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A Sugar-Modified Phosphole Gold Complex with Antiproliferative Properties Acting as a Thioredoxin Reductase Inhibitor in MCF-7 Cells

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Cytosolic thioredoxin reductase (TrxR, EC 1.6.4.5) is a FAD-bound homodimeric oxidoreductase structurally and mechanistically related to glutathione reductase (GR, EC 1.8.1.7). TrxR uses NADPH as an electron donor, and is able to reduce a range of substrates including the physiological thioredoxin (Trx) according to equation (1).

$$Trx(S)_2 + NADPH + H^+ \rightarrow Trx(SH)_2 + NADP^+ \quad (1)$$

TrxR possesses a Cys–SeCys catalytic pair in the more flexible *C*-terminal domain, in addition to the *N*-terminal Cys–XXXX–Cys active site. Trx contains a Cys pair which, in a reduced state, is essential for reducing disulfide bridges critical for protein activity. TrxR and Trx form an important redox system involved in multiple physiological processes.[1] Reduced thioredoxin can provide reducing equivalents to a number of proteins including ribonucleotide reductase, which is involved in DNA synthesis and consequently in cell proliferation. The TrxR/Trx system is also involved in the cellular protection from or repair following oxidative stress, and in the regeneration of antioxidants such as vitamin C, ubiquinone, and lipoic acid.[2] Additionally, the TrxR/Trx system modulates the activity of transcription factors involved in various signaling pathways related to apoptosis and cellular response to growth factors.[3]

TrxR and Trx have been shown to be overexpressed in several tumor types, and increased levels are known to regulate both cancer cell growth and survival.[4] Furthermore, overexpression appears to be correlated with chemotherapeutic resistance, aggressive tumor types and poor prognosis.[5] Taken together, these observations present the TrxR/Trx system as a target of interest for the development of new anticancer agents. Several TrxR inhibitors have already been identified and have shown growth inhibition against tumor cells (for a recent review, see ref. [6]). Among those, metal–ligand complexes are of particular interest because of their combined abilities to irreversibly target the dithiol/selenol catalytic pair essential for TrxR activity and bind DNA.[7]

Nine compounds have been evaluated in the present study for their cytotoxic activities against the human breast cancer MCF-7 cell line using a flow cytometric analysis with 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and propidium iodide (PI) staining.[8] These compounds were selected or designed with the aim of targeting disulfide reductase activity known to be overexpressed in cancer. Analogues **1** and **2** (Figure 1) are metal-free phosphole ligands[9] and derivative **3** has shown slight cytotoxicity in B16 melanoma and P388 leukemia cells.[10] Phosphine-coordinated gold(I) thiosugar complexes such as auranofin (**4**) was developed for the treatment of rheumatoid arthritis, a disorder associated with TrxR overexpression, and reported to inhibit the purified TrxR from human placenta.[11] Phosphole-containing gold and platinum complexes **5**,[12] **6**,[13] **7** and GoPI (**8**, {1-phenyl-2,5-di(2-pyridyl)phosphole}AuCl) are irreversible inhibitors of purified human GR and TrxR.[14,15]

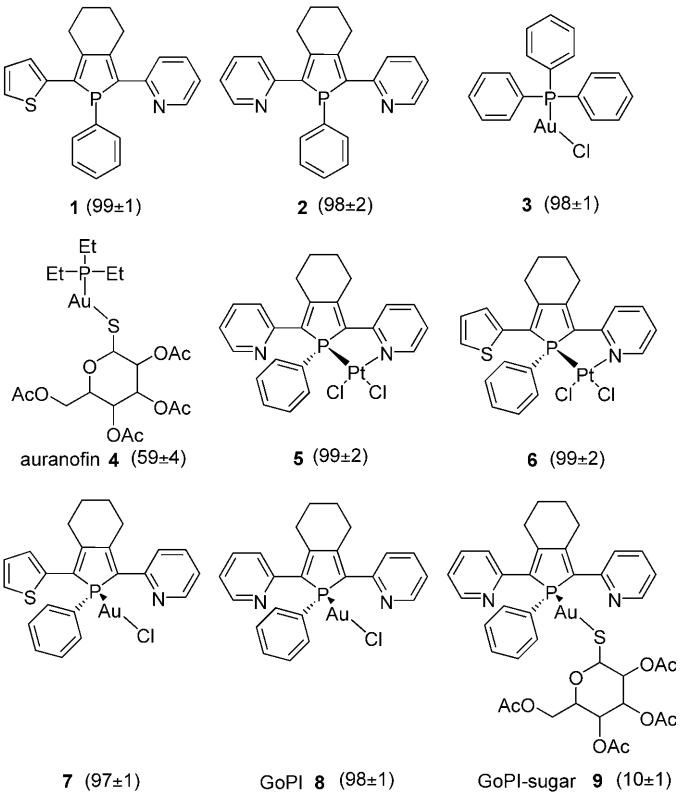

Figure 1. Structures of compounds **1**–**9** and the associated cell survival rates (percent ±SD compared to control) after exposure to the test compound (24 h, 3 μM) evaluated using flow cytometry.

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Complex **8** is a particularly potent inhibitor of these disulfide reductases, however, this compound is unstable; release of the chloride ligand and demetallation in aqueous solutions irrespective of any co-solvent (e.g. dioxan, cremophor, glycerol, ethanol) may preclude its clinical use. The GoPI–sugar derivative (**9**, 1-thio-β-D-glucopyranose 2,3,4,6-tetraacetato-S({1-phenyl-2,5-di(2-pyridyl)phosphole}gold)) is a novel analogue based on the structures of GoPI (**8**) and auranofin (**4**), in an attempt to improve upon the stability and bioavailability of complex **8**.[15] Notably, in contrast to GoPI, the sugar analogue **9** is, 1) very soluble in organic solvents including pentane and ether, 2) soluble in aqueous cremophor (10% *w*/*w*) up to 1.1 mM and 3) stable in these solvents and open to air for prolonged periods as indicated by ^{31}P NMR and UV-vis spectroscopy.

The tetra-acetylated thioglucose moiety of auranofin (**4**), the acetylated groups of which are likely to be intracellularly hydrolyzed, was shown to facilitate the permeability of the drug across the plasma membrane.[16] Auranofin undergoes sequential ligand exchange reactions with cellular thiols through a prodrug effect.[17] It is suggested that this exchange terminates when both thioglucose and triethylphosphine ligands have been displaced by the final dithiol target.[18] Derivative **9** is expected to undergo a similar ligand displacement process. This hypothesis was recently proven; formation of a Cys–Au–Cys coordinate bond was observed in the solved crystal structure of GoPI-alkylated human GR.[7]

A single dose application (3 μM, 24 h incubation) revealed the marked cytotoxicity of auranofin (**4**) and GoPI–sugar (**9**) against MCF-7 cells, with cell survival rates of 59±4 and 10±1%, respectively (Figure 1). As expected, ligand **2**, used in the GoPI–sugar derivative, was not cytotoxic, since the coordinated metal drives the covalent modification and resultant inhibition of the targeted proteins.

The cytotoxic effects of GoPI–sugar (**9**), auranofin (**4**) and GoPI (**8**) were further evaluated in MCF-7 cells using flow cytometric analysis,[8] and displayed IC_{50} values of 2.3±0.1 μM, 3.2±0.2 μM and 6.1±0.3 μM, respectively (Figure 2). GoPI–sugar, the most cytotoxic compound, was therefore chosen for further biological evaluation in MCF-7 cells. Nuclear staining of treated MCF-7 cells with DAPI (4′-6-diamidino-2-phenylindole) revealed that compound **9** triggered a nuclear fragmentation and chromatin condensation, suggestive of late apoptosis (Figure 3).

Our next goal was to identify the possible cellular targets of GoPI–sugar (**9**), which could explain the observed potent cytotoxicity in breast cancer cells. While the chloride has been replaced with a thiosugar ligand, derivative **9** and GoPI (**8**), a known disulfide reductase inhibitor, share the same reactive metal–phosphole moiety. Therefore, GoPI–sugar was evaluated for its ability to inhibit TrxR and GR in MCF-7 cultured cells (Figure 4). A potent concentration-dependent inhibition of TrxR activity by GoPI–sugar was observed with an IC_{50} value of 1.9±0.1 μM. Since the inhibition was observed after cell homogenization and dilution, the inhibition is likely to be irreversible as observed for GoPI on GR.[7] Conversely, GR, although structurally and mechanistically related to TrxR, was not significantly inhibited within the same experimental conditions (Figure 4), suggesting that complex **9** selectively inhibits TrxR in cultured cells. The concentrations needed for a cytotoxic effect to be observed are similar to those required for the inhibition of TrxR in malignant cells, suggesting that cell death arises through the selective inhibition of TrxR by GoPI–sugar. Notably, DTNB (5,5′-dithiobis-2-nitrobenzoic acid) reductase activity in MCF-7 cells was fully inhibited at low micromolar concentrations of the TrxR inhibitor auranofin (**4**) in a cell homogenate (results not shown), suggesting that the DTNB reductase activity was mainly due to TrxR activity, as previously reported.[19]

As mentioned earlier, the TrxR/Trx system is essential to the cell cycle, and various TrxR inhibitors are known to inhibit cell proliferation.[6] To determine the cytostatic effects of GoPI–

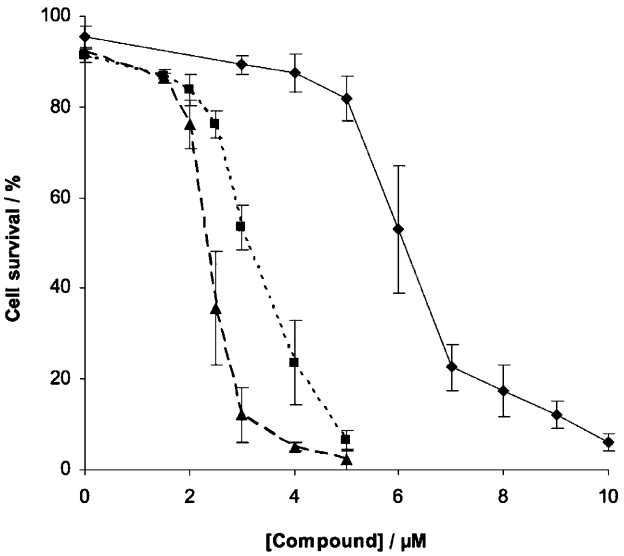

Figure 2. Cytotoxic effects of GoPI–sugar (**9**, ▲), auranofin (**4**, ■) and GoPI (**8**, ◆) on MCF-7 cells after exposure for 24 h to varying concentrations.

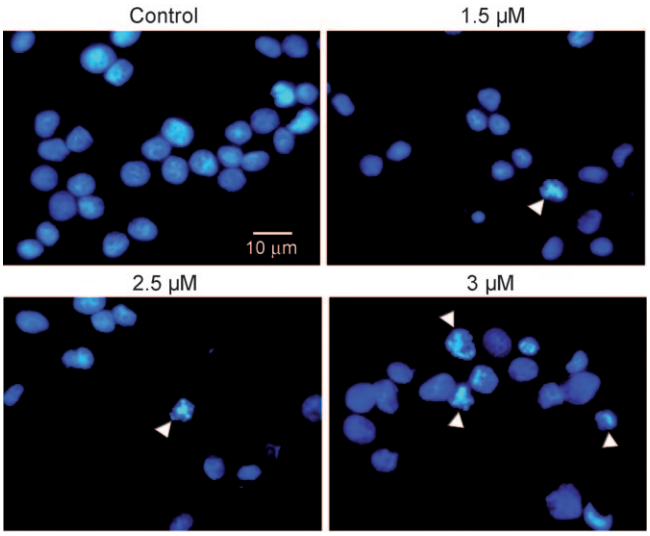

Figure 3. Onset of apoptosis in MCF-7 cells treated with GoPI–sugar (**9**). Representative apoptotic cell nuclei are indicated by white arrowheads.

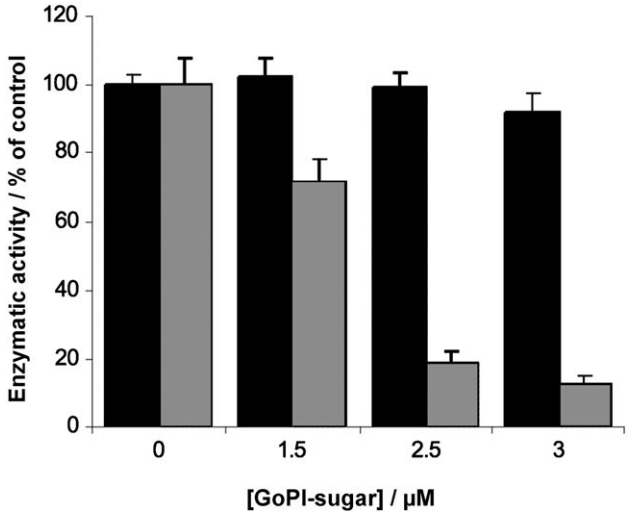

Figure 4. Effects of GoPI–sugar (**9**) on the GR (■) and TrxR (■) activities in MCF-7 cells.

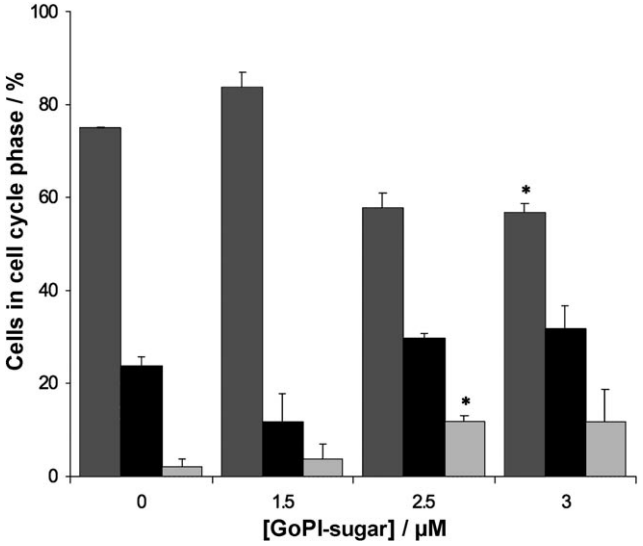

Figure 6. MCF-7 cell cycle phase distribution after exposure to GoPI–sugar (**9**); G0/G1 ■, S ■, G2/M ■. Statistical significance: test concentration vs. control, $p < 0.05$ (*).

sugar (**9**), MCF-7 cells were treated with GoPI–sugar (0.8 and 1.5 μM) and cell growth curves (0–144 h) were plotted. GoPI–sugar appeared to slow down the cell proliferation (Figure 5). Higher concentrations were not used due to the cytotoxicity of this compound. The cell cycle stage of arrest after exposure to compound **9** was determined by flow cytometry (PI staining). Compared with a control (DMSO), MCF-7 cells displayed a dose-dependent G2/M phase arrest after incubation with GoPI–sugar for 24 h (Figure 6). Cell accumulation in the G2/M phase was accompanied by a diminished proportion of cells in the G0/G1 phase at concentrations > 1.5 μM. The perturbation of the cell cycle in MCF-7 cells by GoPI–sugar is in agreement with the antiproliferative activity of this compound observed in the growth curves (Figure 5).

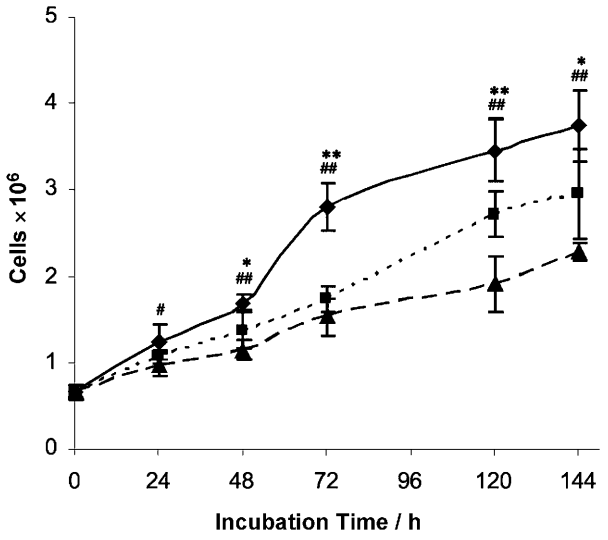

Figure 5. Effect of GoPI–sugar (**9**) on MCF-7 growth at 0.8 μM (■) and 1.5 μM (▲) compared with a control (◆). Statistical significances: 0.8 μM GoPI–sugar vs. control, $p < 0.05$ (*), $p < 0.01$ (**); 1.5 μM GoPI–sugar vs. control, $p < 0.05$ (#), $p < 0.01$ (##).

The effects of GoPI–sugar (**9**) on MCF-7 cells were evaluated, and potent cytotoxic activity was found compared to auranofin (**4**). As reported in recent studies, auranofin inhibits the growth of various cancer cells in vitro, namely human ovarian cancer[20] and acute promyelocytic leukaemia.[21] Auranofin remains under investigation in spite of the modest antitumor activity exhibited in vivo.[22] Moreover, the antitumor activity of other new gold-containing drugs was recently reported against melanoma[23] and breast cancer[24] cell lines, providing further evidence that gold complexes, such as GoPI–sugar, are potential anticancer agents.

In conclusion, GoPI–sugar (**9**) is a novel, potent, irreversible inhibitor of human TrxR in vitro. This inhibition is associated with marked cytotoxic and cytostatic effects in human breast carcinoma cells (MCF-7). Further biological evaluation both in vitro and in vivo is required to fully decipher the potential of GoPI–sugar as an anticancer agent.

Experimental Section

Chemistry: Phospholes and the metal complexes were synthesized according to published procedures and characterized by NMR spectroscopy, MS spectrometry, and X-ray crystallography.[7,9,12–15] [$AuCl(PPh_3)$] and auranofin were purchased from Sigma–Aldrich and ICN, respectively.

Cells: Human mammary adenocarcinoma cells (MCF-7) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 UI mL^{-1}) and streptomycin (100 μg mL^{-1}).

Cytotoxicity assay: Estimation of cell viability by flow cytometry was assessed by dual color fluorescence analysis. MCF-7 cells were plated at 1×10^6 in 100 mm dishes. Following exposure to the test compound (24 h), cells were stained with 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and propidium iodide (PI).[8] H_2DCFDA is a non fluorescent substance that freely enters all cells. In viable cells, H_2DCFDA is converted to the green fluorophore, dichlorofluorescein, by intracellular esterases and oxidation. PI (red

fluorophore) only enters cells that lack membrane integrity. Thus, using this dual color fluorescence staining, viable cells are identified with low red and high green fluorescence. Determination of the IC_{50} values was performed using generalized linear models, as described in the literature.[25]

Enzymatic activity in MCF-7 cells: The inhibitory potency of GoPI–sugar against both GR and TrxR in cell lysates was assessed spectrophotometrically. MCF-7 cells were plated at a density of 1×10^6 in 100 mm dish. After 72 h, the cells were treated with GoPI–sugar (0–3 μM) and incubated for a further 24 h. The cells were then harvested, resuspended in 100 mM potassium phosphate buffer (pH 7), sonicated (×3, 10 s, VibraCell, Fisher Bioblock Scientific, Illkirch, France) and centrifuged (12000 g, 15 min, 4 °C). Supernatants were used for enzyme assays. GR activity assays were carried out at 30 °C in potassium phosphate buffer (pH 7), 0.5 mM EDTA, 1 mM oxidized glutathione (GSSG) and 0.2 mM NADPH.[26] The reaction was started by addition of GSSG and the consumption of NADPH was monitored at 340 nm for 4 min (initial rate conditions). TrxR activity assays were based on the NADPH-dependent DTNB reduction. Formation of the 2-nitro-5- thiobenzoate anion (TNB) was monitored at 412 nm during the initial 2 min (initial rate conditions).[27] The TrxR activity was monitored at 25 °C in potassium phosphate buffer (100 mM, pH 7.4), 2 mM EDTA in the presence of 3 mM DTNB and 0.2 mM NADPH. The rate of the corresponding reaction without NADPH was used to subtract the rate of DTNB reduction by protein free SH groups independently of TrxR activity. Residual activities (%) and IC_{50} values were determined using GLMTox.[25]

Cellular growth assay: MCF-7 cells were seeded at a density of 0.5×10^6 per 100 mm dish. After 24 h, cells were treated with GoPI–sugar (0.8 and 1.5 μM) or solvent alone (DMSO, 1% *v/v*). Cells were quantified by the trypan blue exclusion counting method over the following 6 days.

Cell cycle analysis: MCF-7 cells were plated at a density of 0.75×10^6 per 100 mm dish. After incubation (24 h), cells were exposed to 0–3 μM GoPI–sugar and incubated for a further 24 h. All cells were harvested, stained with PI solution and analyzed for DNA content with a FACSCalibur flow cytometer (Becton-Dickinson, USA) as previously described.[28]

Statistical analysis: Data represent the mean ± SD of at least three independent experiments. Statistical analysis was performed using a Student's t-test.

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