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A gold(I) phosphine complex selectively induces apoptosis in breast cancer cells: Implications for anticancer therapeutics targeted to mitochondria

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ABSTRACT

Bis-chelated gold(I) phosphine complexes have shown great potential as anticancer agents, however, their efficacy has been limited by their high toxicity and lack of selectivity for cancer cells. Here, we have investigated the anticancer activity of a new bis-chelated Au(I) bidentate phosphine complex of the novel water soluble ligand 1,3-bis(di-2-pyridylphosphino)propane (d2pypp). We show that this gold complex [Au(d2-pypp)$_2$]Cl, at submicromolar concentrations, selectively induces apoptosis in breast cancer cells but not in normal breast cells. Apoptosis was induced via the mitochondrial pathway, which involved mitochondrial membrane potential depolarisation, depletion of the glutathione pool and caspase-3 and caspase-9 activation. The gold lipophilic complex was accumulated in mitochondria of cells, driven by the high mitochondrial membrane potential. To address the molecular basis of the observed selectivity between the two cell lines we investigated the effect of the gold complex on the thioredoxin/thioredoxin reductase system in normal and cancer breast cells. We show that [Au(d2pypp)$_2$]Cl inhibits the activities of both thioredoxin and thioredoxin reductase and that this effect is more pronounced in the breast cancer cells. This difference may account for the selective cell death seen in the breast cancer cells but not in the normal cells. Our investigation has led to new insights into the mechanism of action of bis-chelated gold(I) diphosphine complexes and their future development as mitochondria targeted chemotherapeutics.

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Abbreviations: $\Delta\psi_m$, mitochondrial membrane potential; DLC, delocalized lipophilic cation; MPT, membrane permeability transition; Auranofin, 2,3,4,6-tetra-O-acetyl-l-thio-β-D-glucopyranosato-S-triethylphosphine gold(I); TrxR, thioredoxin reductase; Trx, thioredoxin; [Au(dppe)$_2$]Cl, bis[1,2 bis(diphenylphosphino)ethane]gold(I) chloride; [Au(d2pypp)$_2$]Cl, bis[1,3-bis(di-2-pyridylphosphino)propane]gold(I) chloride

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1. Introduction

Gold(I) phosphine compounds have shown early promise as anticancer drugs [1] and can be divided into two distinct classes: neutral, linear, two-coordinate complexes such as triethylphosphine gold(I) chloride and the tetracetylthioglucose derivative, 'auranofin' and cationic, tetrahedral bis-chelated Au(I) diphosphine complexes such as $[Au(dppe)_2]Cl$ (Fig. 1A). Although evidence suggests that the mechanism of antitumor activity of the two classes is different, they both cause mitochondrial dysfunction that leads to cell death [2,3]. A mechanism has been proposed recently for induction of the membrane permeability transition (MPT) by auranofin due to alteration of the thiol redox balance in cells [4,5]. Studies with isolated purified cytoplasmic and mitochondrial thioredoxin reductase (TrxR), enzymes that regulate the cellular redox state, suggest a mechanism of action for auranofin that involves the inhibition of TrxR by binding of Au(I) to the selenocysteine residue at the active site of the enzyme [6–8]. The high affinity of auranofin towards protein thiols has, however, limited its antitumor activity in vivo, with modest activity demonstrated in only one mouse tumor model [9].

It was with this aim of reducing the reactivity with thiols, to obtain a broader spectrum of antitumor activity, that lead to early investigations of Au(I) complexes with chelated diphosphine ligands and the discovery of the antitumor activity of $[Au(dppe)_2]Cl$ [10] and other bis-chelated Au(I) diphosphine complexes [1,11]. These bis-chelated Au(I) diphosphine complexes have unusually low thiol reactivity and, while the cytotoxic mechanism is unknown, their behaviour is consistent with that of other delocalized lipophilic cations (DLCs) which exhibit antitumor activity [12]. Lipophilic cations can pass easily through the lipid bilayer and their positive charge then directs them to the mitochondria at significantly higher concentrations than in the cytoplasm, because of the large negative potential across the inner membrane generated by the respiratory chain [13]. DLCs have been investigated as a new approach to cancer chemotherapy which exploits their selective accumulation in mitochondria of cancer cells as a consequence of the elevated mitochondrial membrane potential ($\Delta\psi_m$) that is a shared feature for many tumor cell lines [14–16].

While early studies showed that $[Au(dppe)_2]Cl$ had significant antitumor activity in vivo [10] its clinical development was halted by the discovery of severe hepatotoxicity in dogs attributable to mitochondrial dysfunction [17,18]. The high lipophilicity of $[Au(dppe)_2]^+$ results in its non-selective concentration into mitochondria causing general membrane permeabilization. Consequently, our more recent approach has been to modify the diphosphine ligands to reduce the lipophilicity and improve the selectivity for cancer cells [19]. Replacing the phenyl substituents with pyridyl groups (with the N atom in either the 2, 3 or 4 position in the ring), provided a series of compounds of type $[Au(dnpype)_2]Cl$ (Fig. 1A) that are structurally similar to $[Au(dppe)_2]Cl$, in which the hydrophilic character covers a very large range. These complexes have been evaluated in subcutaneous colon 38 tumors in mice and whereas the most lipophilic and hydrophilic complexes had no significant tumor growth delay, the 2-pyridyl complex, $[Au(d2pype)_2]Cl$, with intermediate lipophilicity, showed significant antitumor activity which correlated with highest drug concentrations in plasma and tumor tissue [19]. In addition,

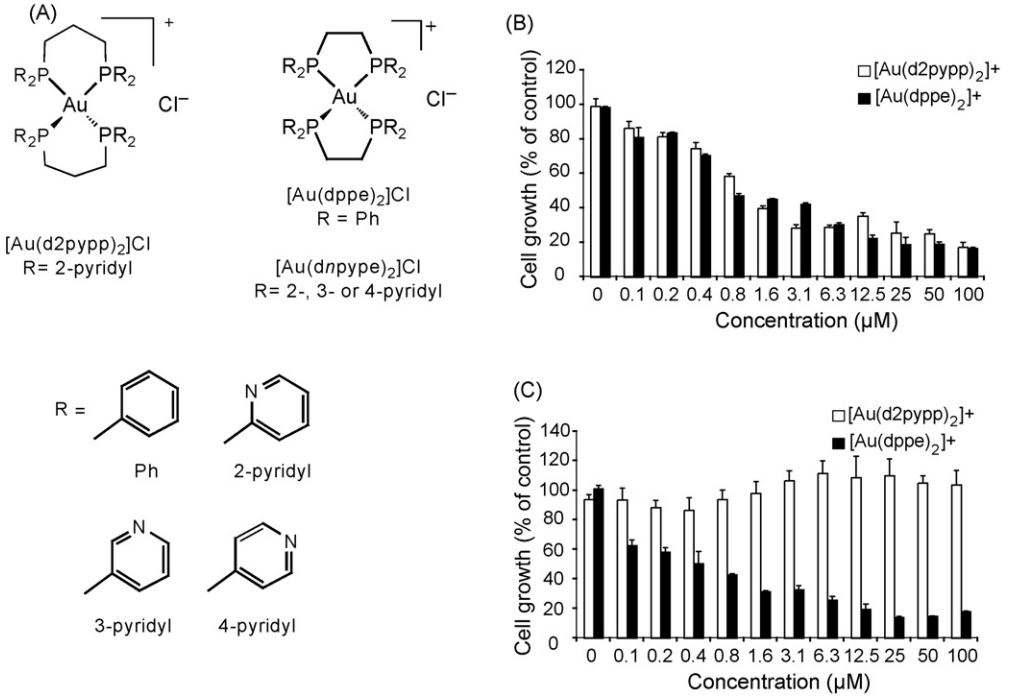

Fig. 1 – (A) Chemical structure of Au(I) complexes with bidentate ligands, $[Au(d2pypp)_2]Cl$, $[Au(dppe)_2]Cl$ and its pyridylphosphine analogs; MDA-MB-468 cells (B) and HMEC cells (C) were treated with increasing concentrations of either $[Au(d2pypp)_2]Cl$ or $[Au(dppe)_2]Cl$ and cell growth was measured after 24 h using a cell titer assay. Data are means ± S.D. of four separate experiments.

this compound has been shown to induce the MPT in isolated mitochondria [2]. These results suggest that by fine-tuning the hydrophilic–lipophilic balance in the optimal range, it may be possible to identify compounds of this class that are able to target the mitochondria of tumor cells with great specificity. However, direct evidence for the accumulation within mitochondria of cells has not been demonstrated to date.

This work investigates the anticancer activity of a new bis-chelated Au(I) complex of the novel water soluble bidentate pyridylphosphine ligand 1,3-bis(di-2-pyridylphosphino)propane (d2pypp) [20]. This compound $[Au(d2pypp)_2]Cl$ (Fig. 1 A) was designed with the idea of combining the features of the two classes of Au(I) phosphines by retaining the lipophilic cationic properties of the tetrahedral bis-chelated complexes that allow accumulation into mitochondria, but enhancing the reactivity towards thiols and selenols that is characteristic of auranofin. The propane bridged complex $[Au(d2pypp)_2]Cl$ has a lipophilicity in the optimal range close to that of the ethane-bridged analog $[Au(d2pype)_2]Cl$, but ligand exchange reactions with thiols/selenols will be more facile, compared to $[Au(dppe)_2]Cl$ and its pyridylphosphine analogs, due to the increased chelate ring size.

Here we show that $[Au(d2pypp)_2]Cl$ is selectively toxic to breast cancer cells but not normal breast cells resulting in programmed cell death mediated via mitochondria. In addition, we have investigated the mechanism for selective toxicity of this complex to breast cancer cells compared to normal cells. Our findings indicate that mitochondria and the thioredoxin system may be the critical targets responsible for the selective toxicity seen in the breast cancer cell line. Together these data provide a new insight into the mechanism of anticancer activity of bis-chelated Au(I) diphosphine complexes.

2. Materials and methods

2.1. Materials

$[Au(d2pypp)_2]Cl$ was synthesized and characterized as recently reported elsewhere [20] and 10 mM stock solutions were prepared in water. The parental compound $[Au(dppe)_2]Cl$ was synthesized and stock solutions prepared according to the published procedures [21,19]. Thioredoxin (human and *Escherichia coli*), thioredoxin reductase (rat) and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) were from Sigma. The fluorogenic substrate 7-amino-4-methylcoumarin-labelled peptide (DEVD-AMC) was obtained from Calbiochem. Annexin V-FLUOS was from Roche Applied Sciences. The caspase-9 colorimetric kit, JC-1 dye, cell culture media and supplements were obtained from Invitrogen.

2.2. Cell culture

A human breast adenocarcinoma cell line MDA-MB-468 (ATCC) was cultured at 37 °C under humidified 95% air/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) without phenol red, containing Earle's balanced salt solution and supplemented with 2 mM Glutamax, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% heat inactivated fetal calf serum (FCS). Human mammary epithelial cells (HMEC, Cambrex) were cultured at 37 °C under humidified 95% air/5% CO_2 in MCDB 170 medium supplemented with MCDB supplement, penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}). To test toxicity, cells were grown to 90% confluence and incubated for 24 h with their growth medium containing increasing concentrations of the gold compounds. Necrotic cell death was determined by the release of lactate dehydrogenase (LDH) in a BMG Labtech Fluostar Optima plate reader as described before [22]. For cell growth studies, cells grown in 96-well plates were incubated for 24 h with 100 μl of their growth medium containing increasing concentrations of $[Au(d2pypp)_2]Cl$ or $[Au(dppe)_2]Cl$. Cell viability was determined using the cell titer assay following the manufacturer's instructions (Promega, G3580). The assay was performed by adding 20 μl of the CellTiter 96® AQueous One Solution Reagent directly to culture wells, incubating for 1 h and then recording the absorbance at 490 nm. The data is expressed as percent of cells grown in the absence of gold compounds. Data are means ± S.D. of four independent experiments.

2.3. Mitochondrial isolation

Mitochondria were prepared from 10^7 cells grown overnight in 15 cm^2 dishes. Cells in PBS were sedimented (150 × *g* for 5 min at 4 °C), resuspended in 4 ml of ice-cold STE buffer (250 mM sucrose, 5 mM Tris–HCl, 1 mM EGTA, pH 7.4) and disrupted with a 7 ml glass homogenizer. The nuclei were sedimented (1000 × *g* for 5 min at 4 °C), the pellet washed and the combined supernatants were centrifuged (10,000 × *g* for 10 min at 4 °C). The mitochondrial pellet was washed once with STE and mitochondrial protein concentrations were determined by the bicinchoninic acid (BCA) assay [23] using bovine serum albumin (BSA) as a standard.

2.4. Measurement of mitochondrial membrane potential

For membrane potential measurements 10^5 cells per well were plated in black 96-well tissue culture plates. The following day the growth medium was removed, the cells were washed twice with medium free of FCS. Increasing concentrations of $[Au(d2pypp)_2]Cl$ were added and incubated up to 6 h. At the end of the incubation the medium was removed and 50 μl of staining solution containing 33 μM JC-1 dye in FCS-free medium was added to each well. For control incubations 50 μM FCCP was added to the staining solution. The cells were incubated for 1 h in the cell incubator, then the staining solution was removed and 200 μl of PBS containing 5% BSA was added to each well. After a further incubation for 5 min the PBS/BSA solution was removed and 100 μl of PBS was added to each well. The ratio of red to green signal was measured in a BMG Labtech Fluostar Optima plate reader ($\lambda_{Excitation}$ = 485 nm, $\lambda_{Emission\ green}$ = 520 nm, $\lambda_{Emission\ red}$ = 590 nm).

2.5. Glutathione recycling assay

Proteins were precipitated from washed cells with 5% sulfosalicylic acid and GSH released into the supernatant was assayed by the recycling assay and compared with GSH

standards (0–0.7 nmol) as described previously [24]. The recycling assay was done using a 96-well plate and consisted of 13.5 μl of sample or standard, mixed with 295 μl of 0.5 mM DTNB, 0.3 mM NADPH in 85 mM NaPi, 3.7 mM EDTA, pH 7.5, and 1 unit of glutathione reductase (Sigma). The formation of thionitrobenzoic acid was measured at 405 nm over 10 min using a kinetic plate reader.

2.6. Apoptosis assays

Apoptosis was induced by incubating MDA-MB-468 and HMEC cells with $[Au(d2pypp)_2]Cl$ or $[Au(dppe)_2]Cl$ (1 or 5 μM). For measuring apoptosis by caspase-3 activation, 10^5 cells were plated overnight in 24-well tissue culture plates, and then incubated with gold compounds for up to 6 h. At the end of the incubation the cells were washed with PBS and lyzed in 50 μl lysis buffer (50 mM HEPES, 10% sucrose, 0.1% Triton X-100, 10 mM dithiothreitol (DTT) pH 7.5) for 20 min on ice. The lysates were clarified by centrifugation (10,000 × *g* for 10 min) and supernatant aliquots (50 μl) were transferred to 96-well plates and incubated with 50 μl of 50 μM DEVD-AMC in 150 μl lysis buffer. Caspase-3 activity was measured fluorometrically by the release of fluorescent AMC upon cleavage of the peptide in a BMG Labtech Fluostar Optima plate reader ($\lambda_{Excitation}$ = 355 nm, $\lambda_{Emission}$ = 460 nm) and calibrated using an AMC standard curve. Caspase-9 activity was measured using a caspase-9 colorimetric kit according to the manufacturer's instructions. To measure apoptosis by annexin V-fluorescein isothiocyanate staining, 10^6 cells grown in 6-well tissue culture plates were incubated in the presence or absence of 5 μM $[Au(d2pypp)_2]Cl$ at 37 °C for up to 6 h and at the end of the incubation were collected by trypsin treatment and centrifugation (1000 × *g* for 5 min). The cells were resuspended in 1 ml of binding buffer (10 mM HEPES/NaOH pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 4% BSA) containing 1.25 μl annexin V-fluorescein isothiocyanate (200 μg/ml) and incubated for 15 min at room temperature in the dark. The cells were collected (1000 × *g* for 5 min) and gently resuspended in 0.5 ml binding buffer containing 10 μl propidium iodide (30 μg/ml). Cells undergoing apoptosis were quantitated using a Becton Dickinson FACS Canto flow cytometer.

2.7. Measurement of mitochondrial uptake

Confluent cells grown on 15 cm^2 dishes were incubated with $[Au(d2pypp)_2]Cl$ (5 μM) at 37 °C for 1 h. At the end of the incubation the cells were washed, pelleted following trypsin treatment and their mitochondria were isolated by the procedure described above. The mitochondrial pellets were resuspended in water (350 μl) and samples (300 μl) were digested in 70% HNO_3 (500 μl) at 70 °C for 2 h. The samples were then diluted 1:10 in water for inductively coupled plasma mass spectrometry (ICP-MS). Gold analysis was carried out using an Agilent 7500cs ICP-MS with a micromist concentric nebulizer, and a double-pass spray chamber maintained at 2 °C. Gold was read at up to 1 ng/ml and the sensitivity limit of detection was 10 pg/ml (95% confidence limit). Protein concentrations of cell fractions were determined by the BCA assay using BSA as a standard.

2.8. Thioredoxin and thioredoxin reductase assays

Fluorescence measurements of reduced thioredoxin ($Trx(SH)_2$) were obtained using a 3 ml stirred cuvette in a Cary Eclipse fluorometer with excitation and emission slit widths of 3 nm. Thioredoxin was used at 1 μM concentration in 3 ml of 50 mM Tris–HCl, 1 mM EDTA, pH 7.5 and was mixed with increasing concentrations of $[Au(d2pypp)_2]Cl$ (1–50 μM) at room temperature for 3 h. For scanning spectra, $\lambda_{Excitation}$ was 290 nm and the $\lambda_{Emission}$ was scanned from 300 to 500 nm, with a fast scan rate and a sampling interval of 1 nm. There was no fluorescence from the buffer under these conditions.

The activity of thioredoxin reductase was determined using the insulin assay according to the published procedure [25]. A mixture of 0.2 μM TrxR and 100 μM NADPH in 50 mM Tris–HCl, 1 mM EDTA, pH 7.5 buffer was pre-incubated at room temperature for 5 min, $[Au(d2pypp)_2]Cl$ was then added and the incubation continued for 2 h. Control incubations were carried out in the absence of the Au(I) compound. To assay enzyme activity, the incubation mixture was added to a cuvette containing 1 mg/ml insulin, 100 μM NADPH, and 10 μM *E. coli* Trx. The decrease in absorbance was recorded at 405 nm with a blank reference using a Beckman UV–vis spectrophotometer. TrxR activity alone was measured as described above in the absence of thioredoxin. TrxR activity was expressed as percent of control.

2.9. Inhibition of thioredoxin and thioredoxin reductase in cells

MDA-MB-468 and HMEC cells were incubated in the presence of either 5 μM $[Au(d2pypp)_2]Cl$ or $[Au(dppe)_2]Cl$ for 2 h at 37 °C, in an incubator with 90% humidified atmosphere containing 5% CO_2. Control incubations were carried out with the same volume of water. At the end of the incubation the cells were washed once in PBS and lyzed in 250 μl of lysis buffer (0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl in TE buffer) supplemented with protease inhibitors (complete, EDTA-free, Roche). The protein concentration of each sample was determined by the BCA assay using BSA as a standard and the Trx and TrxR activities were measured as described before [26]. Briefly, to measure Trx activity, a 50 μl volume corresponding to 10 μg protein from each cell lysate was incubated in 85 mM HEPES, 3 mM EDTA buffer, pH 7.6 containing 0.3 mM insulin, 660 μM NADPH and 50 nM TrxR for 20 min at 37 °C. The reaction was terminated by the addition of 200 μl of 1 mM DTNB in 6 M guanidine hydrochloride (pH 8.0). The blank sample contained everything except TrxR. To measure TrxR activity, the same mixture was prepared where instead of TrxR, 15 μM Trx was used for the experimental sample and this was omitted for the blank. The thionitrobenzoic acid absorbance was measured at 405 nm in a plate reader where the blank value was subtracted from the sample value and the activity was expressed as a percent of the control.

3. Results

To determine whether $[Au(d2pypp)_2]Cl$ can inhibit the growth of a highly tumorigenic breast cancer cell line, MDA-MB-468

cells were chosen as a model cell line. $[Au(d2pypp)_2]Cl$ inhibited more than 50% of MDA-MB-468 cell growth at concentrations as low as 0.8 μM and the profile of activity was similar to that of the parental compound, $[Au(dppe)_2]Cl$ (Fig. 1B). $[Au(d2pypp)_2]Cl$ inhibited the growth of other cancer cell lines at similarly low concentrations (supplementary data, Fig. S1). We compared the effects of these compounds on cell growth in normal human mammary epithelial cells (Fig. 1C). Whereas $[Au(dppe)_2]Cl$ inhibits the growth of these cells at similar concentrations to those seen for the MDA-MB-468 cells (Fig. 1B), HMEC cells treated with $[Au(d2pypp)_2]Cl$ have normal cell growth similar to untreated cells. These data show that $[Au(d2pypp)_2]Cl$ selectively inhibits the growth of MDA-MB-468 breast cancer cells but not the normal breast HMEC cells.

To determine the mechanism by which $[Au(d2pypp)_2]Cl$ causes selective inhibition of cell growth in the MDA-MB-468 cell line, we treated the breast cancer and normal cells with 5 μM concentration of the compound, which inhibits cell growth by more than 50%, and assessed cell death. The amount of LDH released over 12 h was measured as a marker for necrotic cell death, and the amount of caspase-3 activation as a marker of apoptosis (Fig. 2). $[Au(d2pypp)_2]Cl$ causes a marked increase in caspase-3 activation within the first 3 h of incubation with the cells and this event subsequently leads to LDH release and, therefore, cell death (Fig. 2A). In the HMEC cells there is no caspase-3 activation nor LDH release over the same period of time indicating that the effect of $[Au(d2pypp)_2]Cl$ is specific only for the MDA-MB-468 cell line (Fig. 2B). The parental compound, $[Au(dppe)_2]Cl$, is not selective for the breast cancer cell line causing necrotic cell death to both MDA-MB-468 and HMEC cell lines (Fig. 2C and D). In addition, $[Au(dppe)_2]Cl$ is highly toxic and causes LDH release within the first 3 h of incubation, suggesting that it bypasses the apoptotic pathway causing cell necrosis (Fig. 2C and D). These data suggest that $[Au(d2pypp)_2]Cl$ causes selective cell death through the apoptotic pathway in the MDA-MB-468 cells, but not in the HMEC cells.

We used flow cytometry to further investigate whether $[Au(d2pypp)_2]Cl$ leads to selective cell death in breast cancer cells by activating the apoptotic pathway. Adding 5 μM $[Au(d2pypp)_2]Cl$ to MDA-MB-468 cells led to induction of apoptotic cell death 3–6 h later as determined by measuring the proportion of annexin-positive cells (Fig. 3A). Apoptotic cell death was absent in HMEC cells treated with the same concentration of $[Au(d2pypp)_2]Cl$. Control experiments in the absence of the Au(I) compound showed a similar percent of annexin-positive cells in both cell lines, further indicating that $[Au(d2pypp)_2]Cl$ selectively causes apoptotic cell death in MDA-MB-468 cells but not HMEC cells (Fig. 3A).

To ascertain that $[Au(d2pypp)_2]Cl$ causes apoptosis only in the MDA-MB-468 cells we incubated both cell types with the Au(I) compound and measured the total glutathione pool by the glutathione recycling assay. MDA-MB-468 cells treated with 5 μM $[Au(d2pypp)_2]Cl$ show a marked decrease in the total glutathione pool after a 3 h incubation, which is consistent with the onset of apoptosis seen before with the caspase-3 activation and the proportion of annexin-positive cells. There was no change in the total glutathione pool in HMEC cells treated with $[Au(d2pypp)_2]Cl$, consistent with the lack of cell death seen previously (Fig. 3B).

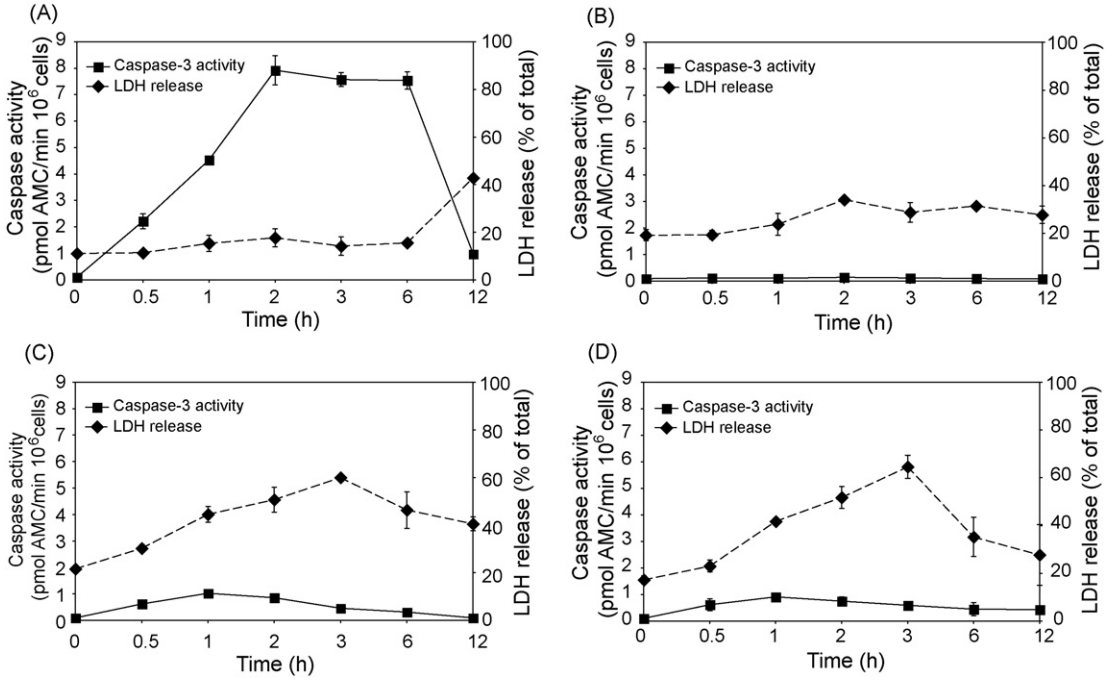

Fig. 2 – $[Au(d2pypp)_2]Cl$ compound induces cell death in cancer cells. MDA-MB-468 cells were treated with either 5 μM $[Au(d2pypp)_2]Cl$ (A) or 5 μM $[Au(dppe)_2]Cl$ (C) for the indicated times. HMEC cells were treated with either 5 μM $[Au(d2pypp)_2]Cl$ (B) or 5 μM $[Au(dppe)_2]Cl$ (D) for the indicated times. After each incubation, cell lysates were harvested and assayed for caspase-3 activity, measured as the rate of DEVD-AMC cleavage. Control incubations were carried out in the absence of Au(I) compounds. The cell medium and the cell lysates were harvested and LDH activity was measured in both. LDH activity is expressed as a percent of total activity. Data are means ± S.D. of four separate experiments.

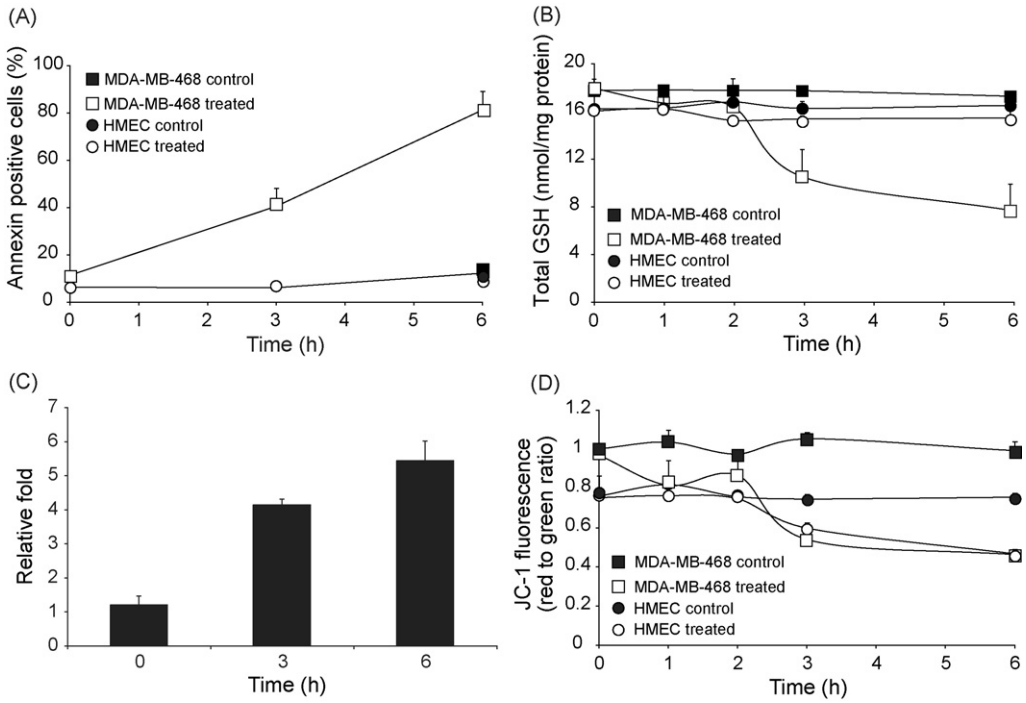

Fig. 3 – Induction of apoptosis by [Au(d2pypp)$_2$]Cl. MDA-MB-468 cells (squares) and HMEC cells (circles) were incubated with no additions (filled squares and circles) or with 5 μM [Au(d2pypp)$_2$]Cl (open squares and circles), then the cells were harvested at the indicated time points. (A) The proportion of annexin V-positive cells at various times after addition of 5 μM [Au(d2pypp)$_2$]Cl in cells was analyzed by flow cytometry. Control incubations in the absence of [Au(d2pypp)$_2$]Cl after 6 h are also shown. The figure shows a typical experiment repeated on three separate cell preparations; (B) total GSH was measured by the glutathione recycling assay in cells treated with 5 μM [Au(d2pypp)$_2$]Cl at the indicated time points. Control measurements in the absence of the compound are also shown. Data are means ± S.D. of three separate experiments; (C) caspase-9 activity was measured using a caspase-9 colorimetric kit in MDA-MB-468 cells treated with 5 μM [Au(d2pypp)$_2$]Cl at the indicated time points. Data are expressed as relative fold of activity compared to control measurements in the absence of the compound and are means ± S.D. of three separate experiments; (D) the effect of 5 μM [Au(d2pypp)$_2$]Cl on $\Delta\psi_m$ in cells was measured using the fluorescent dye JC-1 and expressed as a ratio of red to green fluorescence. Measurements in the absence of [Au(d2pypp)$_2$]Cl are shown. Data are means ± S.D. of four separate experiments.

To determine whether [Au(d2pypp)$_2$]Cl causes apoptosis in the MDA-MB-468 cells via mitochondria we incubated these cells with the Au(I) compound and measured caspase-9 activity. MDA-MB-468 cells treated with 5 μM [Au(d2pypp)$_2$]Cl show a four-fold increase in caspase-9 activity after a 3 h incubation and a five-fold increase in caspase-9 activity after a 6 h incubation suggesting that the Au(I) compound induces apoptosis via mitochondria (Fig. 3C).

DLCs have been shown to dissipate the mitochondrial membrane potential ($\Delta\psi_m$) upon uptake into mitochondria. We have previously shown that the octanol/water partition coefficient of [Au(d2pypp)$_2$]Cl (log $P = -0.41$) [20] lies within the optimal range derived from predictive models for the selective accumulation of DLCs in cancer cells based on lipophilicity [27,28]. The $\Delta\psi_m$ of both HMEC and MDA-MB-468 cells was measured in the absence and presence of [Au(d2pypp)$_2$]Cl using the JC-1 dye. The $\Delta\psi_m$ of both cell lines dissipated over time in the presence of 5 μM [Au(d2pypp)$_2$]Cl, consistent with the uptake of the compound into mitochondria of the cells (Fig. 3D). This decrease suggests that this gold compound has an uncoupling effect on the $\Delta\psi_m$ in both cell types, due to its lipophilicity. Control incubations in the absence of [Au(d2pypp)$_2$]Cl show that MDA-MB-468 cells take up more JC-1 than the HMEC cells suggesting a difference in their $\Delta\psi_m$ (Fig. 3D).

MDA-MB-468 cells were treated with increasing concentrations of [Au(d2pypp)$_2$]Cl and the amount of gold present in mitochondria isolated from these cells was measured by ion coupled plasma mass spectrometry (ICP-MS). The gold found within mitochondria of cells increases with increasing concentrations of [Au(d2pypp)$_2$]Cl (Fig. 4A). At a concentration of 5 μM > 60% of the measured gold is found within mitochondria of cells and this accumulation is driven by the $\Delta\psi_m$ (Fig. 4B). Dissipating the $\Delta\psi_m$ with the uncoupler FCCP, shifts the distribution of the gold to the cytoplasm with only ~20% still remaining in mitochondria (Fig. 4B). To determine the gold distribution within mitochondria we separated them further into membrane and soluble fractions and found that ~40% of the gold was associated with the soluble fraction (Fig. 4C).

A variety of gold compounds including the Au(I) phosphine complex auranofin have been shown to be potent inhibitors of both cytosolic and mitochondrial TrxR [6–8]. The inhibition of thioredoxin reductases is thought to be the key factor for the induction of apoptosis by auranofin and, therefore, it may be

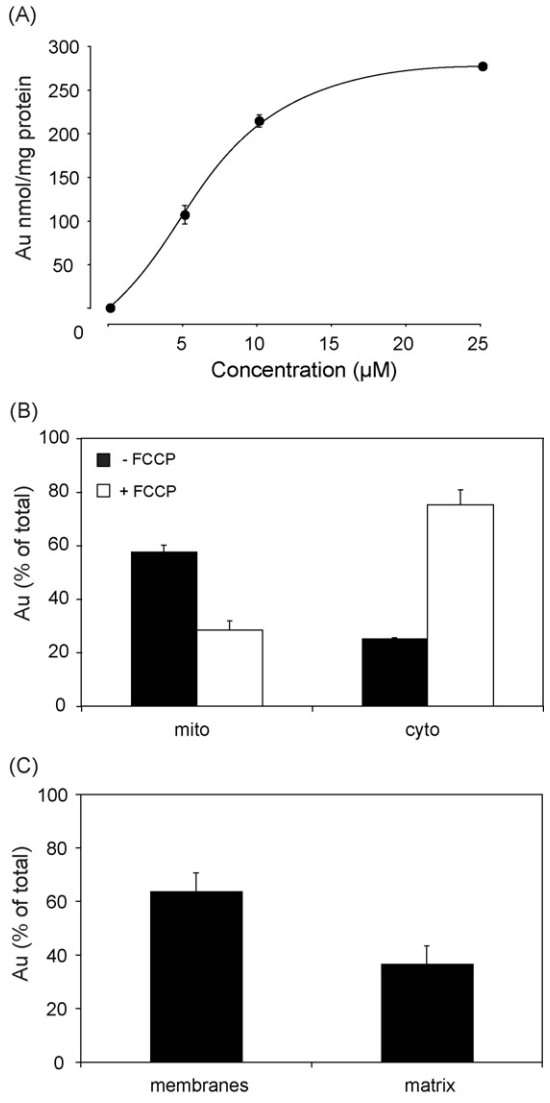

Fig. 4 – [Au(d2pypp)$_2$]Cl uptake into mitochondria within cells. Cells, or mitochondria and mitochondrial fractions, isolated from cells treated with [Au(d2pypp)$_2$]Cl were incubated with 70% nitric acid at 70 °C for 2 h, diluted 10-fold in water and analyzed for gold content by inductively coupled plasma mass spectrometry (ICP-MS). (A) Au(I) uptake in mitochondria of cells treated with increasing concentrations of [Au(d2pypp)$_2$]Cl; (B) Au(I) distribution in mitochondria and cytoplasm of cells treated with 5 μM [Au(d2pypp)$_2$]Cl for 1 h at 37 °C. Control experiments were carried out in the absence of $\Delta\psi_m$ (+FCCP). (C) Au(I) distribution in mitochondrial membrane and soluble fractions. Data are means ± S.D. of two separate experiments.

an important protein target for [Au(d2pypp)$_2$]Cl [29]. The activity of TrxR was lowered to 50 and 5%, respectively, following a 20 min incubation with [Au(d2pypp)$_2$]Cl at concentrations of 1 and 5 μM (Fig. 5A). TrxR recycles Trx to its reduced form, which in turn reduces oxidized protein thiols. To date there appear to be no reported studies on the inhibition of Trx by gold compounds. To investigate whether [Au(d2pypp)$_2$]Cl can inhibit Trx by binding to the active site cysteines we followed the quenching of the fluorescence of tryptophan residues that occurs upon oxidation of Trx(SH)$_2$ to Trx(SS). Thioredoxin gives rise to a distinctive fluorescence spectrum of reduced Trx between 300 and 400 nm that was largely abolished with the addition of 5 μM [Au(d2pypp)$_2$]Cl (Fig. 5B) and the subsequent addition of DTT could not restore the spectrum to that of the reduced protein (Fig. 5B). These data taken together suggest that Trx and TrxR may be potential targets for [Au(d2pypp)$_2$]Cl in cells.

To investigate whether [Au(d2pypp)$_2$]Cl can inhibit Trx and TrxR activity in cells, we treated HMEC and MDA-MB-468 cells with 5 μM [Au(d2pypp)$_2$]Cl for 2 h. We found that [Au(d2-pypp)$_2$]Cl inhibited Trx activity in both cell types, although over 80% of Trx activity was inhibited in the MDA-MB-468 cells compared to ~40% inhibition in the HMEC cells (Fig. 5C). The TrxR activity was also inhibited in the presence of [Au(d2-pypp)$_2$]Cl in both cell types, but to a lesser degree than Trx activity (Fig. 5D). The inhibition of TrxR activity was more pronounced in the cancer cells compared to the normal cells (Fig. 5D). These data demonstrate that [Au(d2pypp)$_2$]Cl can inhibit Trx and TrxR activities in cells.

4. Discussion

Bis-chelated Au(I) diphosphine complexes have shown reproducible antitumor activity in mouse tumor models in vivo [10,11], however, their development as anticancer therapeutics has been hindered by the lack of selectivity between tumor and normal cells. Following on from previous studies, we recently synthesized a new bis-chelated Au(I) complex of 1,3-bis(di-2-pyridylphosphino)propane [20], which has intermediate lipophilic and hydrophilic properties that are critical for improving the toxicity of this class of compounds by finding a balance between cell uptake and binding to extracellular proteins [19]. This new compound [Au(d2pypp)$_2$]Cl is selectively toxic to MDA-MB-468 breast cancer cells, but not to the normal HMEC breast cells. These results are consistent with previous studies on a series of related Au(I) complexes of ethane-bridged bidentate dipyridylphosphines where modification to the hydrophilic–lipophilic balance was shown to influence the cellular uptake, tumor selectivity and host toxicity [19,30]. The selective anticancer activity of [Au(d2-pypp)$_2$]Cl is consistent with trends derived from predictive models for the selective accumulation of DLCs in tumor cells based on their hydrophilic and lipophilic properties [27,28]. The non selective cytotoxicity of the parent compound [Au(dppe)$_2$]Cl has been reported before and is a consequence of its high lipophilicity that leads to generalized disruption of mitochondrial membranes [2]. To account for the selective toxicity of [Au(d2pypp)$_2$]Cl in breast cancer cells, we investigated the mechanism of toxicity and possible protein targets.

The neutral, linear two-coordinate Au(I) phosphine complex, auranofin has been shown to inhibit the growth of cancer cell lines by causing changes in the mitochondrial thiol redox pool that lead to apoptosis [4,5]. However, auranofin has limited anticancer activity in vivo and its toxicity to cultured cancer cells was reduced 10-fold in the presence of serum proteins, suggesting that activity is lost through binding to

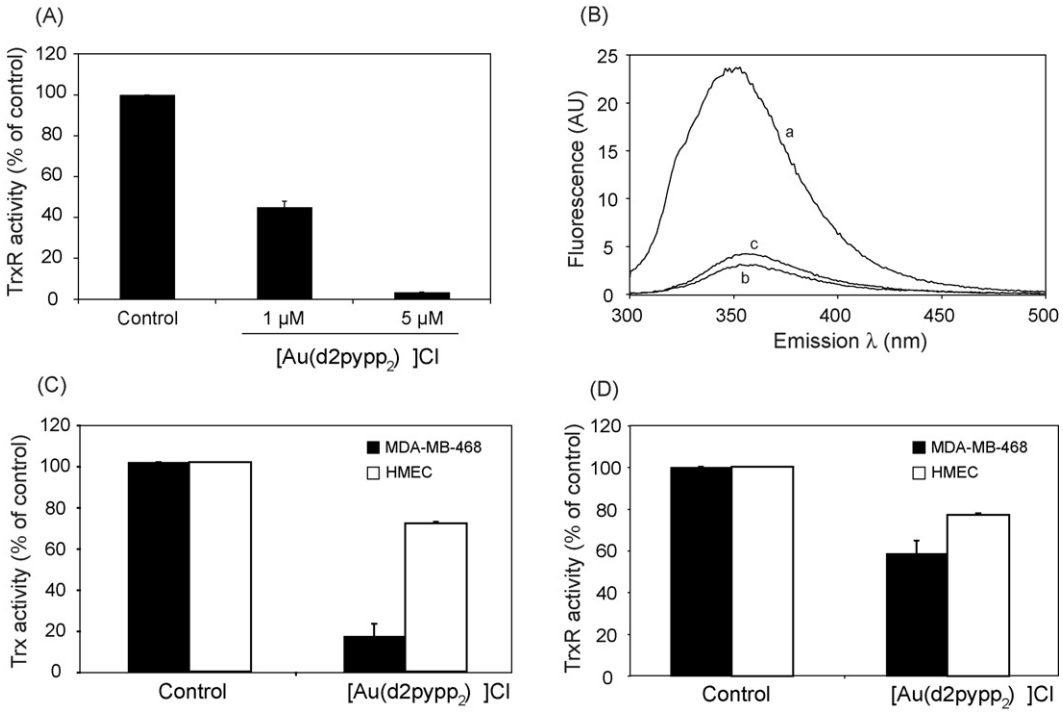

Fig. 5 – Thioredoxin and thioredoxin reductase inhibition of purified proteins and in cells. (A) The activity of purified TrxR enzyme was measured using the insulin assay. Control incubations was carried out in the absence of the Au(I) compound. TrxR activity was expressed as percent of control. Data are means ± S.D. of three separate experiments; (B) direct reaction of [Au(d2pypp)$_2$]Cl with thioredoxin. The fluorescence emission spectrum of 1 μM Trx(SH)$_2$ (trace a) was measured in 50 mM Tris–HCl, 1 mM EDTA, pH 7.5, 24 °C ($\lambda_{Excitation}$ = 290 nm); 5 μM [Au(d2pypp)$_2$]Cl was then added, and the spectrum was recorded 15 min later (trace (b)). A further 5 μM [Au(d2pypp)$_2$]Cl was added, and the spectrum was measured again (data not shown). DTT (10 mM) was then added, and the spectrum was reacquired (trace (c)). [Au(d2pypp)$_2$]Cl with and without 10 mM DTT did not fluoresce in this region. All spectra are corrected for background in the absence of Trx(SH)$_2$. Cells incubated with 5 μM [Au(d2pypp)$_2$]Cl for 2 h at 37 °C were lyzed at the end of the incubation and thioredoxin (C) and thioredoxin reductase activity (D) were measured and expressed as percent of control. Data are means ± S.D. of three separate experiments.

protein thiols [9]. The new bis-chelated Au(I) pyridyl phosphine complex [Au(d2pypp)$_2$]Cl causes apoptosis in a similar manner to auranofin [29], by caspase activation, loss of the total GSH pool and loss of mitochondrial membrane potential suggesting that its mode of action is through mitochondria.

Our data indicate that [Au(d2pypp)$_2$]Cl is taken up into mitochondria of cells driven by the $\Delta\psi_m$. This suggests that the lipophilic–cationic properties of the complex contribute significantly to its concentration within mitochondria. These findings are corroborated by a recent study by McKeage's group of [Au(d2pype)$_2$]Cl (the ethane-bridged analog of [Au(d2pypp)$_2$]Cl) which has been shown to accumulate preferentially in the mitochondrial fractions of cancer cells, driven by the $\Delta\psi_m$ (unpublished data, personal communication). A variety of DLCs have been shown to have anticancer activities in tumor cell lines in culture and when implanted in nude mice by selectively concentrating in mitochondria [31–33]. Although DLCs such as MKT-077 have proven highly toxic in clinical trials, the high selectivity for cancer cells of some DLCs, including our gold complex [Au(d2pypp)$_2$]Cl, suggest that targeting mitochondria of cancer cells may have significant anticancer therapeutic potential that needs to be further explored and developed.

Cytoplasmic and mitochondrial isoforms of TrxR and Trx play a major role in the regulation of the cellular redox state. An increase in Trx and TrxR activity has been correlated with evasion of apoptosis and acceleration of tumor growth [34,35]. In addition, the increased expression of Trx has caused resistance against the cytotoxic effects of some anticancer drugs and it is possible that Trx and TrxR could be a cause of resistance to chemotherapy [36]. The inhibition of the cellular activity of TrxR can lead to apoptosis of cancer cells [37,38], therefore, many emerging anticancer therapies use TrxR as a target for drug development [39–41]. Many compounds, including the Au(I) phosphine complex auranofin, have been shown to be potent inhibitors of purified TrxR enzymes, but only a few studies report its inhibition in cells [42,43]. Trx inhibitors are a more recent innovation and there appear to be no studies on the interaction of gold compounds with Trx. In this study, we have shown that [Au(d2pypp)$_2$]Cl can irreversibly modify Trx and effectively inhibit the activity of purified TrxR and is a significantly more potent inhibitor of Trx than TrxR activity in cells. Inhibiting the activity of Trx and TrxR by [Au(d2pypp)$_2$]Cl in the breast cancer cells may contribute to the onset of apoptosis because they play an important role in the regulation of cell growth.

In addition, the mitochondrial Trx/TrxR system maintains the redox state of mitochondrial protein thiols which is important for the control of the MPT [44]. Inhibition of this system may further contribute to the onset of apoptosis. We have shown that $[Au(d2pypp)_2]Cl$ inhibits Trx and TrxR activity more in the cancer cells than in the normal cells. This difference appears to be in part, a consequence of the increased uptake of the complex into the cancer cells, and in particular into their mitochondria driven by the high $\Delta\psi_m$ as shown with the fluorescent lipophilic dye, JC-1, which also accumulated more in the cancer cells. While Trx and TrxR are targets of $[Au(d2pypp)_2]Cl$, further studies may identify other protein targets that may contribute to its selective toxicity to breast cancer cells.

Previous studies of $[Au(dppe)_2]Cl$ and its pyridylphosphine analogs have demonstrated that these compounds have low reactivity towards protein thiols [9,19,45,46]. The potent inhibition of Trx and TrxR by $[Au(d2pypp)_2]Cl$ suggests a greater propensity to undergo ligand exchange reactions with thiols, consistent with a more facile ring opening of the six-membered chelate ring compared to the five-membered chelates of the parental compound $[Au(dppe)_2]^+$ and other analogs with ethane-bridged diphosphines. For example, NMR studies of Ag(I) complexes of 1,3-bis(diphenylphosphine)propane have shown that equilibria exist in solution between bis-chelated tetrahedral complexes and ring-opened species [47]. Overall the results of this study are consistent with the proposed model illustrated in Fig. 6 in which the reactivity towards protein thiols/selenols is fine-tuned, compared to that of auranofin allowing binding to fewer proteins with greater specificity. While the exact mechanism by which $[Au(d2pypp)_2]Cl$ interacts with Trx and TrxR needs to be further explored, the irreversible modification of Trx could be indicative of Au(I) binding to the active site cysteine residues with displacement of the phosphine ligands. In support of this model, a recent crystal structure of glutathione reductase (GR), an enzyme related to TrxR, modified with a Au(I)phosphole complex shows Au bound to the active center thiols with S–Au–S coordination in the inactive GR product [48].

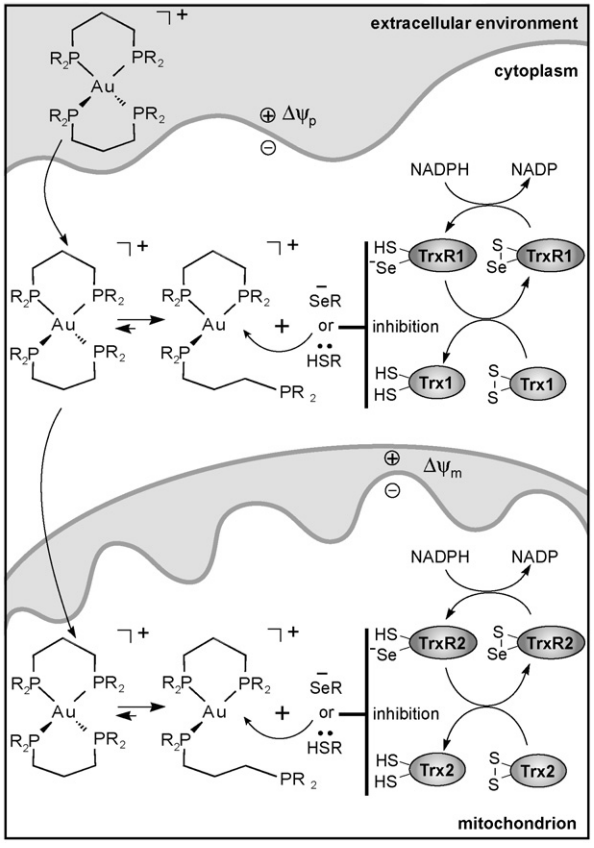

Fig. 6 – Schematic mechanism of $[Au(d2pypp)_2]Cl$ uptake and activity in cells. The gold complex can be selectively enriched in mitochondria driven by the mitochondrial membrane potential although a proportion remains in the cytoplasm. The gold complex can bind the cytoplasmic and the mitochondrial Trx and TrxR through a more facile ring opening of the six-membered chelate ring of this ligand contributing to enhanced reactivity towards the active site thiols and selenols of these proteins.

In summary, we have shown that by fine-tuning the lipophilicity of bis-chelated tetrahedral Au(I) diphosphine compounds we can selectively deliver them to mitochondria of cancer cells. In addition, the inhibitory effect of $[Au(d2pypp)_2]Cl$ on the Trx/TrxR system suggests that the affinity for thiols and selenols of this class of compound can be fine-tuned by manipulating the chelate ring size to control ligand exchange reactions at the Au(I) center. Our results may lead to new approaches for the development of mitochondria targeted chemotherapeutics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.07.022.

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