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Gold(I) complex of *N,N'*-disubstituted cyclic thiourea with *in vitro* and *in vivo* anticancer properties—potent tight-binding inhibition of thioredoxin reductase†

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Coinage metal complexes of an *N,N'*-disubstituted cyclic thiourea exert significant cytotoxicities to cancer cells and, in particular, the gold(I) thiourea complex exhibits a potent tight-binding inhibition of the anticancer drug target thioredoxin reductase with an inhibitory constant at nanomolar level.

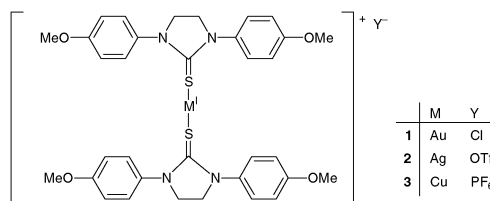
Coinage metal (Au, Ag, Cu) ions, such as d¹⁰ M⁺, exhibit distinct biological activities that could be harnessed to develop effective therapeutic agents including antiarthritic, antimicrobial and anticancer drugs.¹ Under physiological conditions, however, the hydrated d¹⁰ M⁺ ions except Ag(I) are unstable toward precipitation, reduction or aerobic oxidation. Such instability can be circumvented by using appropriate auxiliary ligands. In the literature, phosphine ligands have been employed to develop bioactive complexes of d¹⁰ M⁺ ions.² Recent works have also witnessed other ligand systems, particularly, N-heterocyclic carbenes (NHC) for this endeavor.³

A compelling finding in the anticancer activity of d¹⁰ M⁺ complexes is the remarkable potency of Au(I)–phosphine complexes, such as auranofin (a [Au^I(PEt₃)(SR)] species), in inhibiting thioredoxin reductase (TrxR),⁴ a NADPH dependent selenoenzyme that plays a pivotal role in cancer progression and represents an increasingly attractive target for anticancer drugs.⁵ Recently, Berners-Price and co-workers⁶ reported that a homoleptic Au(I)–NHC complex, [Au^I(NHC)₂]⁺, can selectively inhibit TrxR in cells without inhibiting the closely related non-selenoenzyme glutathione reductase (GR).

We are interested in exploring the possible use of homoleptic d¹⁰ M⁺ thiourea (TU) complexes, [M^I(TU)₂]⁺, as potent inhibitors of TrxR, in view of the well documented coordination chemistry of TU ligands,⁷ including imidazolidine-2-thiones,^{7a} *N*-acyl thioureas^{7b} and thiosemicarbazones.^{7c–e} Despite extensive studies on the anticancer activity of metal thiosemicarbazone complexes^{7c–e} and related Au(III) dithiocarbamate

complexes,⁸ other types of TU ligands are sparsely seen in bioactive metal complexes.⁹ Several Au(III) dithiocarbamate complexes^{8a} and to the best of our knowledge only one metal–TU complex, [Au^I(PEt₃)(S=C(NH₂)₂)]⁺,^{9c} have been documented to inhibit TrxR.

Herein we report the biological activities of homoleptic metal–TU complexes [Au^I(TU)₂]Cl (**1**), [Ag^I(TU)₂]OTf (**2**) and [Cu^I(TU)₂]PF₆ (**3**), of which **1** and **2** are potent inhibitors of purified and cellular TrxR. Interestingly, the TrxR inhibitor **1**, like its NHC analogue,⁶ did not inhibit cellular GR. Also, a significant *in vivo* anticancer activity of **1** has been observed.



Treatment of the previously reported *N,N'*-disubstituted imidazolidine-2-thione¹⁰ with [Au(THT)Cl] (THT = tetrahydrothiophene), AgOTf and [Cu(CH₃CN)₄]PF₆ afforded **1**, **2** and **3**, respectively. The structures of **1** and **2** have been determined by X-ray crystallography (ESI†, Table S1 and Fig. S1 and S2), both featuring a linear two-coordinate M⁺ ion with monodentate S-donor TU ligands. The M–S distances (2.287(3) Å for **1**, average 2.4071(11) Å for **2**) and S–M–S angles (180.0(1)° for **1**, 172.77(4)° for **2**) are similar to those in the [M^I(TU)₂]⁺ complexes with TU = imidazolidine-2-thione (2.2782(15)–2.2873(14) Å and 171.23(5)–174.84(6)° for Au(I);^{11a} 2.4058(8) Å and 180° for Ag(I)^{11b}). The nearest M···M distance in **1** and **2** (>3.2894(7) Å) suggests the absence of significant intermolecular metal–metal interactions. Complexes **1–3** are all stable in the solid state in air and are soluble in DMSO to form a 10 mM stock solution; they also exhibit some solubility and considerable stability in aqueous solution, and no precipitation occurred when the concentration of their solutions in serum supplemented cell culture medium reached up to 30 μM.

We examined the cytotoxicity of **1–3** against a panel of cancer cell lines. The observed half maximal inhibitory concentrations (IC₅₀), together with those observed for the free TU ligand and the benchmark anticancer drug cisplatin, are listed in Table 1. These IC₅₀ values indicate that **1–3** exhibit similar or higher potency compared with cisplatin, whereas the free TU ligand is much less cytotoxic, suggesting that the biological activities of **1–3** are largely metal-mediated.

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† Electronic supplementary information (ESI) available: Synthesis of **1–3**, X-ray crystallographic data for **1** and **2**, experimental procedures for cellular and biochemical assays and animal studies. CCDC 773697–773698. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c0cc01058h

Table 1 Cytotoxicities (IC_{50} in μM , 72 h treatment) of **1–3** against human cancer cell lines

	HeLa ^a	HepG2 ^b	SUNE1 ^c	NCI-H460 ^d
1	14.6 ± 0.7	17.4 ± 1.0	10.8 ± 0.2	3.7 ± 0.3
2	7.2 ± 0.7	4.0 ± 0.4	8.8 ± 1.0	8.9 ± 1.0
3	12.7 ± 0.9	13.0 ± 0.9	8.5 ± 1.0	11.2 ± 0.9
TU	> 100	> 100	> 100	> 100
Cisplatin	4.7 ± 0.3	14.2 ± 1.0	35.2 ± 0.3	38.6 ± 0.4

^a Human cervical epithelioid carcinoma. ^b Human hepatocellular carcinoma. ^c Human nasopharyngeal carcinoma. ^d Human lung carcinoma.

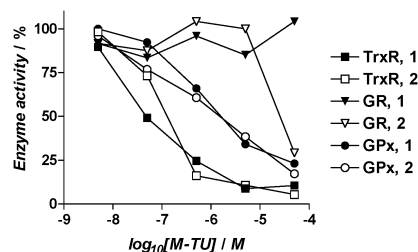
It appears that **1–3** induced cell death through apoptosis, as apoptotic cells were detected by cell permeable DNA fluorescent stain Hoechst 33342 when HeLa cells were treated with **1–3** (10 μM) for 24 h (ESI, Fig. S3†).

The metal uptake by cells upon treatment with 10 μM of **1** or **2** for 2 h was measured by inductively coupled plasma mass spectrometry (ICP-MS), which revealed a Au content of 18 ± 1 fg/cell and a Ag content of 119 ± 3 fg/cell; the latter is 5-fold higher than the Ag content of 22 ± 1 fg/cell in the cells treated with 10 μM of AgNO_3 under the same conditions. Complex **2** also has nearly a 5-fold higher cytotoxicity against HeLa cells (IC_{50} = 7.2 ± 0.7 μM) than AgNO_3 (IC_{50} = 32.1 ± 1.2 μM). These results suggest that the TU ligand serves as a lipophilic carrier of the metal ion to the cells.

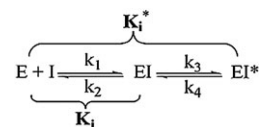
The therapeutic potential of Au(i) complexes has long attracted considerable interest. For example, aurothiomalate (Au(i)-thiolates) and auranofin are disease modifying anti-arthritis drugs and have been studied for their anticancer properties.^{7d,12} We examined the *in vivo* anticancer activities of **1** in mice inoculated with NCI-H460 non-small cell lung cancer cells (ESI†, performed with approval from the Committee on the Use of Live Animals for Teaching and Research, The University of Hong Kong). *Intraperitoneal injection of 1 at 100 mg/kg body weight twice a week resulted in reduction in tumor size by 38 ± 11% (n = 5) compared to vehicle control after a 28-day treatment* (ESI, Table S2†).

The exact molecular mechanism of action of Au(i) complexes has yet to be elucidated, but is generally related to their ability to undergo facile ligand exchange with protein thiol groups, particularly those with low pK_a values.¹³ In this regard, TrxR is a compelling molecular target enzyme of Au(i).^{4,14} We investigated the effect of **1–3** and the free TU ligand on the cellular activities of TrxR, GPx (glutathione peroxidase, another selenoenzyme) and GR. A one-hour treatment of HeLa cancer cells with **1** resulted in an inhibition of the cellular TrxR activity with an IC_{50} value of 50 nM; GPx was also inhibited albeit at a higher concentration (IC_{50} = 1 μM), but GR activity was not affected (Fig. 1). Complex **2** inhibited TrxR and GPx with IC_{50} of 100 nM and 1 μM , respectively, and also significantly suppressed GR activities when added at 50 μM (Fig. 1). Neither **3** nor the free TU ligand (added up to 100 μM) affected the activities of the three enzymes. These data demonstrate that among the coinage metal $[\text{M}^{\text{I}}(\text{TU})_2]^+$ complexes, the Au(i) complex preferentially targets the selenoenzymes.

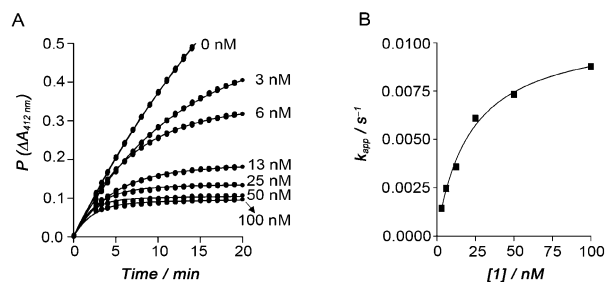
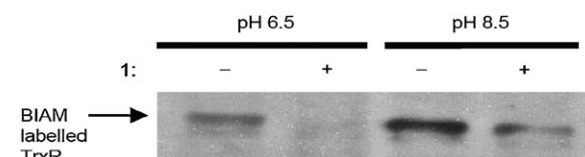
We consider that **1** is particularly useful for understanding the Au(i) inhibition of TrxR. Our *in vitro* enzyme assays

**Fig. 1** TrxR, GR and GPx activities of HeLa cells treated with **1** or **2** for 1 h.

showed that half maximal inhibition of TrxR (recombinant rat TrxR1, 1 nM) was obtained using approximately equal molar concentration of **1**, suggestive of a tight-binding mode of inhibition (ESI, Fig. S4 and S5†). This was further studied by progress curve analysis¹⁵ (ESI†) of the inhibition of TrxR by **1**, performed by adding various amounts of excess **1** (3–100 nM) to a mixture containing 0.2 mM NADPH, 1 nM TrxR and 3 mM disulfide substrate 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in phosphate buffer (pH 7.4). The time course of product concentrations at different concentrations of **1** is depicted in Fig. 2A. The progress curves are non-linear, revealing two-phase equilibria typical of slow-onset tight-binding inhibition. A plot of the apparent first-order rate constants (k_{app} , determined by fit of the data in Fig. 2A) against the concentrations of **1** follows a hyperbolic function (Fig. 2B), indicative of a two-step, tight-binding inhibition mechanism:¹⁵



which involves rapid formation of an initial collision complex (EI) that subsequently undergoes isomerization to the final slow dissociating enzyme–inhibitor complex (EI*). According to this mechanism, the inhibition of TrxR by **1** gave k_3 = 0.011 s⁻¹, k_4 = 0.00014 s⁻¹ and K_i = 1.39 nM.

**Fig. 2** (A) Progress curves of TrxR in the absence or presence of **1** (3–100 nM). (B) Plot of k_{app} against concentrations of **1**.**Fig. 3** Probing the free –SH and –SeH site of TrxR treated with or without **1** by BIAM labelling.

Thus, the tight-binding inhibition is essentially irreversible. Indeed, the enzyme activities could not be recovered after removal of the free inhibitors by centrifugal ultrafiltration. The overall inhibitory constant K_1^* was determined to be 18 pM. These inhibitory constants are close to the values $K_1 = 0.67$ nM and $K_1^* = 36$ pM determined from the steady state rate law established under conditions when EI* was preformed (ESI, Fig. S4; details about the determination of the foregoing k_{app} , k_3 , k_4 , K_1 , K_1^* values are described in ESI†). *Complex 1 is thus among the most potent TrxR inhibitors reported.*^{5b,14a}

The reduced TrxR has free –SH (Cys497) and –SeH (Sec498) groups at the C-terminal active site, making them vulnerable to attack by Au(I).^{5a,b,14a} It has been shown that biotinylated iodoacetamide (BIAM) alkylates both the –SH and –SeH at pH 8.5, but at pH 6.5 alkylates only the –SeH; the resulting adduct can be detected by western blot experiment using streptavidin-linked horseradish peroxidase.^{14d,16} When NADPH-reduced TrxR (0.1 μ M) was preincubated with **1** (4 μ M), the BIAM labelling at pH 8.5 and 6.5 (buffered with 0.1 M Tris-HCl) was inhibited (Fig. 3), suggesting that the Sec or additionally the Cys residue at the active site was involved in the enzyme inactivation. Compared with NADPH-reduced TrxR, the oxidized TrxR having the –S–Se– group, instead of free –SH and –SeH groups, was much less efficiently inhibited by **1** (ESI, Fig. S5†). Moreover, size exclusion chromatography and ICP-MS analysis (SEC-ICP-MS) of the trypsin digest of TrxR treated with **1** showed the appearance of a peptide fraction co-eluted with Se and Au (ESI, Fig. S6†). The formation of the tight enzyme–inhibitor complex (EI*) is likely to involve modification of the redox active Sec/Cys residue *via* coordination with Au(I). This may be congruent with the report of Berners-Price and co-workers on the two-step ligand exchange reactions of $[\text{Au}^{\text{I}}(\text{NHC})_2]^+$ with cysteine and selenocysteine.⁶

In summary, homoleptic d¹⁰ metal thiourea complexes represent a new paradigm in developing metal-based inhibitors of enzymes such as TrxR. In particular, we have demonstrated that the Au(I)–thiourea complex **1** confers specific tight-binding inhibition of TrxR with a potency among the highest reported^{5b} and exhibits effective suppression of the cellular reductase activity. By variation of the thiourea ligand, metal thiourea complexes have the prospect to be a new class of metal-based drugs leads.

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