Biometals (2012) 25:587–598
DOI 10.1007/s10534-012-9547-5

Spectroscopic and electrochemical characterization of gold(I) and gold(III) complexes with glyoxaldehyde *bis*(thiosemicarbazones): cytotoxicity against human tumor cell lines and inhibition of thioredoxin reductase activity

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Received: 10 January 2012 / Accepted: 3 April 2012 / Published online: 17 April 2012

Abstract Complexes $[Au_2(H_2Gy3DH)_2]Cl_2$ (**1**), $[Au(H_2Gy3Me)]Cl_3$ (**2**) and $[Au(H_2Gy3Et)]Cl_3$ (**3**) were obtained with glyoxaldehyde *bis*(thiosemicarbazone) (H_2Gy3DH) and its *N*(3)-methyl (H_2Gy3Me) and *N*(3)-ethyl (H_2Gy3Et) derivatives. The *bis*(thiosemicarbazones) and their gold(I) and gold(III) complexes exhibited anti-proliferative activity against HL-60, Jurkat (leukemia) and MCF-7 (breast cancer) cells at 10 μmol L^{-1}. Complex (**2**) was able to in vitro inhibit thioredoxin reductase (TrxR) activity, which suggests that inhibition of TrxR could be part of its mechanism of action.

Keywords *Bis*(thiosemicarbazones) · Gold complexes · Thioredoxin reductase · Cytotoxicity

Introduction

The favorable pharmacological profile of cisplatin stimulated the search for other metal complexes with different spectra of bioactivities and lower systemic toxicity. Since d^8 gold(III) complexes are isoelectronic and isostructural with platinum(II) complexes, square planar gold(III) compounds soon appeared to be interesting candidates for anticancer evaluation (Gabbiani et al. 2007). However, gold(III) analogs of cisplatin were found to be less stable than the corresponding platinum(II) compounds. During the 1990s, renewed interest for anticancer gold(III) compounds emerged, especially when novel gold(III) complexes, exhibiting improved stability, lower toxicity and favorable in vitro pharmacological properties, were made available for pharmacological testing (Casini et al. 2008; Tiekink 2002).

In addition, the demonstrated in vitro inhibitory effects of the gold(I) antiarthritic drug triethylphosphinegold(I) tetraacetatothioglucose (auranofin) led to systematic evaluation of this and related gold(I) compounds (Tiekink 2008).

Gold complexes are recognized as extremely potent inhibitors of both cytosolic and mitochondrial thioredoxin reductase (TrxR) (Bindoli et al. 2009). The effects of gold(I) and gold(III) complexes on mitochondrial thioredoxin reductase have been examined. The literature reports that both gold(I) and gold(III) complexes can be extremely efficient inhibitors of TrxR (Rigobello et al. 2004).

TrxR is a large homodimeric selenoenzyme which controls the redox state of thioredoxin in the thioredoxin system. Cancer cells often overexpress both thioredoxin and thioredoxin reductase indicating that

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the thioredoxin system may have a crucial role in tumor onset and progression (Bindoli et al. 2009). Hence both thioredoxin and thioredoxin reductase might be considered as suitable targets for the development of new anticancer drug candidates.

Thiosemicarbazones and *bis*(thiosemicarbazones) are well known for their cytotoxic activity against tumor cells both in vitro and in vivo (Beraldo and Gambino 2004; Castiñeiras et al. 2009; Lessa et al. 2010). In many cases coordination to metal ions leads to improvement of the pharmacological properties of this class of compounds (Lessa et al. 2011; Reis et al. 2010; Ferraz et al. 2011). In a previous work we demonstrated that gold(I) complexes with thiosemicarbazones were effective cytotoxic agents against human tumor cell lines and that inhibition of TrxR could be involved in their mechanism of action (Lessa et al. 2011).

Since *bis*(thiosemicarbazones) are tetradentate S–N–N–S ligands, their gold complexes might present improved stability. In the present work we prepared gold(I) and gold(III) complexes with glyoxaldehyde *bis*(thiosemicarbazone) (H_2Gy3DH) and its *N*(3)-methyl (H_2Gy3Me) and *N*(3)-ethyl (H_2Gy3Et) derivatives (Fig. 1). The cytotoxic activity of the studied compounds was evaluated against human HL-60, Jurkat (leukemia), MCF-7 (breast cancer) and HCT-116 (colon cancer) cell lineages. The effect of one of the studied complexes on TrxR's activity was also investigated.

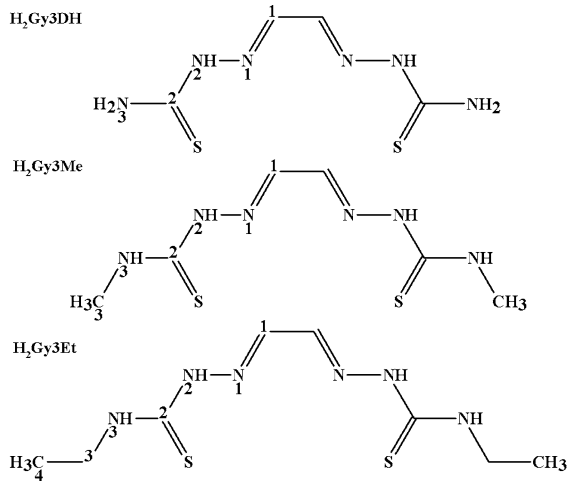


Fig. 1 Generic representation for glyoxaldehyde *bis*(thiosemicarbazone) (H_2Gy3DH) and its *N*(3)-methyl (H_2Gy3Me) and *N*(3)-ethyl (H_2Gy3Et) derivatives

Experimental

Materials and measurements

All common chemicals were purchased from Aldrich and used without further purification. Cisplatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1460, L-glutamine were purchased from Sigma, and antibiotic/antimicotic solution and fetal calf serum were purchased from Gibco (Grand Island, NY).

Partial elemental analyses were performed on a Perkin Elmer CHN 2400 analyzer. An YSI model 31 conductivity bridge was employed for molar conductivity measurements. Infrared spectra were recorded on a Perkin Elmer FT-IR Spectrum GX spectrometer using KBr plates (4,000–400 cm^{-1}) and Nujol mulls between CsI plates (400–170 cm^{-1}). NMR spectra were obtained with a Bruker DPX-200 Avance (200 MHz) spectrometer using deuterated dimethylsulphoxide (DMSO-d_6) as the solvent and TMS as internal reference. Electronic spectra were recorded on a HP8453 diode array—Hewlett Packard spectrophotometer using a 10 mm beam path quartz cuvette and dimethylformamide (DMF) or 0.5 % DMSO/phosphate buffer pH 7.4 as solvent. Electrochemical studies were performed on an EcoChemie μAutolab potentiostat in DMF solution containing tetrabutylammonium tetrafluoroborate ($TBABF_4$, 0.1 mol L^{-1}) supporting electrolyte under argon atmosphere at room temperature. The electrochemical cell employed a standard three electrode configuration: a glassy carbon working electrode, a platinum-wire auxiliary electrode and an Ag/AgCl electrode immersed in a salt bridge containing saturated LiCl in ethanol. Cyclic voltammograms were recorded in the 1,700 to −2,000 mV potential range at 100 mV s^{-1} scan rate. The ferrocene/ferrocenium (Fc/Fc^+) couple (0.400 V vs NHE) was used as an internal standard. Solutions of compounds (1.0×10^{-3} mol L^{-1}) were prepared with $TBABF_4$ 0.1 mol L^{-1} in DMF.

Synthesis of glyoxaldehyde *bis*(thiosemicarbazone) (H_2Gy3DH) and its *N*(3)-methyl (H_2Gy3Me) and *N*(3)-ethyl (H_2Gy3Et) derivatives

The *bis*(thiosemicarbazones) were prepared according to standard procedures (Beraldo et al. 1998).

Synthesis of the gold(I) and gold(III) complexes

The gold(I) and gold(III) complexes were obtained by mixing a methanol solution (20 mL) of the desired *bis*(thiosemicarbazone) (1 mmol) with hydrogen tetrachloroaurate(III) trihydrate ($HAuCl_4$) in 1:1 ligand-to-metal molar ratio under reflux with stirring for 2 h. The resulting solids were filtered off then washed with methanol followed by acetone and dried in vacuo.

Bis{Glyoxaldehyde bis(thiosemicarbazone)gold(I)} chloride $[Au_2(H_2Gy3DH)_2]Cl_2$ (**1**)

Light brown solid. Anal. Calc. ($C_8H_{16}Cl_2N_{12}S_4Au_2$): C, 11.00; H, 1.85; N, 19.24; Found: C, 11.30; H, 2.06; N, 19.42 %. FW: 873.42 g mol^{-1}. IR (KBr, cm^{-1}): *v*(NH) 3344 m, 3244 m, 3152 m, *v*(C=N) 1,606 s, *v*(C=S) 836 m. IR (CsI/Nujol, cm^{-1}): *v*(Au–S.) 232w. ^{1}H NMR [200 MHz, DMSO-d_6, δ (ppm), main signals]: 7.70 [s, 2H, H(1)]; 11.68 [s, 2H, N(2)H)]; 8.31 [s, 1H, N(3)H]; 7.88 [s, 1H, N(3)'H]. Yield 77 %.

Glyoxaldehyde bis[N(3)-methylthiosemicarbazone]gold(III) chloride $[Au(H_2Gy3Me)]Cl_3$ (**2**)

Beige solid. Anal. Calc. ($C_6H_{12}Cl_3N_6S_2Au$): C, 13.45; H, 2.26; N, 15.69; Found: C, 13.89; H, 1.93; N, 15.61 %. FW: 535.66 g mol^{-1}. IR (KBr, cm^{-1}): *v*(NH) 3219 m, 3124 m, *v*(C=N) 1574 s, *v*(C=S) 817 m. IR (CsI/Nujol, cm^{-1}): *v*(Au–S.) 252w, *v*(Au–N) 433w. ^{1}H NMR [200 MHz, DMSO-d_6, δ (ppm)]: 7.94 [s, 2H, H(1)]; 12.29 [s, 2H, N(2)H)]; 9.15 [d, $J = 4.20$ Hz, 2H, N(3)H]; 3.06 [d, $J = 4.46$ Hz, 6H, H(3)]. $\Lambda_M = 32.1\ \Omega^{-1}\ cm^2\ mol^{-1}$ in DMF. Yield 52 %.

Glyoxaldehyde bis{N(3)-ethylthiosemicarbazone}gold(III) chloride $[Au(H_2Gy3Et)]Cl_3$ (**3**)

Beige solid. Anal. Calc. ($C_8H_{16}Cl_3N_6S_2Au$): C, 17.05; H, 2.86; N, 14.91; Found: C, 16.85; H, 2.67; N, 14.63 %. FW: 563.71 g mol^{-1}. IR (KBr, cm^{-1}): *v*(NH) 3342 m, 3120 m, *v*(C=N) 1,565 s, *v*(C=S) 804 m. IR (CsI/Nujol, cm^{-1}): *v*(Au–S.) 241w, *v*(Au–N) 446w. ^{1}H NMR [200 MHz, DMSO-d_6, δ (ppm)]: 7.96 [s, 2H, H(1)]; 12.27 [s, 2H, N(2)H)]; 9.23 [t, $J = 4.96$ Hz, 2H, N(3)H]; 3.60 [t, $J = 6.47$ Hz, 4H, H(3)]; 1.17 [t, $J = 7.06$ Hz, 6H, H(4)]. ^{13}C NMR [200 MHz, DMSO-d_6, δ (ppm)]: 171.71 [C(2)=S)], 143.39 [C(1)=N], 39.44 [C(3)], 14.31 [C(4)]. $\Lambda_M = 26.1\ \Omega^{-1}\ cm^2\ mol^{-1}$ in DMF. Yield 72 %.

Cytotoxic activity against human tumor cell lines

Cell lines

Jurkat (human immortalized line of T lymphocyte), HL-60 (wild type human promyelocytic leukemia), MCF-7 (human breast adenocarcinoma) and HCT-116 (colorectal carcinoma) cell lines were kindly given by Dr. Gustavo Amarante-Mendes (São Paulo University, Brazil). All lineages were maintained in the logarithmic phase of growth in RPMI 1640 medium supplemented with penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) (GIBCO BRL, Grand Island, NY) enriched with 2 mmol L^{-1} of L-glutamine and 10 % of fetal bovine serum. All cultures were maintained at 37 °C in a humidified incubator with 5 % CO_2 and 95 % air. The media were changed twice weekly and they were regularly examined.

Evaluation of the cytotoxic effect against human tumor cell lines

Jurkat and HCT-116 cell lines were inoculated at 100,000 cells per well, while HL-60 and MCF-7 cell lines were inoculated at 50,000 and 40,000 cells per well, respectively. The plates were pre-incubated for 24 h at 37 °C to allow adaptation of cells prior to the addition of the test compounds. Freshly prepared solutions of the different substances were tested at 10 μmol L^{-1}. Subsequently, the plates were inoculated for 48 h in an atmosphere of 5 % CO_2 and 100 % relative humidity. Control groups included treatment with 0.1 % DMSO (negative control) and 10 μmol L^{-1} of cisplatin and auranofin (positive controls). Cell viability was estimated by measuring the rate of mitochondrial reduction of MTT. All substances were dissolved in DMSO, prior to dilution. All compounds were tested in triplicate, in three independent experiments.

In vitro cell viability assay: MTT assay

The MTT assay is a standard colorimetric assay, in which mitochondrial activity is measured by splitting tetrazolium salts with mitochondrial dehydrogenases

from only viable cells (Mosmann 1983). Briefly, after 4 h of the end of incubation of cells with different compounds, 20 μL of MTT solution (2.5 mg mL^{-1} in phosphate-buffered saline) were added to each well, the supernatant was removed and 200 μL of 0.04 mol L^{-1} HCl in isopropyl alcohol were added to dissolve the formazan crystals. The optical densities (OD) were evaluated in a spectrophotometer at 570 nm. Controls included drug-containing medium (background) and drug-free complete medium. Drug-free complete medium was used as control (blank) and was treated in the same way as the drug-containing media. Results were expressed as percentage of cell proliferation, comparing with 0.1 % DMSO control and were calculated as follows: viability (%) = (mean OD treated − mean OD background)/mean OD untreated cultured, i.e. 0.1% DMSO − mean OD blank wells) × 100. Interactions of compounds and media were estimated on the basis of the variations between drug-containing medium and drug-free medium to escape from false-positive or false-negative (Ulukaya et al. 2004).

Statistical analysis

All experiments were performed in at least three replicates per compound and results shown are the average of three independent experiments. Data are represented as mean ± SEM. Significance was tested by the Student's test *t*.

Inhibition of TrxR activity

Rat liver TrxR (Sigma) was used to determine TrxR inhibition by the compounds. The studies were carried out by means of the DTNB (dithiobisnitrobenzoic acid) reduction assay. This assay makes use of the fact that TrxR reduces the disulfide bonds of DTNB with formation of 5-thionitrobenzoic acid (TNB), which can be detected photometrically.

The assay was performed according to the manufacturer's instructions (Sigma product information sheet T9698) and Ott and co-workers (2009) with appropriate modifications. Initially, the TrxR rat liver solution was diluted with potassium phosphate buffer, pH 7.0. To 20 μL aliquots of this solution (each containing approximately 0.12 units of the enzyme) 20 μL of 5 % DMF in potassium phosphate buffer pH 7.0, containing the complex (**2**) (ten different concentrations in the 0.05–50 μmol L^{-1} range) or vehicle without compound (control) were added, and the resulting solutions were incubated for 1 h at 37 °C with moderate shaking. For H_2Gy3Me, $HAuCl_4$ and auranofin the experiments were performed at three concentrations (0.5, 5.0 and 10 μmol L^{-1}) in order to compare their inhibitory activities with that of complex (**2**). The solutions were transferred quantitatively to 96-well plates, and each 200 μL of reaction mixture (10 mL of reaction mixture consisted of 1.0 mL of 1.0 mol L^{-1} potassium phosphate buffer, pH 7.0; 0.20 mL of 500 mmol L^{-1} EDTA solution pH 7.5; 0.80 mL of 63 mmol L^{-1} DTNB in ethanol; 0.10 mL of 20 mg mL^{-1} bovine serum albumin; 0.05 mL of 48 mmol L^{-1} NADPH and 7.85 mL of water) were added. To correct for non-enzymatic product formation, 40 μL of 1.0 mol L^{-1} potassium phosphate buffer, pH 7.0, and 200 μL of reaction mixture were processed simultaneously (blank value). After proper mixing the formation of TNB was monitored in a microplate reader (Thermo Scientific Multiskan® Spectrum) at 412 nm in 2 s intervals for 4 min. The absorbance of the blank was subtracted from that of the control and treated wells. The enzymatic activities were calculated as the maximum absorbance produced in 4 min in each well. The experiments were performed in triplicate. The IC_{50} values were calculated as the concentration of **2** which decreased the enzymatic activity by 50 % in relation to the untreated control.

Results and discussion

Formation of the gold(I) and gold(III) complexes

Microanalyses are compatible with the formation of either $[Au(H_2Gy3DH)Cl]$ or $[Au_2(H_2Gy3DH)_2]Cl_2$ (**1**), $[Au(H_2Gy3Me)]Cl_3$ (**2**) and $[Au(H_2Gy3Et)]Cl_3$ (**3**) in which a neutral *bis*(thiosemicarbazone) is attached to the metal center (Fig. 2).

The molar conductivity of **1** was not determined due to its low solubility in commonly used solvents (Geary 1971). Complex (**1**) could be a monomer in which the *bis*(thiosemicarbazone) would probably be attached to the gold(I) center through the sulfur atom along with a chloride ion. It could as well be a dimer of gold(I), with two neutral *bis*(thiosemicarbazones) attached to two gold(I) centers and two chloride ions

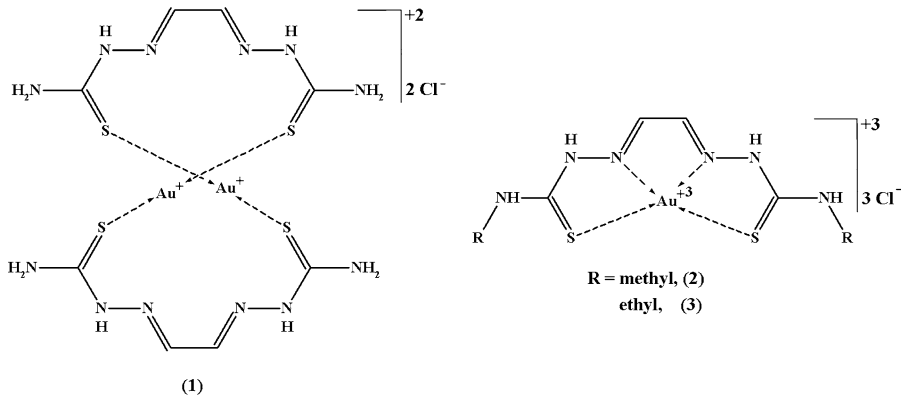


Fig. 2 Proposed structures for complexes $[Au_2(H_2Gy3DH)_2]Cl_2$ (**1**), $[Au(H_2Gy3Me)]Cl_3$ (**2**) and $[Au(H_2Gy3Et)]Cl_3$ (**3**)

acting as counter ions. Reduction of gold(III) to gold(I) was observed for other gold complexes with thiosemicarbazones (Lessa et al. 2011; Santos et al. 2004). Reduction of gold(III) by the thiosemicarbazones could occur considering that these compounds exhibit reducing properties, as shown by previous reports (Borges et al. 1997). However, since **1** was obtained in good yield (77 %) reduction of gold(III) with oxidation of methanol could also take place.

As shown in "Spectroscopic characterization", the proposed formulation for **1** is $[Au_2(H_2Gy3DH)_2]Cl_2$.

Complexes (**2**) and (**3**) are monomers in which a neutral *bis*(thiosemicarbazone) is attached to the gold(III) center. There are three chlorides acting as counter ions. Although molar conductivity data for **2** and **3** suggested the presence of neutral compounds, our assumption of these complexes being 1:3 electrolytes was based on findings by other groups, which attributed molar conductivities lower than the expected in gold(III) complexes with thiosemicarbazones to hydrogen bond association between the chloride ions and some hydrogen donor group from the ligand (Casas et al. 2004).

Spectroscopic characterization

The ν(C=S) absorption observed at 836–808 cm^{-1} in the infrared spectra of the *bis*(thiosemicarbazones) shifts to 819–804 cm^{-1} in the spectra of **1**–**3**, indicating coordination of the sulfur (Castiñeiras et al. 2008; Lobana et al. 2008). The small shift is compatible with complexation of thione sulfur (Khanye et al. 2010).

The vibrations attributed to ν(C=N) at 1554 and 1521 cm^{-1} in the infrared spectra of H_2Gy3Me and H_2Gy3Et, respectively, shift to 1574 and 1565 cm^{-1} in the spectra of complexes (**2**) and (**3**), in agreement with coordination of the imine nitrogen (Reis et al. 2010; Ferraz et al. 2011; Lessa et al. 2011).

The absorption attributed to ν(C=N) at 1606 cm^{-1} in the infrared spectrum of H_2Gy3DH is not modified in the spectrum of complex (**1**) indicating that the imine nitrogens are not attached to the gold(I) center.

The absorptions at 252–232 cm^{-1} in the infrared spectra of **1**–**3** were attributed to the ν(Au–S) vibrations (Kouroulis et al. 2009; Afanasieva et al. 2005; Casas et al. 2006). In the spectra of **2** and **3**, additional absorptions at 433 and 446 cm^{-1} were attributed the $\nu(Au–N_{imine})$ vibration (Kouroulis et al. 2009; Afanasieva et al. 2005; Casas et al. 2006). These absorptions were not observed in the spectrum of **1**, indicating that the imine nitrogens are not attached to the metal.

Therefore the results suggest that in **1** the *bis*(thiosemicarbazone) is attached to gold(I) through the sulfur atom whereas in **2** and **3** the ligands are coordinated to gold(III) through the N_2S_2 chelating system.

In the ^{1}H NMR spectra of complexes (**1**–**3**) the signals of all hydrogens undergo significant shifts in relation to their positions in the free bases. The *bis*(thiosemicarbazones) are attached to the metal ion in the neutral form. In all complexes the two arms of the *bis*(thiosemicarbazones) are equally coordinated to the metal, as shown in the ^{1}H NMR spectrum of complex (**3**) (Fig. 3). Hence complex (**1**) should most probably be a dimer (see Fig. 1). Considering that gold(I) complexes are generally linear and that the metal ion is a soft acid, it was proposed that the coordination sphere of each metal center is filled only

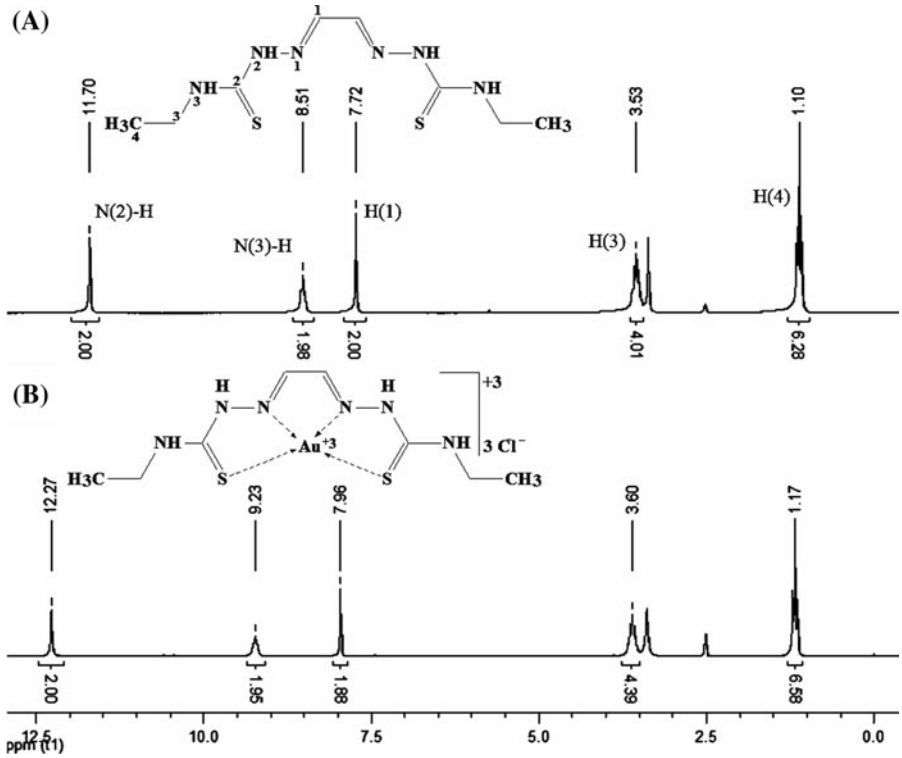


Fig. 3 ^{1}H NMR spectra of H_2Gy3Et (**A**) and $[Au(H_2Gy3Et)]Cl_3$ (**3**) (**B**)

by the sulfur atoms from two *bis*(thiosemicarbazones) H_2Gy3DH. Thus, the two chloride ions present in complex (**1**) would act as counter ions. In fact, the literature reports a gold(I) complex (Castiñeiras et al. 2009) with pyridil *bis*{3-hexamethylene-iminylthiosemicarbazone} whose crystal structure is similar to that proposed for complex (**1**).

Only the ^{13}C NMR spectrum of **3** could be recorded due to the low solubility of the other complexes in DMSO-d_6. The signals of C=N and C=S undergo significant shifts, suggesting coordination of the *bis*(thiosemicarbazone) through the N_2S_2 chelating system.

Electronic spectra of the *bis*(thiosemicarbazones) and their complexes (**1–3**) were recorded in DMF. The molar absorption coefficient (ε) was not determined for **1**, due to its low solubility. The electronic spectrum of **1** is shown for comparison with the spectral profiles of **2** and **3** (Fig. 4A). Two bands around 349 and 359 nm ($\varepsilon = 4.13 \times 10^4$ to 4.85×10^4 mol^{-1} L cm^{-1}) were observed in the electronic spectra of H_2Gy3Me and H_2Gy3Et, which are assigned to $n \rightarrow \pi^*$ transitions of the azomethine and thioamide groups, respectively (West et al. 1997).

A bathochromic shift of the $n \rightarrow \pi^*$ transitions, along with a hypochromic effect ($\varepsilon = 2.45 \times 10^4$ mol^{-1} L cm^{-1} to 2.99×10^4 mol^{-1} L cm^{-1}) were observed in the spectra of complexes (**2**) and (**3**). In addition, S → Au(III) and N → Au(III) ligand-to-metal charge-transfer transitions were observed in the 390–480 nm region in the spectra of **2** and **3**. These bands were not observed in the spectrum of **1**, in accordance with the presence of gold(I).

Spectra of the *bis*(thiosemicarbazones) and complexes (**1–3**) were also recorded in 0.5 % DMSO/phosphate buffer pH 7.4 to simulate physiological conditions (data not shown). The spectra of **1–3** did not alter from $t = 0$ to $t = 60$ min, indicating that the complexes are stable.

Cyclic voltammetry

During the synthesis of complex (**1**) the gold(III) ion was reduced to gold(I), which was not observed for complexes (**2**) and (**3**). Therefore, we investigated the electrochemical behavior of the *bis*(thiosemicarbazones) in order to verify their influence on the reduction of gold(III). In addition, the cyclic voltammograms of

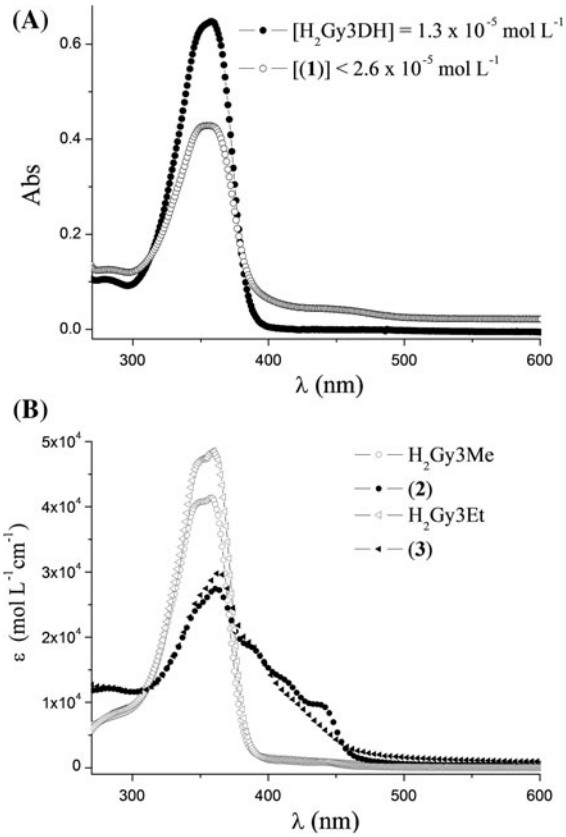


Fig. 4 Electronic spectra of H_2Gy3DH and **1** (**A**) and of H_2Gy3Me, H_2Gy3Et, **2** and **3** (**B**), recorded in DMF

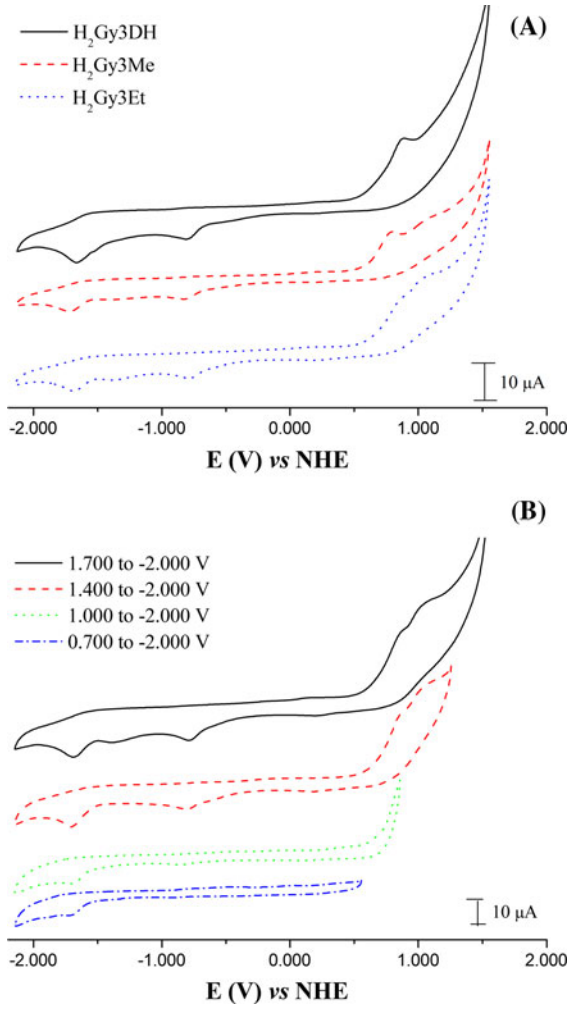


Fig. 5 Cyclic voltammograms of H_2Gy3DH, H_2Gy3Me and H_2Gy3Et (**A**) and of H_2Gy3Et, recorded in four potential ranges, evidencing de dependence among the redox processes (**B**). Scan rate: 100 mV s^{-1} (DMF)

1–**3** were recorded in order to verify the distinct behaviors of gold(I) and gold(III) complexes.

Figure 5A presents the cyclic voltammograms of H_2Gy3DH, H_2Gy3Me and H_2Gy3Et. Three cathodic peaks were observed for all *bis*(thiosemicarbazones). In addition, two anodic peaks were observed for H_2Gy3Me and H_2Gy3Et, while only one anodic peak was observed in the cyclic voltammogram of H_2Gy3DH. For all *bis*(thiosemicarbazones) the oxidation processes are correlated to the reduction processes at −0.789 to −1.522 V versus NHE whereas the reduction process at −1.668–1.722 V versus NHE is irreversible, as illustrated by the cyclic voltammograms of H_2Gy3Et recorded in four ranges (Fig. 5B).

Oxidation of *bis*(thiosemicarbazones) has been reported and may be related to the generation of ring-cyclized products, such as thiadiazoles (Refaey et al. 2008). The successive cathodic steps observed in the cyclic voltammogram of all compounds may be assigned to reduction processes centered on the C=S moiety as previously reported in the literature (Evans et al. 2009; Zhang et al. 1995).

The cyclic voltammograms of **1**–**3** are shown in Fig. 6. The processes associated to the ligands in complexes (**1**–**3**) are slightly shifted in comparison to those in the free *bis*(thiosemicarbazones).

Complexes (**2**) and (**3**) present similar electrochemical behavior in solution, which differs from the electrochemical behavior of complex (**1**), suggesting in principle that the oxidation state of the metal in **1** is different from that in **2** and **3**.

The electrochemical behaviors of **1**–**3** were compared with that of $HAuCl_4$ in order to obtain information about the oxidation state of the metal in these

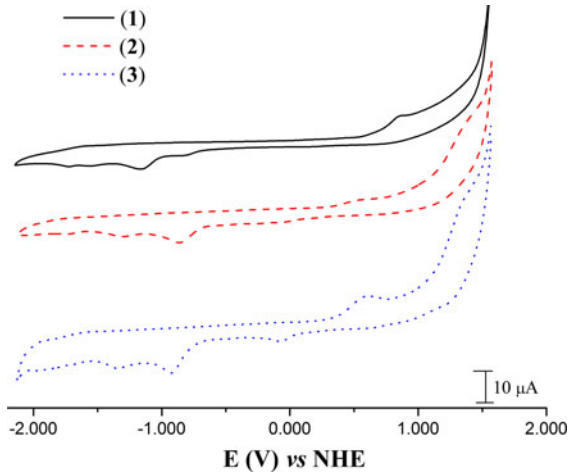


Fig. 6 Cyclic voltammograms of $[Au_2(H_2Gy3DH)_2]Cl_2$ (**1**), $[Au(H_2Gy3Me)]Cl_3$ (**2**) and $[Au(H_2Gy3Et)]Cl_3$ (**3**). Scan rate: 100 mV s^{-1} (DMF)

complexes. There were no similarities between the voltammograms of **1** and $HAuCl_4$ (Fig. 7A). In addition, we observed only one additional cathodic peak at −1.168 V versus ENH in the voltammogram of complex (**1**), which may be attributed to Au(I) → Au(0). Thus, the results suggest the presence of gold(I) in the structure of **1**, in accordance with other analysis carried out in this work.

In the cyclic voltammograms of complexes (**2**) and (**3**) one *quasi*-reversible process at 0.236 and 0.268 V versus NHE for **2** and **3** respectively, which is absent in the cyclic voltammograms of H_2Gy3Me and H_2Gy3Et but is present in $HAuCl_4$, was attributed to Au(II) → Au(I). The other cathodic and anodic peaks assigned to gold(III) in $HAuCl_4$ were not observed in the cyclic voltammograms of **2** and **3** probably because they may appear in the same region as the redox processes of the ligands. Hence although we were unable to see the Au(III) → Au(II) processes, the observation of the Au(II) → Au(I) process indicates that Au(III) was present. Thus, the results suggest the presence of a gold(III) center in the structure of **2** and **3**. Figure 7B shows, as example, the electrochemical behaviors of H_2Gy3Et, **3** and $HAuCl_4$.

Cyclic voltammetry data of *bis*(thiosemicarbazones) and their gold complexes (**1–3**) are collected in Table 1. Unfortunately, according to Table 1, it is not possible to correlate the oxidation potential of the *bis*(thiosemicarbazones) with their abilities or disabilities to reduce gold(III) during the synthesis of **1–3**.

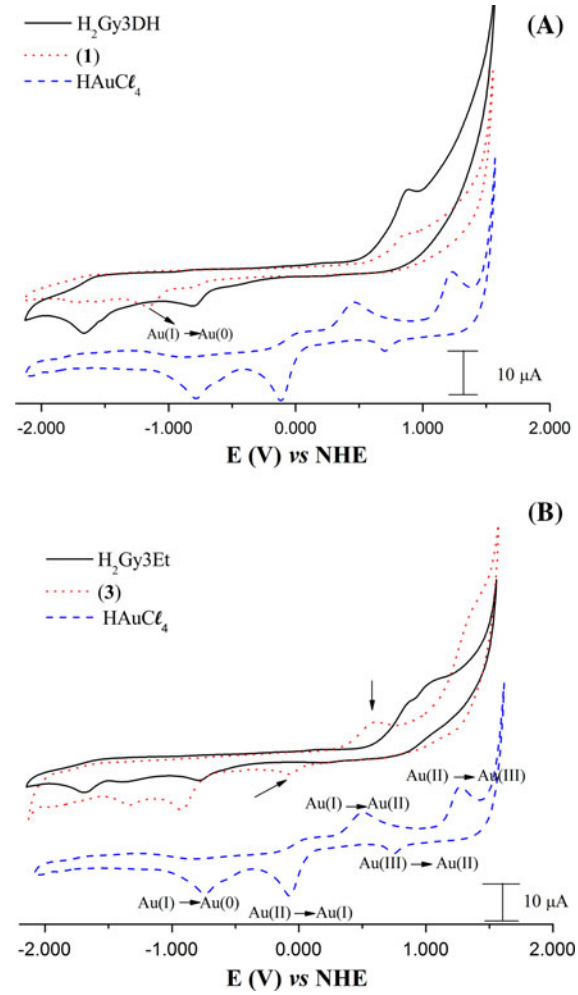
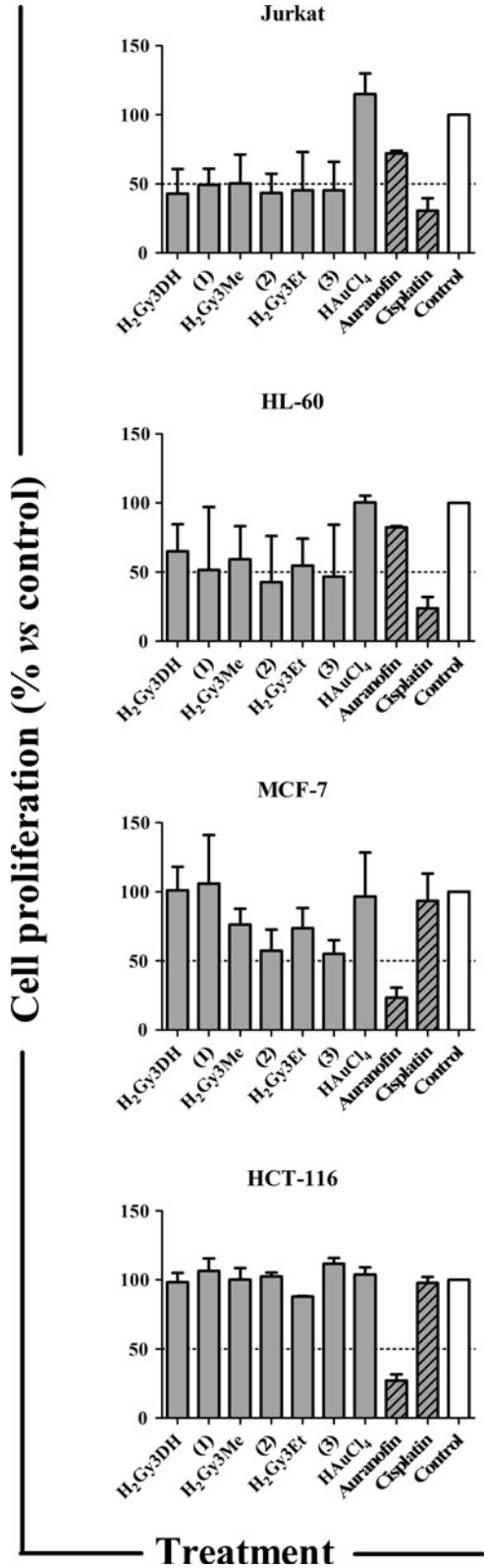


Fig. 7 Electrochemical behaviors of H_2Gy3DH, **1** and $HAuCl_4$ (**A**) and of H_2Gy3Et, **3** and $HAuCl_4$ (**B**). Redox processes attributed to the metal center in cyclic voltammograms of complexes are indicated by arrows. Scan rate: 100 mV s^{-1} (DMF)

However, the electrochemical studies were important to confirm the oxidation state of gold in the structure of complexes (**1–3**).

Cytotoxicity

Figure 8 shows the percentage of proliferation of Jurkat, HL-60, MCF-7 and HCT-116 cells treated with the *bis*(thiosemicarbazones), their complexes (**1–3**) and $HAuCl_4$ (10 μmol L^{-1}) versus control (cells treated with 0.1 % DMSO). The drugs cisplatin and auranofin were used as positive controls. The former is widely used in clinics as an anticancer agent, while the



◀**Fig. 8** Cytotoxic effect of *bis*(thiosemicarbazones) H_2Gy3DH, H_2Gy3Me and H_2Gy3Et and of complexes (**1**–**3**) on Jurkat, HL60, MCF-7 and HCT-116 cell lines. Cells were treated with compounds (10 μmol L^{-1}) for 48 h and the cell proliferation/cell survival was measured by the MTT assay as described in the experimental section. Data are expressed as mean ± SEM of percentage of cell proliferation compared to control (cells treated with DMSO 0.1 %). Representative data of at least three independent experiments performed in triplicate

latter is employed for the treatment of rheumatoid arthritis. Auranofin was used as a control because it contains gold in its structure. In addition, there are reports in the literature demonstrating that auranofin presents cytotoxic activity against certain tumor cell lines (Tiekink 2002; Cox et al. 2008).

In general, the *bis*(thiosemicarbazones) and their complexes (**1**–**3**) showed cytotoxic effects against the studied tumor cell lines, except against HCT-116 cells. Jurkat and HL-60 were the most sensitive cells to the compounds. **1**–**3** were as or more cytotoxic than auranofin against Jurkat and HL-60 cells, but they were less active than auranofin against MCF-7 and HCT-116 solid tumor cells.

H_2Gy3Me, H_2Gy3Et and their complexes (**2**) and (**3**) were more active against MCF-7 cells than cisplatin. Moreover, complexation to gold(III) resulted in increased cytotoxicity against MCF-7 cells in complexes (**2**) and (**3**).

Auranofin proved to be less cytotoxic against leukemic cells than against the solid tumor MCF-7 and HCT-116 cells. Opposite behavior was observed for cisplatin. $HAuCl_4$ showed no cytotoxic effect against all cell lines.

Inhibition of TrxR activity

Inhibition of TrxR was considered as one potential mechanism for the anti-proliferative activities of **1**–**3**. The effect of complex (**2**) on the activity of TrxR was evaluated. Thus, the in vitro inhibitory activities of **2**, H_2Gy3Me, $HAuCl_4$ and auranofin were evaluated using isolated rat liver TrxR, by means of the DTNB reduction assay.

Complex (**2**) inhibited the enzyme's activity with $IC_{50} = 2.90$ μmol L^{-1} (95% confidence interval = 2.49–3.37 μmol L^{-1}) (Fig. 9). This IC_{50} value is lower than the dose used in the cytotoxic activity assay of **2** (10 μmol L^{-1}), suggesting TrxR as a possible biological target. The complex's ability to

Table 1 Cyclic voltammetry data of the *bis*(thiosemicarbazones) and their gold complexes

E (V vs NHE)	H_2Gy3DH	(**1**)	H_2Gy3Me	(**2**)	H_2Gy3Et	(**3**)	$HAuCl_4$
E_{pa}	0.890	–	1.076	–	1.042	–	***0.508***
			0.797	–	0.857	–	***1.274***
				0.539		***0.610***	
E_{pc}	−0.814	−0.826	−0.829	−0.867	−0.789	−0.928	***0.747***
	−1.522	−1.553	−1.361	−1.307	−1.394	−1.338	***−0.742***
	−1.668	−1.724	−1.722	−1.712	−1.687	−1.968	***−0.074***
		−1.168		***−0.067***		***−0.074***	

Processes attributed to the metal center are in italic-bold face type

E_{pa} anodic peak potential, E_{pc} cathodic peak potential

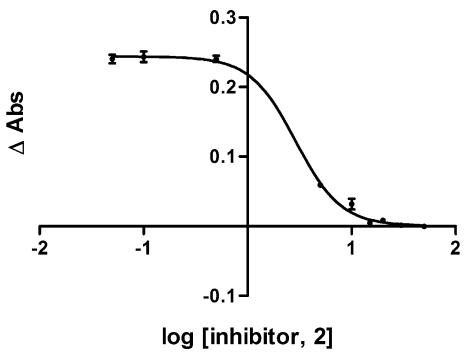


Fig. 9 Inhibitory effect of $[Au(H_2Gy3Me)]Cl_3$ (**2**) on TrxR activity: log(inhibitor, **2**) versus response (absorbance) curve obtained for IC_{50} determination. Enzyme (0.12 unit) was treated without (control) and with **2** (ten different concentrations in the 0.05–50 μmol L^{-1} range) for 1 h and TrxR activity was evaluated by DTNB assay as described in the experimental section. Representative data of experiments performed in triplicate

inhibit the TrxR enzyme was compared with that of H_2Gy3Me, $HAuCl_4$ and auranofin at three concentrations: 0.5, 5.0 and 10 μmol L^{-1} (Fig. 10), being the last one the same compound concentration used in the anti-proliferative activity assays.

We observed that at 0.5 μmol L^{-1}, H_2Gy3Me and **2** did not inhibit the enzyme's activity, whereas $HAuCl_4$ and auranofin inhibited 46.3 and 71.0 % of TrxR's activity, respectively. At 5.0 and 10.0 μmol L^{-1}, **2** inhibited 76.5 and 87.4 % of TrxR's activity, respectively, while the *bis*(tiosemicarbazone) was not active. At 5.0 μmol L^{-1}, $HAuCl_4$ and auranofin inhibited 94.2 and 95.6 % of the enzyme's activity. At 10.0 μmol L^{-1}, $HAuCl_4$ and auranofin inhibited 97.3 and 96.4 % of TrxR's activity, respectively.

Since not only complex (**2**) and auranofin but also $HAuCl_4$ strongly inhibited the enzyme's activity, the inhibitory effect on TrxR may be related to the presence of gold. However, although $HAuCl_4$ is a potent inhibitor of TrxR's activity, it did not exhibit cytotoxic effect against HL-60, Jurkat, MCF-7 and HCT-116 tumor cell lines. The hydrophilic character of $HAuCl_4$ probably hinders its passage through the cell membrane. Unlike $HAuCl_4$, **2** not only inhibited TrxR's activity but also presented cytotoxic effect, which suggests that the *bis*(thiosemicarbazone) acted as carrier of gold into the cells.

Conclusions

The *bis*(thiosemicarbazones) and their gold(I) and gold(III) complexes presented anti-proliferative activity against Jurkat, HL-60 and MCF-7 human cancer cell lines. Upon coordination to gold(III) the anti-proliferative effect increases in some cases. Most of the studied compounds proved to be equally or more active than auranofin against Jurkat and HL-60 cells and some of them were more effective than cisplatin against MCF-7 cells. The compounds were not effective against HCT-116 cells at 10 μmol L^{-1}. $HAuCl_4$ proved to have no anti-proliferative effect.

Complex (**2**) was able to inhibit the activity of thioredoxin reductase (TrxR), an enzyme which is involved in DNA syntheses. Since $HAuCl_4$ proved to be a strong inhibitor of the enzyme's activity but does not present cytotoxicity against the studied tumor cell lines, we may suggest that its high hydrophilicity probably hinders its passage through the cell membrane. Unlike $HAuCl_4$, **2** not only inhibited TrxR activity but also presented cytotoxic effect, which suggests that the *bis*(thiosemicarbazone), besides its

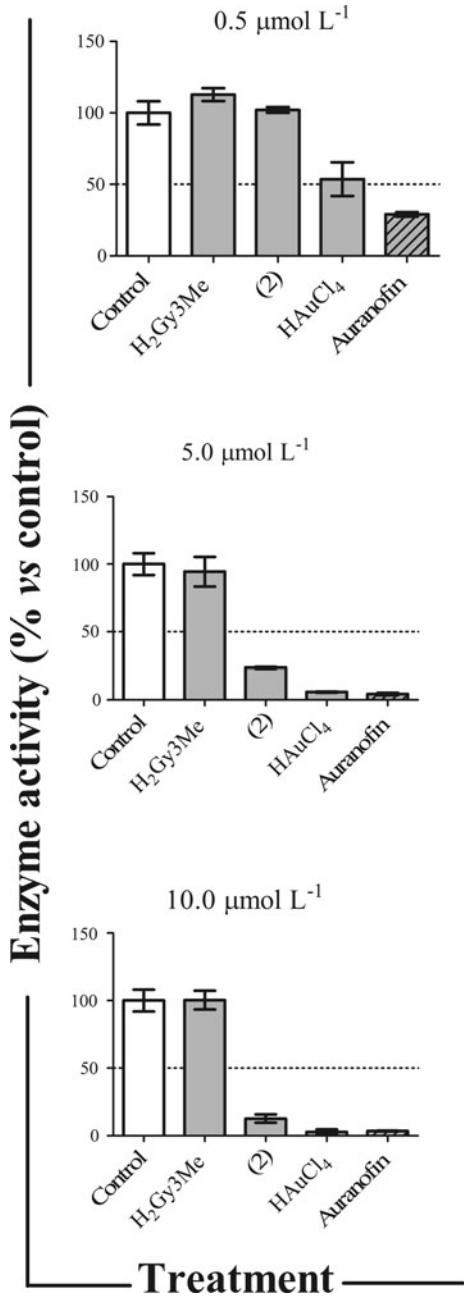


Fig. 10 Inhibitory effect of H_2Gy3Me, **2**, $HAuCl_4$ and auranofin on TrxR. TrxR (0.12 unit) was incubated without (control) and with compounds at 0.5, 5.0 and 10.0 μmol L^{-1} concentrations for 1 h at 37 °C. TrxR activity was evaluated by DTNB assay as described in the experimental section. Data are expressed as mean ± SD of percentage of cell proliferation compared to control. Representative data of experiments performed in triplicate

own cytotoxic effect, acted as carrier of gold into the cells.

Acknowledgments This work was supported by CNPq and INCT-INOFAR, Proc. CNPq 573.364/2008-6).

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