Thioredoxin reductase and cancer cell growth inhibition by organogold(III) compounds

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Thioredoxin (Trx) expression is increased in several human primary cancers associated with aggressive tumor growth and decreased patient survival, and the Trx/Trx reductase (TrxR) system therefore provides an attractive target for cancer drug development. Various gold(III) compounds with none, one, two or three carbon–gold bonds were evaluated for their capacity to inhibit TrxR and the growth of MCF-7 cancer cells *in vitro*. Compounds with up to two carbon–gold bonds were often potent inhibitors of TrxR with IC_{50} values as low as 2 nmol/l. In the presence of Trx and insulin the inhibiting capacity was much lower. However, the inhibitory concentrations of the compounds did not correlate with the ability to kill cells. Out of the organometallics tested, only compound 8 with two carbon–gold bonds was able to inhibit colony formation by MCF-7 breast cancer cells at low micromolar concentrations (IC_{50} = 1.6 μmol/l). Unfortunately, the compound did not show any anti-tumor activity against MCF-7 breast cancer and HT-29 colon cancer xenografts in scid mice. *Anti-Cancer Drugs* 17:539–544 © 2006 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2006, **17**:539–544

Keywords: anti-tumor activity, organogold compounds, thioredoxin, thioredoxin reductase

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Sponsorship: Financial support from Cancerfonden, the Swedish Research Council and the NIH (CA 77204 and CA 17094) is gratefully acknowledged.

Received 21 December 2005 **Accepted** 26 January 2006

Introduction

Auranofin and aurothioglucose (Fig. 1) are two coordinate gold(I) thiolates that have been used for the treatment of rheumatic diseases. The thiolate and phosphine ligands stabilize these complexes against disproportionation to zerovalent and trivalent gold. They also allow exchange reactions with biological ligands. However, extensive investigation of the pharmacological properties of other organogold compounds has been restricted by the poor stability of the materials under physiological conditions. Among organometallic compounds carrying gold in various oxidation states (–I, 0, I, II, III, IV and V), only the 0, I and III valent ones are stable in biological environments. The potential anti-tumor activities of several of these have been demonstrated in various experimental models [1–4]. To the best of our knowledge, however, no gold compound has yet entered clinical trials for the treatment of cancer [5,6].

Thioredoxin (Trx)-1 is a ubiquitously expressed small redox protein with a conserved catalytic site that undergoes reversible NADPH-dependent reduction by selenocysteine-containing flavoprotein Trx-1 reductase (TrxR) [7]. Trx-1 has multiple effects in the cell that includes the regulation of the DNA binding and *trans*-activating activity of redox-sensitive transcription factors such as the glucocorticoid receptor [8], NF-κB [9], p53 [10], HIF-1 [11] and, indirectly through redox factor (Ref)-1/HAP1, AP-1 (Fos/Jun heterodimer) [12]. Trx-1 also binds in a redox-dependent manner to enzymes to regulate their activity including apoptosis signal-regulated kinase-1 [13], protein kinases C [14] and the tumor suppresser PTEN (phosphatase and tensin homolog deleted on chromosome 10) [15]. In a third function, Trx-1 provides reducing equivalents to cytoplasmic Trx peroxidases that protect cells against oxidant-induced apoptosis by scavenging H_2O_2 and organic hydroperoxides [16]. Trx-1 expression is increased in several human cancers, including lung, colon, cervix, liver, pancreatic, colorectal and squamous cell cancer [7,17–19]. Clinically, increased Trx-1 levels have been linked to aggressive tumor growth, inhibition of apoptosis and decreased patient survival [17–21]. This makes the Trx/TrxR redox pathway an attractive target for new cancer drug development [7].

Trx-1 reduction by human TrxR in its NADPH-reduced [22] form has been found to be strongly inhibited by auranofin with a K_i of 4 nmol/l when measured in the presence of 50 μmol/l Trx. Aurothioglucose had a similar effect on mouse TrxR [23]. Since inhibition is reversed by gold-chelating agents and thioglucose itself is not an inhibitor of TrxR, it is likely that binding of these compounds to Trx entails interaction of the gold atom with the C-terminal redox active Cys495/SeCys496

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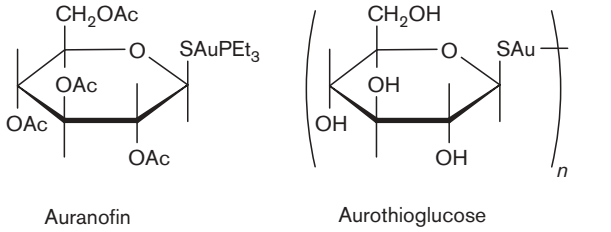
Fig. 1



Auranofin Aurothioglucose

Structure of auranofin and aurothioglucose

moiety of the enzyme. Auranophin also exhibits antineoplastic activity and inhibits DNA synthesis [24].

Compounds with a carbon–gold bond are less likely to be decomposed under physiological conditions than the compounds mentioned above and are also more lipophilic. Since these gold compounds are isoelectronic with platinum(II) derivatives (e.g. those used clinically in the anti-cancer drugs cisplatin and carboplatin), we thought it would be interesting to evaluate a series of gold(III) compounds with an increasing number ($n = 0$–3) of carbon–gold bonds for their Trx/TrxR-inhibiting activity and cancer cell-killing capacity as potential antitumor agents.

Materials and methods

^{1}H- and ^{13}C-NMR spectra were recorded at 400 and 100 MHz respectively, on a Varian 400 MHz Unity instrument. For proton spectra, the residual peak of $CHCl_3$ was used as the internal reference (7.26 ppm), while the central peak of $CDCl_3$ (77.0 ppm) was used as the reference for carbon spectra. Elemental analyses were carried out by Analytical Laboratories (Lindlar, Germany). [2-[(Dimethylamino)methyl]phenyl]lithium [25], chloro[2-[(dimethylamino)methyl]-phenyl]mercury(II) [26] and the organogold compounds: $AuClPPh_3$ [27], **1** [28], **2**, **3** and **5** [29], **6** [4], **7** [30], and **8** [31] were prepared according to literature methods. $Na[AuCl_4]$ and all other reagents were purchased from Sigma-Aldrich Sweden AB, and used as supplied. Compound **1** shown in Table 1 is formed via deprotonation of $Au(en)_2Cl_3$ [28] above pH 4 [32]. Human recombinant Trx-1 was obtained from Sigma (St Louis, Missouri, USA) and human TrxR-1 purified from human placenta as previously described [15].

Dimethyl[2-[(dimethylamino)methyl]phenyl-C,N]gold(III) (9)

Methyllithium (0.52 ml, 1.6 mol/l in Et_2O, 0.84 mmol) was added dropwise to an ethereal suspension of dichloro[2-[(dimethylamino)methyl]phenyl-C,N]gold(III) (0.112 g, 0.28 mmol) at 0°C under argon. The reaction mixture was stirred for 20 min at this temperature. After further stirring for 20 min at room temperature, methyl iodide (0.05 ml, 0.84 mmol) was slowly added at 0°C to the reaction mixture. The solution was stirred for 20 min at 0°C and for an additional 20 min at room temperature. Distilled water was added (3 ml), the ether layer was separated and the solvent evaporated *in vacuo*. The violet powder was dried under vacuum to afford 62 mg (61.6%) of the title compound. Analysis calculated for $C_{11}H_{18}Cl_2Au$: C, 36.59; H, 4.98. Found: C, 36.43; H, 5.05. ^{1}H-NMR ($CDCl_3$) δ, ppm: 7.60 (dd, 1H, $J = 7.2$, 1.2 Hz) 7.27 (d, 1H, $J = 1.2$ Hz), 7.20 (dd, 1H, $J = 7.2$, 7.2 Hz), 7.13 (ddd, 1H, $J = 7.2$, 1.2 Hz), 4.07 (s, 2H), 2.75 (s, 6H), 1.30 (s, 3H), 0.18 (s, 3H); ^{13}C-NMR ($CDCl_3$) δ 123.1, 125.5, 126.5, 132.0, 147.9, 171.9, 74.5, 49.2, 13.2, 2.1.

Trimethyl[triphenylphosphine]gold(III) (10)

Methyllithium (0.41 ml, 1.6 mol/l in Et_2O, 0.66 mmol) was added dropwise to an ethereal suspension of $AuClPPh_3$ (0.108 g, 0.22 mmol) at 0°C under argon and stirred for 1 h at 0°C. After an additional 1 h at room temperature, the mixture was cooled in an ice bath and methyliodide (0.04 ml, 0.66 mmol) was slowly added. The solution was stirred for 1 h at 0°C and another 1 h at room temperature. The reaction mixture was filtered and evaporated *in vacuo*. The white powder was dried under vacuum to afford 78 mg (41.8%) of the title compound. Spectral data were in good agreement with the literature [33].

TrxR/Trx assay

TrxR/Trx-dependent insulin-reducing activity was measured in an incubation with a final volume of 60 µl containing 100 mmol/l HEPES buffer, pH 7.2, 5 mmol/l EDTA (HE buffer), 1 mmol/l NADPH, 1.0 µmol/l TrxR, 0.8 µmol/l Trx, 2.5 mg/ml bovine insulin and inhibitor. Incubations were for 30 min at 37°C in flat-bottom 96-well microtiter plates. The reaction was stopped by the addition of 50 µl of 6 mol/l guanidine–HCl, 50 mmol/l Tris, pH 8.0, and 10 mmol/l dithionitrobenzoic acid (DTNB), and the absorbance measured at 412 nm.

TrxR assay

Assays of TrxR were carried out in flat-bottom 96-well microtiter plates. TrxR activity was measured in a final incubation volume of 60 µl containing HE buffer, 10 mmol/l DTNB, 1.0 µmol/l TrxR, 1 mmol/l NADPH and inhibitor. Compounds were diluted in HE buffer and added to the wells as 20-µl aliquots, and TrxR was then added, also as 20-µl aliquots in HE buffer. To ensure uniform coverage of the bottom of the well the plate was spun briefly at 3000 *g*. To start the reaction, NADPH and DTNB were added as a 20-µl aliquot in HE buffer and the plate was moved to the multiwell microplate spectrophotometer (Molecular Device, Menlo Park,

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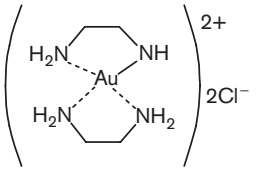
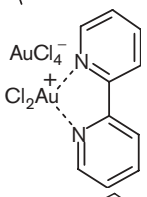
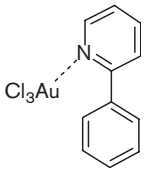
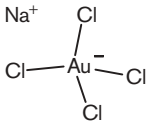
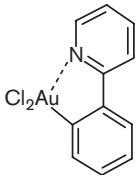
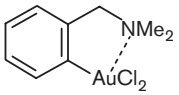
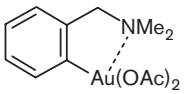
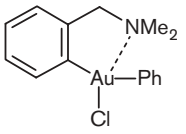
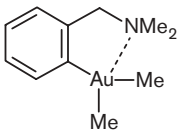
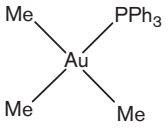
Table 1 Inhibition of TrxR and cancer cell growth by organogold compounds

Compound		IC_{50}(μmol/l)		
		TrxR/Trx[a]	TrxR[b]	MCF-7[c]
	**1**	0.32	0.2	36.0
	**2**	0.18	0.012	78.0
	**3**	0.18	0.03	28.0
	**4**	0.18	0.0058	52.0
	**5**	2.1	0.036	12.0
	**6**	0.72	0.18	34.0
	**7**	0.52	0.03	30.0
	**8**	2.1	0.0022	1.6
	**9**	42.0	1.8	9.0
	**10**	>50	0.68	24.0

[a]Inhibition of TrxR in the presence of Trx and insulin.
[b]Inhibition of TrxR.
[c]Inhibition of colony formation by human MCF-7 breast cancer cells.

California, USA) which had been pre-heated to 37°C. The optical density at 412 nm was measured every 10 s and initial linear reaction rates measured.

Growth inhibition assay

Compound cytotoxicity was measured using modifications of the MTT assay as described by Mosmann [34]

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and Carmichael [35]. The MCF-7 human breast cancer cell line was chosen for its known growth dependence on Trx-1 [36]. MCF-7 cells were seeded at 3000 cells/well into 96-well plates in DMEM supplemented with 10% FBS. After 24 h at 37°C/5.5% CO_2, drugs were added to the wells at concentrations ranging from 0.1 to 100 μmol/l. The cells were further incubated for 72 h, after which 40 μl/well of a 2.5-μg/μl MTT solution was added and an additional 3-h, 37°C/5.5% CO_2 incubation was performed. At the end of the incubation time, the untransformed MTT was removed from each well by aspiration and 150 μl/well of DMSO was added. The plate was shaken to ensure full solubilization of the formazan dye followed by dual optical density readings of 595 and 655 nm. Cytoviability of control cells was considered to be 100%. For the treated cells, viability was expressed as a percentage of control cells. All determinations were carried out in triplicate.

Anti-tumor studies

MCF-7 human breast cancer cells were obtained from the ATCC (Manassas, Virginia, USA). All cells were tested to be mycoplasma free using a PCR ELISA kit (Roche Diagnostics, Indianapolis, Indiana, USA) and grown in 95% humidified air with 5% CO_2 at 37°C in McCoy's 5A medium supplemented with 10% FBS. Approximately 10^7 cells in log cell growth were injected s.c. in 0.1 ml PBS into the flanks of female scid mice (SHP-77 and MCF-7 cells). Female mice that were to receive the estrogen-dependent MCF-7 breast cancer cell line were implanted s.c. in the back with a 90-day 17β-estradiol release pellet (Innovative Research of America, Sarasota, Florida, USA) a day before the tumor cells. The animals were weighed weekly, and tumor diameters measured twice weekly at right angles (d_{short} and d_{long}) with electronic calipers and converted to volume by the formula volume = $(d_{short})^2 \times (d_{long})/2$ [37]. When the tumors reached volumes between 100 and 170 mm^3, the mice were stratified into groups of eight animals having approximately equal mean tumor volumes and administration of compound **8** was started. When the tumor volume reached 2000 mm^3 or above, or became necrotic, the animals were euthanized.

Results and discussion

Trx-1/TrxR activity was measured as the post-incubation reduction of added DTNB by insulin free thiol formed in an incubation containing Trx-1, TrxR, NADPH and insulin (Table 1: Trx-1/TrxR activity). TrxR activity was measured as the direct reduction of DTNB by TrxR, NADPH (Table 1: TrxR activity). Surprisingly, the gold compounds were often extremely potent inhibitors in the TrxR assay, but much less potent inhibitors in the Trx-1/TrxR assay (see, e.g. Table 1, compounds **4** and **8**). This may be due to reaction of the gold compound with excess insulin present in the assay reducing its effective concentration. The potency of the compounds as inhibitors of MCF-7 breast cancer cell growth was less than that of inhibition of TrxR or Trx-1/TrxR (Table 1). We can only speculate about the reasons for this, but the reaction of the compounds with media serum and cell proteins is certainly a possibility. With these caveats, conclusions can be made of the activity of the compounds as inhibitors of Trx-1/TrxR and cell growth inhibitors.

Like phosphines, amines serve as good complexing agents in various organometallics. Gold(III) is known to form stable chelating 1:2 (compound **1**) and 1:1 complexes (compound **2**) with ethylenediamine and 2,2′-bipyridyl, respectively, and a square-planar *N*-bonded complex **3** with 2-phenylpyridine (Table 1). We reasoned that the stabilization brought about by this complexation might make the compounds more effective as TrxR and cancer cell growth inhibitors. As shown in Table 1, these compounds, all lacking carbon–gold bonds, were potent inhibitors of TrxR activity (IC_{50} = 30–200 nmol/l), weaker inhibitors of Trx-1/TrxR activity (IC_{50} = 0.18–0.32 μmol/l) and were relatively weak inhibitors of MCF-7 cancer cell growth (IC_{50}s = 28–78 μmol/l). This could be a feature of reactivity discussed above or, for cell growth inhibition considering that these materials are essentially ionic, due to poor cellular uptake.

In the evaluation of these data, there is a problem with the tetrachloroaurate anion present in complex **2**. The inhibiting capacity of the compound could also be due to this counterion. Therefore, sodium tetrachloroaurate (**4**) was also included in the study as a reference. As shown in Table 1, it has rather similar inhibition characteristics to complex **2**. It therefore seems that the cationic part of complex **2** is at most a poor inhibitor of TrxR. Also, the similar results with complex **3** and sodium tetrachloroaurate (**4**) suggest that introduction of a pyridine entity is not enough for the two compounds to behave differently in the in-vitro systems investigated.

Compounds **5–7** were prepared and tested as representatives of organogold(III) compounds with one carbon–gold bond. In addition, the materials carry pyridine and amine ligands that would stabilize them via intramolecular coordination. All three compounds inhibited TrxR in micromolar concentrations in the combined assay. Again, the inhibiting capacity in the absence of Trx was much better. The effect was remarkably large with compound **5** (IC_{50} = 2.1 μmol/l in the presence and 36 nmol/l in the absence of the disulfide). As was observed above with the compounds lacking a carbon–gold bond, the ability of organogolds **5–7** to inhibit colony formation by MCF-7 breast cancer cells was not exceptional (IC_{50}s = 12–34 μmol/l). The similar inhibition characteristics of compounds **6** and **7** seem to indicate that the nature of the two non-carbon ligands bonded to gold (chloride or acetate) are not critical to the biological activity.

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2-(Dimethylaminomethyl)phenyl phenyl gold(III)-chloride (**8**) was included in the study as an example of an organogold compound with two carbon–gold bonds. The role of the dimethylaminomethyl moiety is obviously to provide extra stabilization via intramolecular coordination. As shown in Table 1, compound **8** was the most potent inhibitor of TrxR found in this study (IC_{50} = 2.2 nmol/l). In the mixed assay, though, it was almost 3 orders of magnitude less potent. In addition, the compound was also the most efficient inhibitor of in-vitro colony formation by MCF-7 cells (IC_{50} = 1.6 μmol/l). Introduction of an increasing number of carbon–gold bonds in the gold (III) compounds under investigation would make them more lipophilic. This is likely to facilitate cellular uptake. Also, with two carbon–gold bonds in the molecule, there is still a possibility for covalent bonding of gold to essential selenol or thiol groups in the enzyme via displacement of the remaining ligand. Unfortunately, compound **8** showed only minimal anti-tumor activity against MCF-7 breast cancer and HT-29 colon cancer xenografts in scid mice.

Two organogold compounds **9** and **10** with three carbon–gold bonds were prepared and their inhibiting capacity evaluated. The former is stabilized by intramolecular coordination, the latter by complexation to triphenylphosphine. As shown in Table 1, the compounds are notably poorer inhibitors of TrxR than the other compounds evaluated. This is true both in the presence and absence of Trx. However, as was often the case above, the inhibiting capacity is much better in the absence of the disulfide substrate of the enzyme/insulin. Organogold(III) compounds with three carbon–gold bonds can no longer bind thiols or selenols covalently. However, there is still a possibility for binding a fourth ligand via coordination. This may be the reason why these compounds are effective as TrxR inhibitors. In the cell culture experiments, the two compounds **9** and **10** perform equally as well as many of the other organogolds tested (IC_{50} = 9.0 and 24.0 μmol/l, respectively). It may be that the better cellular uptake of the lipophilic compounds **9** and **10** compensates for their poorer inhibiting capacity.

Conclusions

Various gold(III) compounds with none, one, two or three carbon–gold bonds were evaluated for their capacity to inhibit TrxR and the growth of MCF-7 cancer cells *in vitro*. Compounds with up to two carbon–gold bonds were often potent inhibitors of TrxR with IC_{50} values as low as 2 nmol/l. In the presence of Trx and insulin, however, the inhibiting capacity was much lower. The inhibitory concentrations of the compounds did not correlate with the ability to kill cells. In general, cell killing required much higher concentrations than required for TrxR inhibition. Although we can only speculate about the reasons for this, it seems likely that the compounds could react with the growth media or undergo metabolism. Out of the organometallics tested, only compound **8** with two carbon–gold bonds was able to inhibit colony formation by MCF-7 breast cancer cells at low micromolar concentrations (IC_{50} = 1.6 μmol/l). Unfortunately, the compound did not show any anti-tumor activity against MCF-7 breast cancer and HT-29 colon cancer xenografts in scid mice. Introduction of an increasing number of carbon–gold bonds into gold(III) compounds makes them more lipophilic and thus facilitates cellular uptake. The ability to interact with thiol or selenol groups via covalent bonding, however, is reduced. Based on the series of gold(III) compounds studied in this work, it seems that two carbon–gold bonds and one exchangeable group that could interact with biological ligands are optimal for obtaining gold-based inhibitors of Trx. Based on this finding, it could be worthwhile to try to find other organogold compounds suitable for clinical use.

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