Superoxide anion and Bax/Bak seem to be highly implicated in the toxicity of 3, inducing predominantly apoptotic cell death. However, the role of superoxide and Bax/Bak is only partial for 1 and very limited for 2, which induce necrotic cell death. As previously pointed out, we believe that the different behavior displayed by 1 and 2 versus 3 comes from the fact that 1 and 2 are not reduced to gold(I) derivatives in vitro while compound 3 may be reduced to \([\text{Au}]\text{(PR}_3\) species. We are currently working on the design of new iminophosphorane ligands with different hydrophobic/lipophilic ratios and their coordination to d\(_6\) metal centers for the preparation of novel more potent cytotoxic compounds with a lower systemic toxicity in humans.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>annexin buffer</td>
<td>annexin binding buffer</td>
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<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
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<tr>
<td>auranozin</td>
<td>2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyrasonato-S-triethylphoshine gold(I)</td>
</tr>
<tr>
<td>bipy</td>
<td>6–(1,1-dimethylbenzyl)-2,2′-bipyridine</td>
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<tr>
<td>bipy-Me</td>
<td>6,6′-dimethyl-2,2′-bipyridine</td>
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<tr>
<td>BCL</td>
<td>B-cell chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>dpp</td>
<td>1,2-bis(diphenylphosphino)ethane</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ESATD</td>
<td>ethylsarcosinedithiocarbamate</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>Hepes</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>L-NAC</td>
<td>N-acetyl-L-cysteine</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MnTBAP</td>
<td>Mn(III)tetakis (4-benzoic acid) porphyrin</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
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<td>PS</td>
<td>phosphatidylserine</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>TrxR</td>
<td>thioredoxin reductase</td>
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