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Gold(III) bis(dithiolene) complexes: from molecular conductors to prospective anticancer, antimicrobial and antiplasmodial agents

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Dedication: D. Fontinha, M. Prudêncio - antiplasmodial activity studies; S. A. Sousa, J. H. Leitão - antimicrobial activity studies; Y. Le Gal, D. Lorcy: synthesis of complexes 1-4 and redox properties studies; M. Velho, R. Silva, D. Belo, M. Almeida: synthesis of complexes 5 and 6 and stability studies in solution; T. Morais, J. Guerreiro, T. Pinheiro, F. Marques: anticancer activity and mechanistic studies.

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Abstract

The anticancer, antimicrobial and antiplasmodial activities of six gold(III) bis(dithiolene) complexes were studied. Complexes (**1-6**) showed relevant anticancer properties against A2780/A2780cisR ovarian cancer cells (IC_{50} values of 0.08-2 µM), also being able to overcome cisplatin resistance on A2780cisR cells. Complex **1** also exhibited significant antimicrobial activity against *Staphylococcus aureus* (minimum inhibitory concentration (MIC) values of 12.1±3.9 µg/mL) and both *Candida glabrata* and *Candida albicans* (MICs of 9.7±2.7 and 19.9±2.4 µg/mL, respectively). In addition, all complexes displayed antiplasmodial activity against the *Plasmodium berghei* parasite liver stages, even exhibiting better results than the ones obtained using primaquine, an anti-malarial drug. Mechanistic studies support the idea that thioredoxin reductase, but not DNA, is a possible target of these complexes. Complex **1** is stable under biological conditions, which would be important if this compound is ever to be considered as a drug. Overall, the results obtained evidenced the promising biological activity of complex **1**, which might have potential as a novel anticancer, antimicrobial and antiplasmodial agent to be used as an alternative to current therapeutics.

Introduction

The worldwide increase in the incidence of cancer and in the number of infections caused by microorganisms resistant to available antibiotics led to the urgent need to develop novel efficient antitumor and antimicrobial agents.[1-4] In fact, microbial drug resistance is responsible for the increasing mortality rate observed for infectious diseases, due to the lack of effective drugs and methods for prevention and treatment. Therefore, novel antimicrobial drugs, possibly acting by different mechanisms and/or on non-classical targets may have the potential to overcome antimicrobial resistance.[5,6]

The incorporation of transition metal ions into rationally designed ligands offers new opportunities to develop unique metal-containing compounds aimed at enhancing their biological activity and bioavailability, overcoming drug resistance and toxicity, and improving their specificity.[7,8] In this context cisplatin's biological ability to inhibit *Escherichia coli* division, paved the way to the first studies of its antitumor activity and sparking an interest in its use in cancer chemotherapy becoming the most powerful drug prescribed against different types of solid tumors.[9-11] Cisplatin's mode of action has been attributed mainly to its ability to bind DNA, causing DNA damage and subsequently triggering cell cycle arrest and apoptosis in cancer cells.[11-13] However, due to the development of drug resistance and the severe side effects, novel classes of anticancer drugs based on other transition metals (*e.g.*, Ru, Cu, V) have been proposed as prospective alternatives to cisplatin as antitumor agents[14] or exhibiting multifunctional properties as antitumor, antimicrobial, antiviral, and anti-inflammatory agents.[15-18]

Gold complexes, have been considered as potential alternatives to overcome resistance to cisplatin. Nevertheless, their clinical use has been limited due to their toxicity and poor physiological stability. Phosphines, N-heterocyclic carbenes (NHCs) and thiosemicarbazonates became increasingly employed to form stable gold(I) complexes for clinical applications, in particular as potential anticancer agents.[19-23] One of the lead compounds, auranofin, a thiolate-phosphine gold(I) complex, initially used to treat rheumatoid arthritis, was found to have a broad spectrum of activity, thus paving the way to the development of other gold(I) complexes with potential clinical interest.[24,25] Auranofin showed potent antitumor activity, in particular towards some types of solid tumors and leukaemia,[26-28] representing a reference compound when new gold compounds are studied as prospective therapeutic drugs.

More recently efforts have focused upon the development of gold(III) complexes through stabilization of the gold higher oxidation state bearing for instance carbon-,

nitrogen- and oxygen- atoms to prevent gold(III) reduction under physiological conditions. Sulphur-containing ligands are often introduced to improve the stability of gold(III) complexes, with dithiocarbamates receiving considerable attention due to the substantial stabilizing effect of these bidentate chelating molecules.[29] Cyclometallation has been particularly explored, as an effective strategy to increase the stability of gold complexes in physiological conditions as a result of the presence of an Au-C bond. This strategy has been successfully applied to the synthesis of a plethora of gold complexes containing C^N, C^N^N and C^N^C ligands with potential use as anticancer agents.[30]

Gold(III) complexes are isostructural and isoelectronic with platinum(II) complexes, both forming four-coordinated square-planar complexes. Therefore, it was initially expected that gold(III) would exhibit a mode of action similar to that of cisplatin for which DNA is the main target. Results reported so far suggest that the interactions of cytotoxic gold(III) complexes with DNA are weaker and different than those of platinum analogues, which indicates that DNA might not represent the sole biological target.[31-34] The mechanisms of action displayed by the gold(III) complexes are still under investigation to unveil their cellular targets as well as their usefulness as a versatile class of metal-based compounds with antitumor, antimicrobial, antiviral, and antiparasitic properties.[32, 35-39]

Contrasting with the various studies exploring the use of gold complexes as anticancer agents, their antimicrobial activity remains poorly investigated. However, the steady increase of microbial resistance to conventional antibiotics posed a serious threat to public health, and a huge financial burden to public health systems.[40]

The 2014 World Health Organization (WHO) report on Antimicrobial Resistance [41] highlighted 9 bacteria as deserving concern from the international community. This included *Escherichia coli* resistant to third-generation cephalosporins or to fluoroquinolones, *Klebsiella pneumoniae* resistant to third-generation cephalosporins or to carbapenems, *Staphylococcus aureus* resistant to methicillin, *Streptococcus pneumoniae* non-susceptible to penicillin, nontyphoidal *Salmonella* and *Shigella* resistant to fluoroquinolones, and *Neisseria gonorrhoeae* resistant to cephalosporin.[41] Fungal infections, particularly those caused by *Candida* spp., are also an increasing public health problem, particularly due to increased resistance to azoles and the emergence of resistance to echinocandins.[41]

The above context prompted us to explore a series of mononuclear gold(III) bis (dithiolene) complexes in their monoanionic state as prospective antitumor, antibacterial

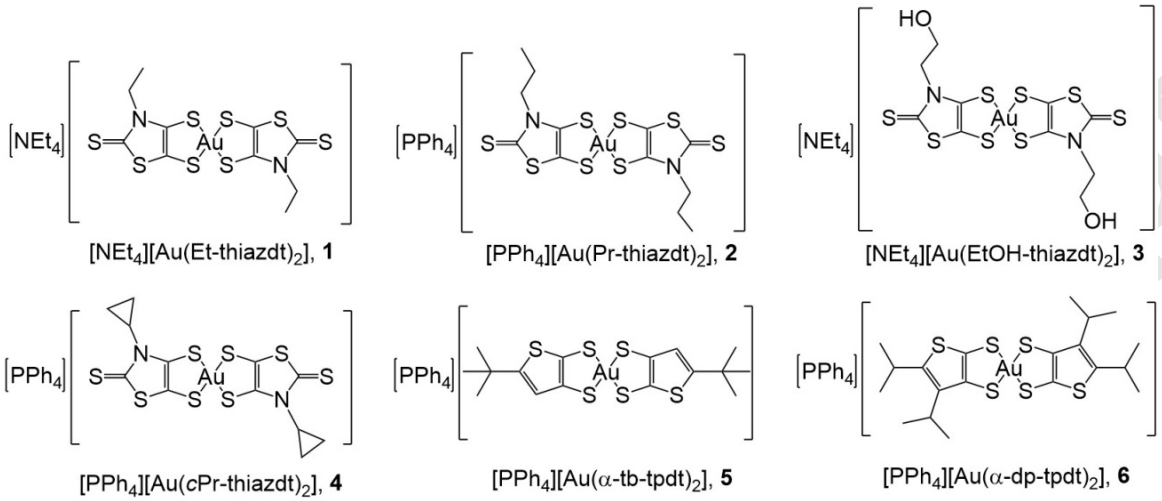
and antiplasmodial drugs. Usually, the interest on this class of monoanionic gold(III) complexes is related to their use as precursors of neutral single-component molecular conductors and magnetic materials.[42-46] As found for cisplatin, these compounds feature square planar coordination geometries, and, as such, a similar mechanism of action, *e.g.*, antitumor activity in cells sensitive to cisplatin and interaction with DNA, should be expected for these compounds. Reports on the biological activity of similar gold(III) compounds, however, are relatively scarce in the literature. Recently, a publication on gold(III) 1,2-dithiolene complexes described the potential of this type of compounds against Gram-positive bacteria.[47]

Gold bis(1,2-dithiolene) complexes can exhibit several oxidation states from dianionic, monoanionic, neutral to monocationic ones, depending on the electron donating or withdrawing character of the dithiolene ligand. Due to the non-innocent character of the dithiolene ligand,[48] thanks to the mixing of the metal and ligand orbitals generating an electron delocalization over the metallacycle, it is possible to deliberately modulate their redox properties. So far, only the biological activity of gold complexes with electron withdrawing dithiolene ligands has been investigated, leading to monoanionic complexes which can be easily reduced to the dianionic state (gold(II) complexes).[49] Herein we want to take advantage of the non-innocent character of the ligand by investigating complexes which can be easily oxidized (rather than reduced).

In this study, we investigated monoanionic gold bis(dithiolene) complexes containing either N-alkyl-1,3-thiazoline-2-thione dithiolate ligands, with different alkyl groups such as ethyl, propyl, hydroxyethyl and cyclopropyl (R = Et, Pr, EtOH, cPr), namely $[Au(R\text{-thiazdt})_2]^{-1}$ [50-52] or alkyl substituted thiophenedithiolate ligands [53] (Scheme 1). These compounds were assessed towards cisplatin sensitive and resistant ovarian cancer cells, clinically relevant bacteria such as *S. aureus* and *E. coli*, and two strains of the species *Candida albicans* and *C. glabrata*, the most important fungal opportunistic pathogens.[54]

In order to overcome the scarcity of compounds targeting the liver stage of *Plasmodium* infection, an obligatory and clinically silent phase of the malaria parasite's life cycle before symptoms arise, we also screened our gold(III) complexes for their ability to inhibit the infection of human hepatoma cells (Huh7) by *Plasmodium* (*P.*) *berghei*. The biological activities of the gold complexes were studied and compared with those of auranofin, the reference drug currently being explored for potential therapeutic

application in cancer, bacterial and parasitic infections.[27] The effect of alterations of the ligand skeleton on the biological properties of theses complexes is also discussed.



Scheme 1: Structures of the complexes studied in this work.

Experimental

Materials

The monoanionic gold bis(dithiolene) complexes **1-6** were synthesized as tetraethylammonium salts in the case of **1** [50] and **3** [52], and as tetraphenylphosphonium salts in all other cases.[51] Complexes **1-6** were prepared according to previously reported procedures, starting from the corresponding cyanoethylthio protected dithiolene ligands in the case of **1-4** or from the hydrolytic cleavage of the precursor ketones, in the case of **5** and **6**.[53]

Methods

Cyclic voltammetry studies

Cyclic Voltammetry investigations were carried out at room temperature, on a 10^{-3} M solution of the complexes **1-6** in CH_2Cl_2-$[NBu_4][PF_6]$ 0.1 M. Cyclic Voltammograms were recorded on a Biologic SP-50 instruments at 0.1 Vs^{-1} on a platinum disk electrode. Potentials were measured *versus* KCl Saturated Calomel Electrode (SCE).

Cytotoxic activity of gold complexes

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The ovarian cancer cell lines A2780 and A2780cisR, sensitive and resistant to cisplatin, respectively, were obtained from Sigma-Aldrich. V79 normal lung fibroblasts were obtained from the American Type Culture Collection (ATCC). Cells were grown in RPMI-1640 (A2780/A2780cisR) or DMEM containing GlutaMax I (V79) supplemented with 10% fetal bovine serum and were maintained in a 5% CO_2 incubator in a humidified atmosphere at 37ºC. All compound stocks were initially prepared in DMSO, and then diluted in culture medium to a final DMSO concentration that did not exceed 1% (v/v) and had no cytotoxic effect in cells. The reference drugs auranofin and cisplatin were first diluted in DMSO and water, respectively, followed by their dilution in medium to perform the assays. Cell viability was measured by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay which estimates the number of metabolically active cells that are able to reduce tetrazolium into a purple formazan product.[55] For the assays, cells were seeded in 96-well plates at a density of 2×10^4 cells (ovarian cells) or $1\text{x}10^4$ cells (V79) in 200 µL of the appropriate medium and incubated overnight for optimal adherence. After careful removal of the medium, 200 µL of a serial dilution of compounds in fresh medium were added to the cells and incubation was carried out for 24 and 48 h at 37°C. At the end of the treatment, the medium was discarded and the cells were incubated with 200 µL of an MTT solution in PBS (0.5 mg/mL). After 3 h at 37°C the medium was removed and 200 µL of DMSO were applied to each well to solubilize the purple formazan crystals formed. The absorbance at 570 nm was measured using a plate spectrophotometer (Power Wave Xs, Bio-Tek). The IC_{50} values were calculated from dose-response curves analyzed with the GraphPad Prism software (version 5.0). Results are shown as the mean ± SD of two independent experiments done with six replicates.

Bacterial and fungal strains

The bacterial strains *Staphylococcus aureus* Newman and *Escherichia coli* ATCC25922, and the fungal strains *Candida glabrata* CBS138 and *Candida albicans* SC5134 were used. Bacterial and fungal strains were maintained, respectively, in Lennox Broth (LB) solid medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl and 15 g/L agar) and Yeast Extract-Peptone-Dextrose (YPD) solid medium (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract and 15 g/L agar). Stock solutions of the tested compounds were prepared in DMSO, at final concentrations of 10 mg/mL (complexes **1**

and **2**), 5 mg/mL (complexes **3**, **4** and **6**, and Auranofin) or 2.5 mg/mL (complex **5**), depending on their solubility.

Determination of gold complexes antimicrobial activity

Antibacterial susceptibility to the compounds was carried out according to standard methods (NCCLS) and as previously described.[49,56] Briefly, 96-well polystyrene microtiter plates (Greiner Bio-One) were filled with 100 µL of Mueller-Hinton (MH) broth (Fluka Analytical). Sequential 1:2 dilutions of each compound were prepared from the stock solutions in order to obtain final concentrations ranging from 0.49 µg/mL to 125 µg/mL, except when testing auranofin activity towards *S. aureus* Newman. In this case, the concentrations used ranged from 0.06 µg/mL to 125 µg/mL. Six-hours grown bacterial cultures (carried out in MH broth at 37°C and 250 rev.min^{-1}) were diluted with MH fresh medium to a final optical density of 0.02, measured at 640 nm (OD_{640}) in a Hitachi U-2000 UV/Vis spectrophotometer. The wells of the microplates were then inoculated with 100 µL of the diluted bacterial suspension (*S. aureus* Newman or *E. coli* ATCC25922) and incubated for 24 h at 37ºC. After incubation, the wells were examined for turbidity (growth), resuspended by pipetting, and their optical density was measured in a SPECTROstar Nano microplate reader (BMG Labtech) at 640 nm.

Antifungal susceptibility testing was carried out according to the standardized microdilution method recommended by EUCAST (European Committee on Antimicrobial Susceptibility Testing) for *Candida* spp. and as previously described.[40,57] Briefly, 96-well microtiter plates (Greiner Bio-One) were filled with 100 µL of RPMI-1640 liquid medium (Sigma) buffered to pH 7.0 with 0.165 M morpholinepropanesulphonic acid (MOPS; Sigma). Sequential 1:2 dilutions of each compound solution were carried out in order to obtain final concentrations ranging from 0.49 µg/mL to 125 µg/mL. Overnight grown fungal cultures (carried out in YPD broth at 30°C and 250 rev.min^{-1}) were diluted with RPMI-1640 fresh liquid medium to a final optical density of 0.025, measured at 530 nm (OD_{530}) in a Hitachi U-2000 UV/Vis spectrophotometer. The wells were then inoculated by the addition of 100 µL of the fungal suspensions (*C. glabrata* CBS138 or *C. albicans* SC5134) and incubated for 24 h at 35ºC. After incubation, the wells were examined for turbidity (growth), ressuspended

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and their optical density was measured in a SPECTROstar Nano microplate reader (BMG Labtech) at 530 nm.

All the compounds were tested in three independent experiments and in duplicate wells. Minimum inhibitory concentrations (MICs) were estimated after data fitting of the OD_{640} or OD_{530} mean values using a modified Gompertz equation, using the GraphPad Prism software (version 6.07).[58] In each experiment, positive (without compound) and negative controls (no organism inoculum) were carried out. The effect of 5% (v/v) or 1.25% (v/v) DMSO on bacterial or fungal growth, respectively, was also assessed.

In vitro activity of gold complexes against the hepatic stage of P. berghei infection

The *in vitro* activity of the compounds against infection of a human hepatoma cell line (Huh7) by the rodent *P. berghei* malaria parasite was assessed by a bioluminescence method, as previously described.[59] Briefly, Huh7 cells were routinely cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, 1% (v/v) glutamine, 1% (v/v) nonessential amino acids, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7, and maintained at 37°C with 5% CO_2. Compound stock solutions were prepared in DMSO at 10 mM concentration. In order to assess their activity against the hepatic stage of *P. berghei* infection, compounds were serially diluted in infection medium, *i.e.* culture medium supplemented with 50 µg/mL of gentamicin and 0.8 µg/mL of amphotericin B. One day prior to infection, Huh7 cells were seeded at $1x10^4$ cells/well in 96-well plates. On the day of infection, the culture medium was replaced by the adequate compound dilutions and cells were incubated for 1 h at 37ºC, 5% CO_2. Firefly luciferase-expressing *P. berghei* sporozoites were freshly isolated from the salivary glands of female *Anopheles stephensi* mosquitoes and added to the cells at a 1:1 ratio, followed by centrifugation at 1800 x g for 5 min, and subsequent incubation for 48 h at 37ºC, 5% CO_2. The effect of each compound concentration on the viability of Huh7 cells was assessed by the CellTiter-Blue assay (Promega, USA), according to the manufacturer's protocol. Infection load was measured by a bioluminescence assay (Biotium, USA) following the manufacturer's instructions, using a multi-plate reader Infinite M200 (Tecan, Switzerland).

Analysis of cellular uptake of gold complexes

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The concentrations of gold (Au) were determined by particle-induced X-ray emission (PIXE) technique, installed at the Van de Graaf accelerator of Instituto Superior Técnico, in A2780 cells incubated with the complexes **1**, **2**, **3**, **4**, and the reference drug, auranofin at equimolar concentrations of 50 µM for 2 h. The cell pellets were obtained by centrifugation after washing the cells with PBS to remove the culture medium, freeze-dried and digested using suprapure reagents, nitric and hydrochloric acids (1:3 molar ratio) together with yttrium (Y) (100 mg/l) as an internal standard. The procedure combined ultrasound cycles of 30 min at 60°C and microwave-assisted acid digestion (350 W, 15 s). The detailed methodology encompassing PIXE analysis, concentration calculation and quality control, was previously described.[60] The elemental concentrations were obtained in µg/g (of dry material) and converted to µg/10^6 cells.

In vitro thioredoxin (TrxR) activity assay

To determine the inhibition of TrxR by the gold complexes, a commercially available Thioredoxin Reductase Assay Kit (Sigma-Aldrich) was used with minor modifications for a 96-well plate format assay. The compounds were first dissolved in DMSO at various concentrations in the range of 0.1 nM-20 µM. In a 96-well plate 2 µL of the enzyme solution were added to 14 µL of assay buffer (phosphate buffer pH 7.0 containing 50 mM EDTA), 180 µL of working buffer (phosphate buffer containing 0.25 mM NADPH) and 2 µL of the complexes' solutions. The enzymatic reaction was started with the addition of 6 µL DTNB (0.1 M in DMSO). A blank sample (without enzyme) and a positive control (without compounds) were included in the assays. After proper shaking for 15 min, the formation of TNB was monitored at 412 nm with a plate spectrophotometer (Power Wave Xs, Bio-Tek). The IC_{50} values were calculated using the GraphPad Prism software (version 5.0).

Gold complexes - DNA interactions

Sample preparation for spectrofluorometric experiments

Stock solutions of CT-DNA were prepared by dissolving *ca*. 1 mg of CT-DNA fibers in 1 mL of 10 mM Hepes buffer pH 7.4, leaving it at 4°C on an orbital stirrer with slow gentle shaking for 3 days. Concentration of DNA solutions were expressed in nucleotide units (nuc), determined spectrophotometrically using the molar absorption coefficient of 6600 $M^{-1}cm^{-1}$ at 260 nm.[61] Purity of the CT-DNA solutions was checked by monitoring the ratio of UV absorbance at 260 and 280 nm, and the ratio $A_{260nm}/A_{280nm} \geq 1.8$

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indicating that the DNA was sufficiently free of proteins.[61] DNA solutions were stored at 4°C and used after 4 days at most. Ethidium bromide (EB) stock solutions were prepared dissolving a known amount of the probe in Millipore® water and kept in dark for sample preparation. CT-DNA (80 μM.nuc^{-1}) and EB (20 μM) were mixed in 10 mM Hepes buffer pH 7.4 and were incubated in the dark for 1 h at 37ºC, in the dark. Due to the limited solubility of gold complexes in aqueous media, DMSO was used to prepare concentrated stock solutions of each complex. The competitive interaction between EB and gold complexes was studied by adding different concentrations of gold complexes in DMSO (0 - 40 μM) to the EB-DNA solution. After addition of each gold complex solution to the EB-DNA solution, the final DMSO concentration was 1%. The mixtures were stirred to ensure the formation of a homogeneous solution and then stood in an incubator at 37ºC for 24 h in the dark. The reference solutions were also prepared according to the above procedures and without EB and DNA. Spectroscopic measurements were carried out on individually prepared samples to ensure the same pre-incubation time at 25°C for all samples in each assay.

Fluorescence spectroscopic measurements

Steady state fluorescence measurements were carried out at 25ºC on a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon) equipped with double grating monochromators in both excitation and emission light paths from Horiba Jobin Yvon. The excitation wavelength was set at 510 nm (with 4 nm/4 nm slit widths for excitation and emission) and emission spectra were recorded in the range of 520-650 nm. Buffer solutions of the gold complexes in corresponding concentrations were used as reference when measuring the fluorescence spectra of {EB-DNA}-complex mixtures. The fluorescence intensities were corrected for the self-absorption of the exciting light and reabsorption of the emitted light to decrease the inner filter effect.[62,63] Using UV-visible absorption, data were recorded for each sample on a Jasco V-660 spectrophotometer in the range of 500 to 900 nm with 1 cm path quartz cells. Corrections were carried out according to Eq. (1)

$$I_{corr} = I_{meas} \times 10^{(A_{EX} + A_{EM})/2} \qquad \text{Eq. (1)}$$

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where I_{corr} and I_{meas} are the corrected and measured fluorescence intensities, and the A_{EX} and A_{EM} are the absorbance values at the excitation and emission wavelengths in the samples, respectively.[64]

Results and Discussion

Redox properties of the gold complexes

The redox properties of the six gold(III) bis(dithiolene) complexes with structurally related manifolds (**1-6**) have been previously described in several publications under different conditions.[50-53] However, in order to achieve a better comparison of all the complexes, their redox properties were investigated by cyclic voltammetry using the same conditions (dichloromethane solutions and NBu_4PF_6 as supporting electrolyte). The oxidation potentials are shown in Table 1 together with those of $[Au(mnt)_2]^{-1}$ for comparison purposes. For all the investigated complexes upon anodic scan two oxidation waves were observed corresponding to the oxidation of the monoanionic species into the neutral one and to the monocationic complex as shown in Figure 1 for **1** and **6**. Comparison of the redox potentials shows that complexes **5** and **6** are easier to oxidize into the neutral species than complexes **1-4** while, on the contrary, neutral complexes **1-4** are easier to oxidize to the monocationic species than complexes **5** and **6**. As a consequence, the potential stability window of the neutral species is higher for **5** and **6** than for **1-4**. This difference is assigned to the nature of the dithiolene ligand as the thiazoline core in **1-4** is not aromatic while the thiophene one in **5** and **6** is aromatic and stabilizes the neutral complex. Another difference between these two complex families is that all the **1-4** complexes present adsorption phenomena of the oxidized species at the electrode followed by sharp desorption reduction peak while this is not the case for **5** and **6** complexes. Another interesting feature is the influence of the ligands on the electrochemical properties within these series of complexes compared to the derivative previously studied, $[Au(mnt)]^{-1}$.[49] Indeed, all new ligands have a strong electron donating effect that results in easier oxidation (by 0.75 to 1V) of the gold complexes to the neutral state. Besides this effect on the -1/0 oxidation potential, the electron donating character of the ligand makes all the investigated complexes more difficult to reduce to the Au(II) species than $[Au(mnt)]^{-1}$.

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Table 1. Redox potentials of complexes **1-6** (E in V *vs*. SCE) in CH_2Cl_2, NBu_4PF_6, Pt, scan rate 100 mV.s^{-1}.

Complexes	$E_{1/2}^{-2/-1}$	$E_{1/2}^{-1/0}$	$E_{pa2}/E_{pc2}^{0/+1}$
$[Au(Et\text{-}thiazdt)_2]^{-1}$ **1**	-0.90**	0.55/0.49*	0.71/0.61*
$[Au(Pr\text{-}thiazdt)_2]^{-1}$ **2**	-0.85**	0.56/0.53*	0.73/0.64
$[Au(EtOH\text{-}thiazdt)_2]^{-1}$ **3**	-0.99**	0.47/0.35*	0.67/0.46*
$[Au(cPr\text{-}thiazdt)_2]^{-1}$ **4**	-0.95**	0.52/0.46*	0.65**
$[Au(\alpha\text{-}tb\text{-}tpdt)_2]^{-1}$ **5**	-	0.35	0.96/0.89
$[Au(\alpha\text{-}db\text{-}tpdt)_2]^{-1}$ **6**	-	0.32	0.92/0.85
$[Au(mnt)_2]^{-1(a)}$	-0.705	1.325**	

* Adsorption ** Irreversible process. (a) the redox potentials were given in V vs Fc,[65] E in V vs SCE were estimated by adding +405 mV to the given redox potentials.

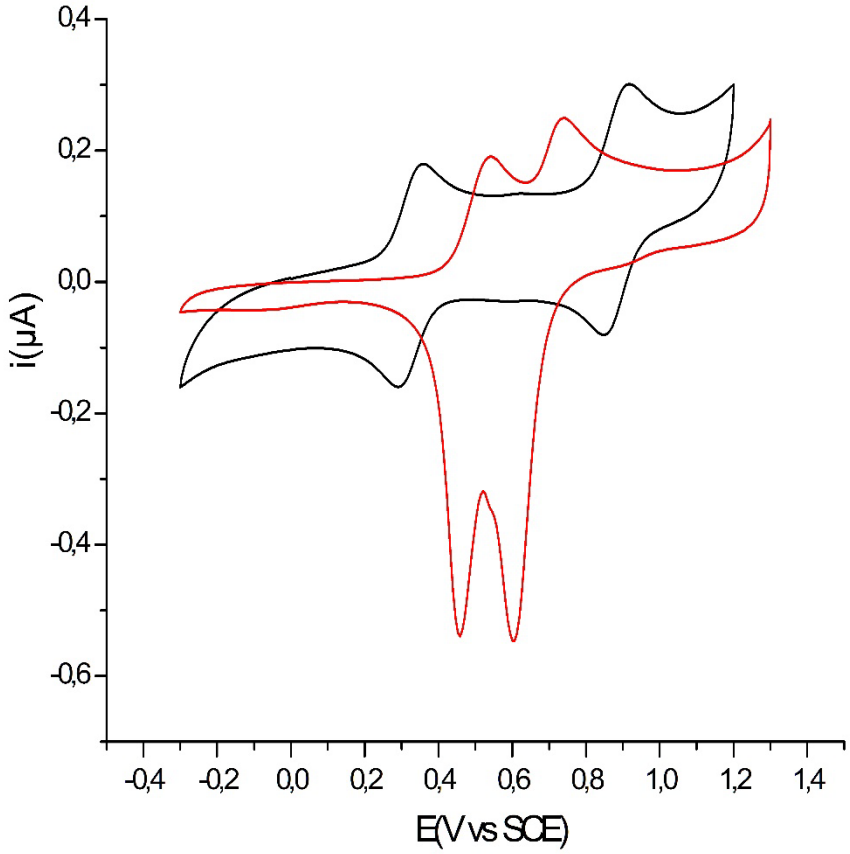


Figure 1. Cyclic voltammograms of $[Au(Et\text{-}thiazdt)_2]^{-1}$ **1** (red) and $[Au(\alpha\text{-}db\text{-}tpdt)_2]^{-1}$ **6** (black) in 0.1 M Bu_4NPF_6/CH_2Cl_2, scan rate 100 mVs^{-1}.

Anticancer activity of the gold complexes

The anticancer activity of complexes **1-6** was evaluated in the A2780 and A2780cisR ovarian cancer cells. Cells were exposed to increasing concentrations of the complexes (10^{-8} - 10^{-4} M) for 24 and 48 h, at 37°C. The IC_{50} values were calculated from dose response curves using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Results show that at 48 h incubation the complexes displayed high cytotoxic activity for both cancer cell lines ranging from 0.4 - 1.3 µM (A2780) and 0.08 - 2.0 µM (A2780cisR). Interestingly, these gold complexes **1-6**, with electron donating ligands, display higher cytotoxic activity than the previously studied gold complexes with electron withdrawing dithiolene ligands (0.9 - 4.4 µM (A2780) and 1.3 - 5.5 µM (A2780cisR)).[49] Remarkably, the gold complexes exhibited a more favorable cytotoxic profile than cisplatin and, most importantly, were able to overcome resistance to this reference chemotherapeutic drug in the resistant A2780cisR cell line. In relation to auranofin, the cytotoxic activity of the gold complexes, in particular **3**, **4** and **5**, was found to be similar to this reference drug (Table 2).

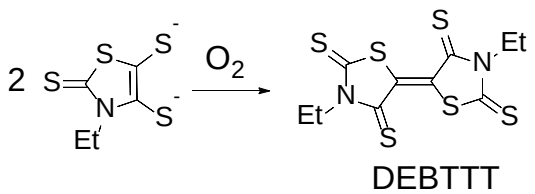
All compounds were also evaluated against normal fibroblasts in order to determine their selectivity for cancer cells, in particular against the ovarian cancer cells used in this study. The results obtained showed that the gold complexes were more active against the ovarian cancer cells than the normal fibroblasts, presenting selectivity indexes (SI) of 4-7 for **1-4**, 2 for **5** and **6** and ≤ 2 for cisplatin and auranofin. Clearly, these gold complexes can be divided into two groups: those having SI close to the reference drugs (**5** and **6**) and those having a higher selectivity against cancer cells (**1-4**) which constitutes a clear advantage of these gold compounds relative to the reference drugs.

Table 2. IC_{50} values (µM) calculated for the gold complexes **1-6** and the reference drugs on the ovarian cancer cells (A2780 and A2780cisR) and normal fibroblasts (V79) upon 24 and 48 h incubation. Results are shown as the mean ± SD of two independent experiments done with six replicates.

Complexes	**A2780**	**A2780cisR**	**V79**

	24 h	48 h	24 h	48 h	24 h	48 h
1	4.23±0.85	1.30±0.34	1.46±0.88	1.36±0.27	12.2±2.5	5.40±1.09
2	4.60±1.15	0.52±0.23	3.81±1.07	1.96±0.50	5.36±0.9	3.05±0.70
3	0.42±0.01	0.44±0.05	0.54±0.02	0.66±0.15	3.60±0.8	3.27±1.15
4	4.38±0.07	0.52±0.15	1.00±0.38	0.77±0.18	13.6±5.5	3.39±0.96
5	3.90±2.7	0.70±0.10	0.93±0.06	0.08±0.02	4.34±1.8	1.34±0.80
6	5.24±1.2	1.24±0.29	4.80±1.0	0.96±0.37	10.3±2.0	2.75±0.91
Cisplatin	----	3.60±1.25	----	35.8±13.5	----	6.70±2.70
Auranofin	----	0.43±0.27	----	0.52±0.17	----	0.66±0.35

It has been previously demonstrated that if decoordination of the dithiolene ligand occurs in **1**, the free ligand can be easily oxidized into an organic acceptor, the DEBTTT, as depicted in Scheme 2.[66] Thus, DEBTTT's activity against the A2780 cells after 48 h treatment was tested, in order to rule out the possibility that, if formation of this species was occurring, it was contributing to the anticancer activity observed for complex **1.** The oxidized form of the free ligand of complex **1** was found to be nontoxic in A2780 cells (IC_{50} values > 100 μM), which demonstrates that the relevant cytotoxic effects observed for the complex could be ascribed to the coordination of gold with the two dithiolene ligands.



Scheme 2. Oxidation of the ligand to give DEBTTT.

Complexes **1-6** are known to be redox active molecules which can be oxidized into the neutral gold complexes.[50-53] Therefore, we also analysed the cytotoxicity of the correspondent neutral gold complexes. However, these neutral complexes presented low

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activity in accordance with their poor solubility even in DMSO. For that reason, they were excluded from this study.

Antimicrobial activity of the gold complexes

The antimicrobial properties of complexes **1-6** were assessed based on the determination of the MIC values towards the gram-positive *S. aureus* Newman, the gram-negative *E. coli* ATCC25922, and the fungal strains *C. glabrata* CBS138 and *C. albicans* SC5134, using the microdilution method. The final concentrations of DMSO used to dissolve the compounds under study did not affect the growth of bacterial or fungal strains (data not shown).

Complex **1** was able to inhibit *S. aureus* Newman growth, with an estimated MIC value of 12.1 μg/mL. The other five complexes tested did not inhibit the growth of the *S. aureus* strain tested for concentrations ≤ 125 μg/mL. However, at 125 μg/mL, complex **3** reduced 87% of the *S. aureus* Newman growth. Contrastingly, no *E. coli* growth inhibition was detected for all the compounds tested at concentrations up to 125 μg/mL. At concentrations higher than 125 μg/mL the compounds precipitated during the assay and the results were thus not considered.

As a control, the antibacterial activity of auranofin was evaluated. Auranofin was very active in inhibiting the growth of the Gram-positive *S. aureus* Newman, with a MIC of 0.2 μg/mL (Table 3). In contrast, the MIC value for the Gram-negative *E. coli* ATCC25922 was 35.6 μg/mL (Table 3). These results are in accordance with previous reports describing a high potency of auranofin against Gram-positive clinical isolates tested, with inhibitory activities in the range 0.12 – 2 μg/mL and higher MIC values for Gram-negative bacteria (> 16 μg/mL),[67,68] and suggest a higher antibacterial activity of complex **1** towards Gram-positive bacteria.

Table 3. Estimated minimum inhibitory concentration (MIC) towards the gram-positive bacteria *S. aureus* Newman, the gram-negative *E. coli* ATCC25922, and the *Candida glabrata* and *C. albicans* fungal strains. Results are shown as the mean ± SD of three independent experiments done with two replicates.

	MIC (μg/mL)

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Compound	*S. aureus* Newman	*E. coli* ATCC25922	*C. glabrata* CBS138	*C. albicans* SC5134
1	12.1 ± 3.9	> 125	9.7 ± 2.7	19.9 ± 2.4
2	> 125	> 125	> 125	> 125
3	> 125	> 125	> 62.5	> 62.5
4	> 125	> 125	> 62.5	> 62.5
5	> 62.5	> 62.5	> 31.3	> 31.3
6	> 125	> 125	> 62.5	> 62.5
Auranofin	0.2	35.6 ± 0.5	15.3 ± 0.4	7.9 ± 0.6

We have also assessed the antimicrobial activities of the gold(III) complexes against the pathogenic strains *C. glabrata* CBS138 and *C. albicans* SC5134 (Table 3). The results obtained show that complex **1** was able to inhibit these fungi, with MIC values of 9.7 and 19.9 μg/mL, respectively. The other five compounds tested were unable to inhibit growth of these *Candida* strains for concentrations below those indicated in Table 3. Higher concentrations were not tested since the final concentrations of DMSO necessary to solubilize the complexes were higher than 1%, which has been reported to affect fungal growth.[69] These results indicate that complex **1** has the highest antifungal activity towards the *Candida* clinical isolates. As a control, the antifungal activity of auranofin was also tested against the clinical *Candida* spp. isolates. Auranofin was very active in inhibiting the growth of the *Candida* spp. strains tested, with the MIC ranging from 7.9 to 15.3 μg/mL (Table 3). These results are in good agreement with a recent study on auranofin activity against several clinical *Candida* spp. isolates.[70]

Among the complexes tested, all the $[Au(R\text{-thiazdt})_2]^{-1}$ complexes, **1-4**, present similar redox activities [50-52] while the ones with a thiophene backbone are easier to oxidize.[53] The fact that only complex **1** is the most efficient of the series suggests that the antibacterial and antifungal activity is not uniquely dependent on the redox properties of these complexes. The complexes differ in their geometry and in the presence of substituents of variable bulkiness on the ligands. The lack of bulky substituents facilitates intermolecular interactions between the complexes and molecular

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components of target cells. Indeed, all these complexes are sulfur-rich molecules, especially $[Au(R\text{-}thiazdt)_2]^{-1}$ complexes **1-4**, and intermolecular chalcogen·chalcogen interactions, S···S, are susceptible to occur.[50-52] In this sense, complex **1** is the less sterically hindered of the whole series, which could contribute to its higher effectiveness.

The MIC values determined for complex **1** towards *S. aureus*, *C. albicans* and *C. glabrata* (16.3 ± 5.3, 26.1 ± 3.2, and 13.1 ± 3.6 µM, respectively) are similar or higher than the IC_{50} values determined for normal fibroblasts (12.2 ± 2.5 and 5.4 ± 1.09 µM, at 24 and 48 h, respectively). These results indicate that additional chemical modifications should be introduced in complex **1,** in order to either lower its cytotoxicity or increase its antimicrobial activity.

Antiplasmodial activity of the gold complexes

The hepatic stage of *Plasmodium* infection is a privileged target for antimalarial intervention strategies, not only because of its asymptomatic nature, but also because the liver can serve as a reservoir for dormant parasite forms that may lead to relapses after the elimination of the initial blood infection.[71] Primaquine (PQ) is the only clinically approved drug known to eliminate liver forms of *Plasmodium* and to prevent relapse of malaria and, for that reason, it was used as a positive control in this study.[72] Although the mechanism of action of this drug remains to be fully elucidated, it has been attributed in part to the reduction of PQ active metabolites by the liver cytochrome P450 NADPH oxidoreductase (CPR), leading to the generation of hydrogen peroxide, and consequent killing of the parasite.[73,74] However, PQ often induces severe side effects, associated to its toxic metabolites, and causes complications such as hemolytic anemia.[75]

As depicted in Figure 2, the six compounds evaluated in these assays, in particular **2**, **4** and **6**, as well as auranofin, were active against *P. berghei* infection at 1 µM. At 10 µM the effect observed was masked by the cytotoxicity of the compounds against the Huh7 hepatic cells, as observed by the decreased confluency of cells treated with this concentration of the compounds, when compared to that of cells incubated with the drug vehicle control (0.1% DMSO).

The gold compounds displayed better antiplasmodial activity than PQ, which was inactive at 1 µM and partially active at 10 µM. Comparing complexes **1**, **2** and **3**, which

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bear different alkyl chain lengths, the one having the shorter alkyl chain length had the lowest activity on Huh7 cells (**1<2<3**). The cytotoxicity of the complexes also increased in the same order, thus diminishing their potential to reduce infection without compromising the viability of the hepatic host cells.

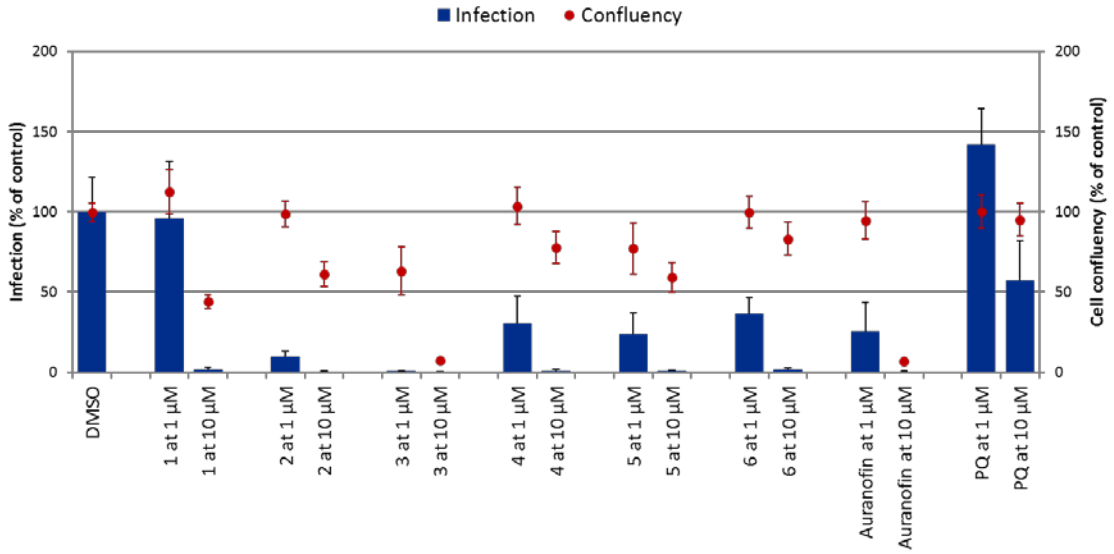

Figure 2. – *In vitro* activity of gold(III) bis(dithiolene) complexes against *P. berghei* hepatic stages. Huh7 cells were infected with luciferase-expressing *P. berghei* sporozoites in the presence of 1 and 10 µM of each compound or in the presence of equivalent amounts of DMSO, employed as a negative control. Primaquine (PQ) was used as a positive control. Infection load (bars) and cell confluence (dots) were assessed at 48 h post infection. Results are shown as the mean ± SD from two independent experiments.

Mechanistic insights

The mode of action of cytotoxic gold complexes, even for the most promising ones, remains to be fully elucidated. Gold complexes show a much lower affinity for DNA than cisplatin, and are often found to overcome resistance to cisplatin. Therefore, it has been assumed that the apoptotic cell death observed would result from DNA-independent processes, suggesting a mode of action substantially distinct from that of cisplatin. Instead of targeting the DNA most studies of gold compounds have revealed that mitochondria is the most likely biological target among several cellular organelles, as cancer cells present highly activated mitochondrial activity. However, the molecular mechanism of gold compounds towards mitochondria is deeply dependent on the nature

of the ligands. For instance, gold complexes bearing sulphur-containing ligands possess a higher association with cellular components that are rich in sulfhydryl groups like thioredoxin reductase (TrxR).[34,76]

Inhibition of thioredoxin reductase (TrxR) by the gold complexes

Cancer cells often overexpress thiol-dependent antioxidant systems such as the thioredoxin and thioredoxin reductase (TrxR) that control the intracellular redox balance. Gold, as well as other transition metals, is a selective inhibitor of thioredoxin reductase, binding with high affinity to the Cysteine (Cys) or Selenocystein (Sec) residues of the two catalytically important redox sites of the enzyme. Accordingly, several different classes of gold complexes, including auranofin, are recognized as potent inhibitors of this enzymatic system.[76-78]

The effect of the gold complexes studied in this work on the activity of TrxR was measured by a colorimetric DTNB assay. This assay is based on the reduction of DTNB (5,5′ dithiobis(2-nitrobenzoic acid)) into TNB (5-thio-2-nitrobenzoic acid) with the concomitant oxidation of NADPH, in a reaction catalyzed by TrxR. The reduction produces a strong yellow color that is measured at 412 nm.[79] Table 4 show the IC_{50} values obtained for the complexes tested in a concentration range of 0.1 nM - 20 µM. The inhibitory effect of complexes **1-3** were similar, with low IC_{50} values (0.2 - 0.4 µM), suggesting TrxR as a possible biological target of these complexes. On the contrary, complexes **5** and **6** showed a weak inhibition of TrxR, for the range of concentrations used. As expected, auranofin displayed the lowest IC_{50} value, one order of magnitude lower than complexes **1-3**. Altogether, these results support the idea that the thioredoxin system is a possible target for some of the gold complexes under study. Indeed, all of these complexes are sulfur-rich molecules, especially $[Au(R\text{-thiazdt})_2]^{-1}$ complexes (**1-4)**, and interactions with the two redox sites of the enzyme are susceptible to occur. Therefore, in the case of complexes **1-4**, the peripheral thione group can potentiate S···S short interactions, while in the case of complexes **5** and **6**, the bulkier substituents may prevent intermolecular interactions.

Table 4. Inhibition of TrxR (IC_{50} values) by the gold complexes **1-6** and auranofin. Data are shown as the mean ± SD of two independent experiments done with four replicates for each complex concentration.

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Complexes	IC_{50} (µM)
1	0.39 ± 0.13
2	0.54 ± 0.15
3	0.18 ± 0.08
4	2.13 ± 1.11
5	28.0 ± 9.90
6	24.3 ± 10.1
Auranofin	0.016 ± 0.006

Cellular uptake of the gold complexes

The uptake of selected gold complexes, and of the reference drug auranofin, was evaluated with the aim to correlate the cytotoxic activity against the ovarian cancer cells with the elemental contents (Au) in bulk cell pellets. The A2780 cells were used to assess the uptake of four complexes, **1**, **2, 3** and **4**, and auranofin at equimolar concentrations of 50 µM for 2 h.

A high uptake of complexes **1**, **2** and auranofin by A2780 cells was observed that contrasted with a low uptake of complexes **3** and **4** as is depicted in Figure 3. These results highlight two features: i) the magnitude of uptake of complex **3**, which showed high cytotoxic activity against A2780 cells (comparable to auranofin), was low; ii) the Au concentration in auranofin treated cells was comparable to the less cytotoxic complexes **1** and **2**. These findings point out that the Au concentration values did not seem to follow the same trend as the cytotoxicity estimations, assessed by the MTT assay (Table 2). However, comparing the uptake/IC_{50} ratios, complex **3** and auranofin were clearly discriminated from the remaining complexes: **4** (0.05) < **1** (0.33) ≤ **2** (0.30) < **3** (1.7) < auranofin (3.4). This tendency compares to the cytotoxic activity found for the complexes and reference drug auranofin against A2780 cells. In addition, the cellular uptake of complexes **1**, **2**, and **3** follows a similar trend to the one found for their TxrR inhibition, as can be inferred from data on Table 4 and Figure 3. The low uptake of complex 4 by A2780 cells is in line with its low cytotoxicity and low TxrR inhibition.

These results suggest that different parameters may be influencing the interaction of the different complexes with cells, such as the time required for the complex to interact

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with the cell (rate of uptake of the compound) and/or the differential cellular components involved in the capture of the complexes.

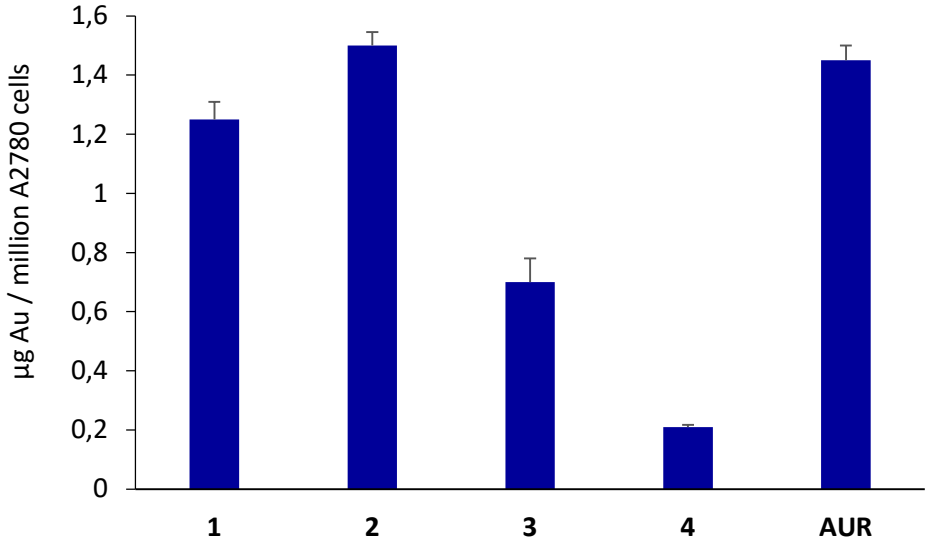

Figure 3. Total cellular accumulation of gold in the A2780 cells upon treatment with 50 µM of each compound for 2 h. Values are the mean ± SD of two independent experiments.

Interactions of gold complexes with CT-DNA

Fluorescence quenching of EB-DNA by gold complexes

Fluorescence spectroscopy is a technique currently used to explore the interactions between small molecules and biomolecules, such as DNA and proteins. The fluorescent DNA probe ethidium bromide (EB) was used to characterize the interaction of the four gold complexes (**1-4**) with CT-DNA, since under our experimental conditions no fluorescence emission was displayed by the gold compounds and DNA. The fluorophore EB is widely used since when it binds to DNA, it shows an intense emission band as the result of its strong intercalation between DNA base pairs.[80]

Probe displacement studies with the four gold complexes were carried out using constant DNA and EB concentrations and varying the metal complex concentration. The effect of selected gold complexes **1-4** on the fluorescence intensity of the {EB-DNA} adduct is shown in Figure 4. The fluorescence intensity of the {EB-DNA} adduct showed only a slight decrease upon the increase on the concentration of complexes **1** and **2**, while for complexes **3** and **4** this decrease was more pronounced. Upon addition of complexes **1-4** at final concentrations of 40 µM, the fluorescence intensity of the

{EB-DNA} adduct decreased by 1.5%, 7.6%, 17.6% and 40.8%, respectively. The observed quenching suggests that complexes **2**, **3** and **4** can displace the intercalated EB from its DNA binding site. The quenching observed for complex **1** was considered negligible. It is worth mentioning that the small differences on the complexes structures led to great differences in their ability to displace EB. Furthermore, the addition of complex **4**, at a final concentration of 40 μM, red-shifted the maximum wavelength of the {EB-DNA} by 7 nm.

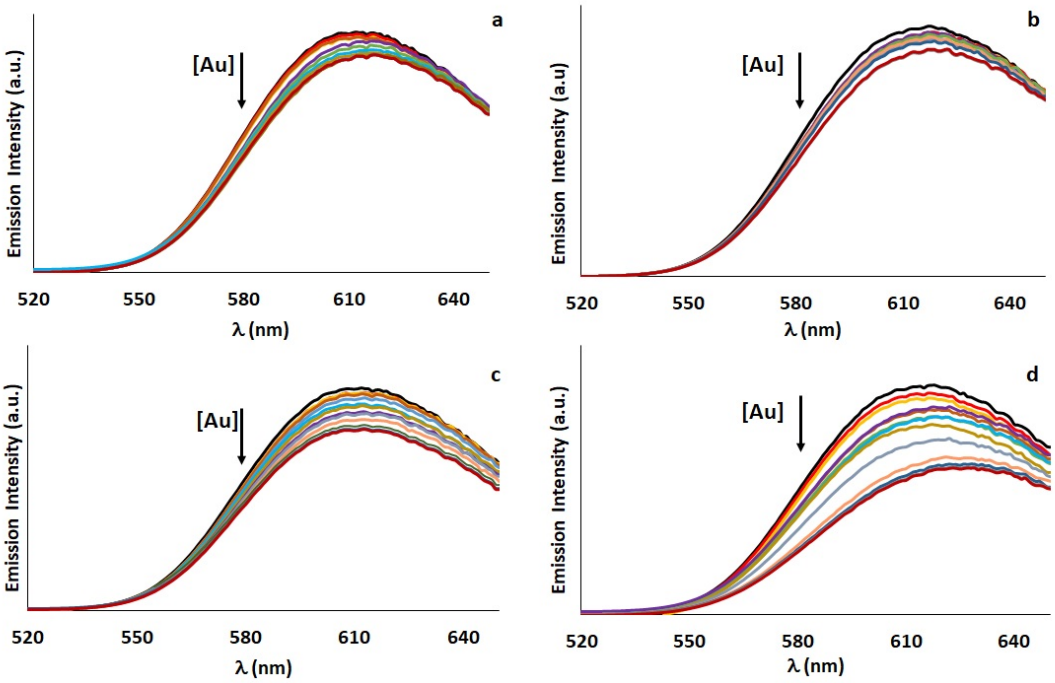

Figure 4. – Fluorescence emission spectra of EB bound to DNA in the absence (black lines) and presence of Au compounds a) **1**; b) **2**; c) **3** and d) **4**. Experimental conditions: [DNA] = 80 μM; [EB] = 20 μM; [Au] = 0 – 40 μM; 10 mM Hepes buffer, pH 7.4; incubation at 37°C during 24 h; λ_{exc} = 510 nm.

As mentioned above, the main difference between all these four complexes is related to the steric strain hindrance generated by the R side chain (R = Et, Pr, EtOH, cPr). For the complexes **1-3**, all the alkyl chains are flexible (R = Et, Pr, EtOH) and the difference observed for **3** compared to **2** could be tentatively assigned to the hydroxyl group and its high potential to generate intermolecular interactions by hydrogen bonding.[52] On the other hand, the cyclopropyl group in complex **4** provides additional constraints compared with the flexible alkyl chains since it is located above the plane of the

complex, preventing close intermolecular interactions and generating additional steric hindrance.[51]

Complexes-DNA binding constants

The mechanism of fluorescence quenching can be described by means of the following Stern-Volmer equation.[64]

$$\frac{I_0}{I} = 1 + k_q \tau_0 [Q] = 1 + k_{SV}[Q] \qquad (1)$$

where I_0 and I are defined as the emission intensity in the absence and presence of a quencher, respectively, and [Q] is the concentration of the quencher. In this study, compounds **2-4** were used as a quencher. K_q is the bimolecular quenching rate constant; τ_0 is the lifetime of the fluorophore in the absence of quencher and K_{SV} is the Stern–Volmer quenching constant whose values where obtained from the slopes of the plots of I_0/I *vs.* [Q]. Figure 5 depicts the plots of I_0/I *vs.* [Q] for the competition studies between compounds **2-4** with EB.

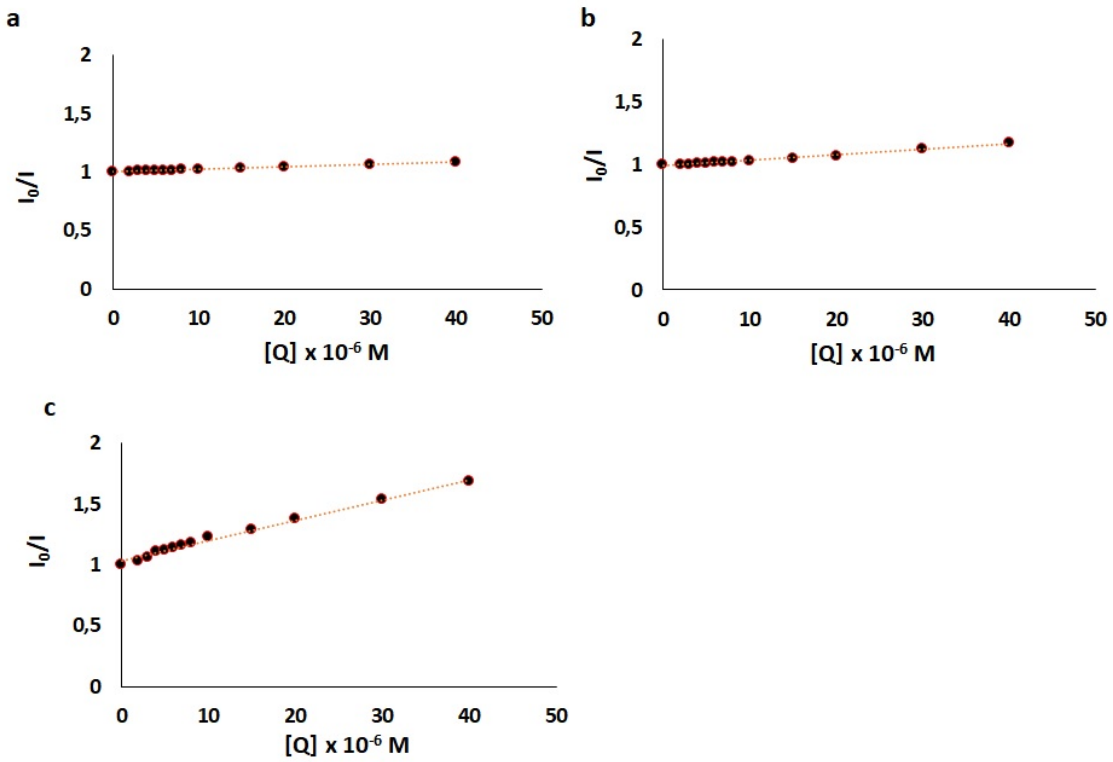

Figure 5. – Stern-Volmer plots for complexes a) **2**; b) **3** and c) **4**.

The binding constants determined from Stern-Volmer equation, presented in Table 5, indicate that the complexes studied have a weak capacity to substitute EB from the [EB-DNA} adduct.

Table 5. - Stern–Volmer constant (Ksv), bimolecular quenching rate constant (kq), and correlation coefficient (R) for the quenchers **2**, **3** and **4**.

Compound	K_{SV} (x 10^3 $Lmol^{-1}$)	K_q (x 10^{11} $Lmol^{-1}s^{-1}$)	R^2
2	2.1 ± 0.02	2.1 ± 0.02	0.9919
3	4.3 ± 0.01	4.3 ± 0.01	0.9912
4	16.8 ± 0.02	16.8 ± 0.02	0.9904

Stability studies in solution by UV-visible spectroscopy

The stability in solution of complex **1** was assessed by UV-visible spectroscopy along the time up to 48 h. The spectra of **1** was recorded in DMSO and in colourless MEM medium in the absence and presence of fetal bovine serum (FBS). Results from this study suggested that complex **1** (Figure 6, a-c) is very stable in DMSO, except in MEM medium in the absence of FBS, after t0, where a slight shift and a decrease intensity of the maxima are observed. In medium with 10% FBS, to simulate the conditions of the cellular studies, the solutions of **1** remained translucent at least until 48 h, with no visible precipitation. The stability of **1** in the presence of cellular antioxidants glutathione (GSH) and ascorbate (AsA) was also evaluated. Both reducing agents were assayed at 100 µM in the presence of the gold complex at 1:1 molar ratio. Results presented in Figure 6 d) and e) suggested that neither GSH nor AsA are able to reduce the gold complex, since no shape modification nor maximum shift of the band at 365 nm was observed.

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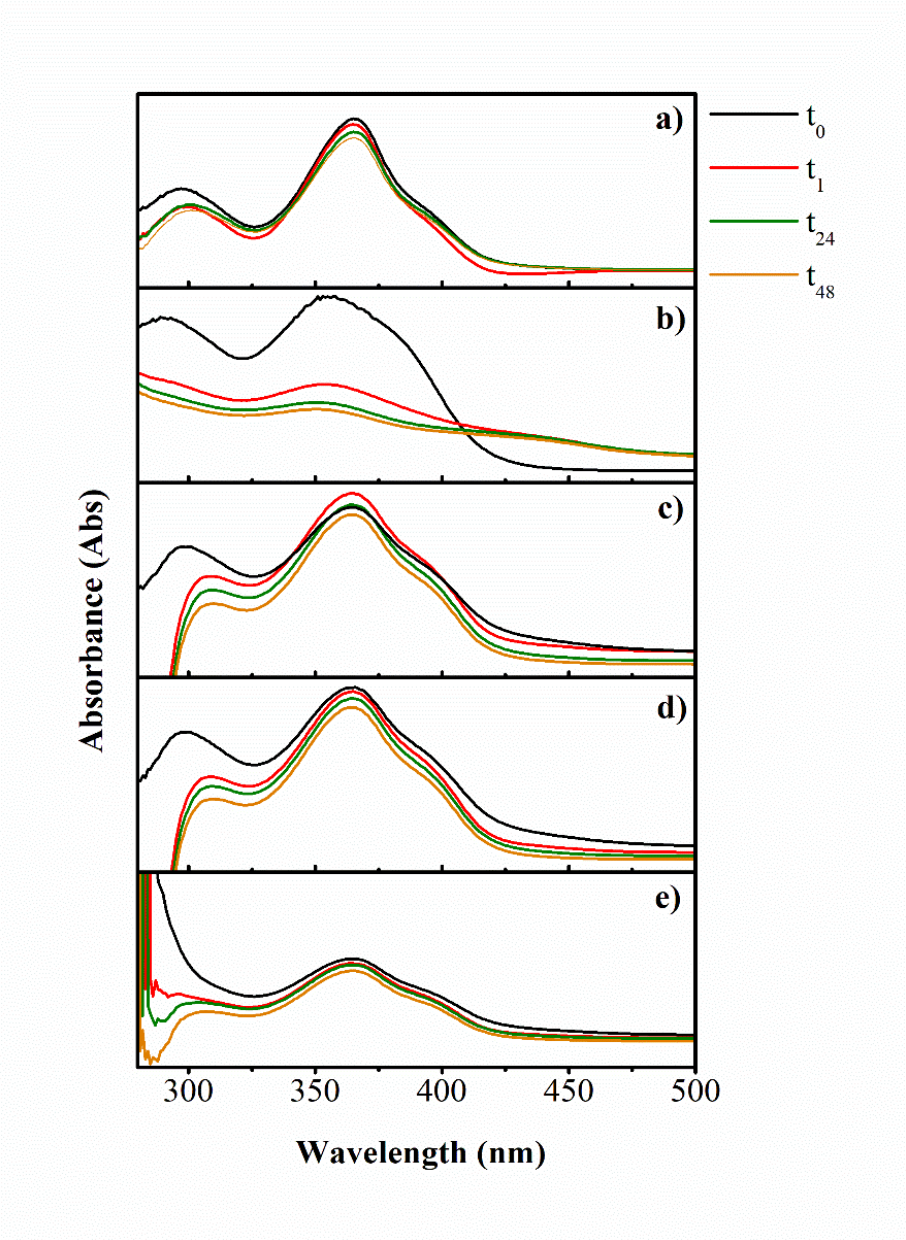

Figure 6. Time-dependent UV-vis spectra of complex **1** in DMSO (a), in the cellular MEM medium (b), in the MEM medium in the presence of FBS (c), in the MEM medium in the presence of FBS with GSH (d) and in the MEM medium in the presence of FBS with AsA (e) at 0 h (t_0), 1 h (t_1), 24 h (t_{24}) and 48 h (t_{48}). The concentration of **1** and the antioxidants was 100 µM.

Conclusions

The present study reports the screening of 6 gold(III) bis(dithiolene) complexes for their potential anticancer, antimicrobial and antiplasmodial activities. This is the first report

dealing with this type of complexes as antiplasmodial agents. Although the synthesis and structures of this class of monoanionic compounds has already been described for their importance as precursors of neutral molecular conductors and magnetic materials, their antiplasmodial activity has not been assessed before, and studies on their antimicrobial and anticancer properties are scarce. These monoanionic gold complexes present some similarities such as their redox activity, as they can all be oxidized to the neutral species. These complexes also share the feature of being composed of heteroatoms like sulfur, not common, which are able to participate in chalcogen interactions. The main structural differences among the complexes under study correspond to the alkyl groups grafted on their backbones, resulting in distinct steric hindrances and different abilities to establish intermolecular interactions. Another difference relies on the aromatic character of the thiophene dithiolene ligand (tpdt) at variance with the non-aromatic character of the thiazoline dithiolene ligand (thiazdt).

The cytotoxic properties of this class of gold complexes have attracted attention only recently. In particular, gold(III) dithiolenes can be considered an emerging class of metal complexes with potential antitumor properties alternative to cisplatin due to their exceptional cytotoxic properties, involving both common and specific mechanisms. In addition, they can reduce the susceptibility to opportunistic microbial infections, since they also present antimicrobial activity. This work also highlights the importance of this type of complexes as prospective precursors for the development of antimalarial drugs.

Both experimental and clinical studies support the notion that TrxR status has a close relationship to the onset and development of several diseases including cancer. Therefore, there is an increasing interest in the development of novel TrxR modulators. Results from this work also highlight the relevance of gold(III) bis-dithiolenes as promising TrxR inhibitors. In summary, the gold complexes presented herein exhibit the ability to overcome resistance on cisplatin-resistant cells, presenting lower cytotoxicity compared to auranofin in noncancerous cells, and possessing the potential to selectively inhibit thiol-containing enzymes, such as TrxR. Complex **1**, in particular, displays a favourable biological profile that warrants its further exploitation as a drug lead for the development of novel molecules with antitumor, antimicrobial and antiplasmodial activities, as alternatives to current therapeutics.

Conflict of interest

The authors declare no conflict of interest.

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Acknowledgements

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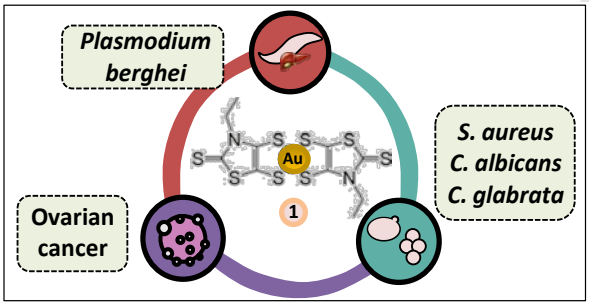

Graphical Abstract (synopsis).

Gold(III) bis(dithiolene) complexes were evaluated as therapeutic drugs. [Net4][Au(Et-thiazdt)$_2$], **1**, exhibit interesting anticancer, antibacterial, antifungal and antimicrobial properties.

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