Electronic supplementary information (ESI)

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

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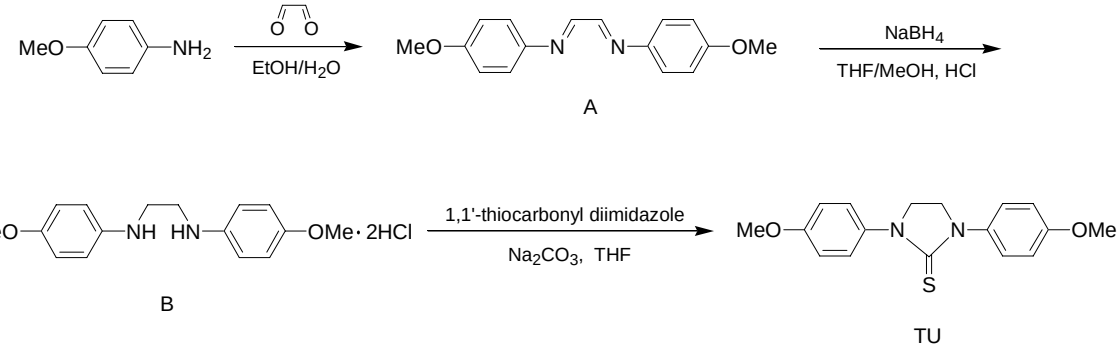
Experimental

Materials

All chemicals were purchased from Sigma-Aldrich Chemical Co. (St Louis, MI, USA), unless otherwise stated. [Au(THT)Cl] and $[Cu(CH_3CN)_4]PF_6$ were prepared according to literature procedures.[1,2]

Synthesis of *N,N'*-disubstituted cyclic thiourea ligand (TU)

This ligand was synthesized via compounds A and B depicted in the following scheme by modifications of the literature procedures.[3,4]



Glyoxal-bis(4-methoxyphenyl)imine (A).[3] To a solution of *p*-anisidine (12.3 g, 0.1 mol) in EtOH (50 ml) was added a mixture of 40% aqueous solution of glyoxal (7.3 g, 0.05 mol), EtOH (10 ml) and water (10 ml) at 25 °C. The mixture was stirred overnight. Upon addition of water (30 ml), a yellow solid precipitated, which was collected by filtration and dried *in vacuo*. Yield: 5.5 g (82%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.42 (s, 2H), 7.32 (d, J = 9.0, 4H), 6.96 (d, J = 9.0, 4H), 3.84 (s, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 160.2, 158.0, 143.4, 123.4, 115.0, 55.9.

***N,N'*-Bis(4-methoxyphenylamino)ethane dihydrochloride (B).**[3,4] A suspension of glyoxal-bis(4-methoxyphenyl)imine (1.34 g, 5 mmol) in a mixture of THF (30 ml) and MeOH (5 ml) was treated at 0 °C with sodium borohydride (0.76 g, 20 mmol). The mixture was stirred overnight at 25 °C and subsequently heated for 2 h under reflux. Upon addition of ice-water (30 ml) and 3 M HCl (30 ml), a white solid precipitated, which was collected by filtration and dried *in vacuo*. Yield: 1.2 g (88%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.79 (d, J = 8.92, 4H), 6.62 (d, J = 8.92, 4H), 3.75 (s, 6H), 3.34 (s, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 152.8, 142.7, 115.4, 114.9, 56.2, 44.9.

1,3-Bis(4-methoxyphenyl)imidazolidine-2-thione (TU).[4] To a solution of *N,N'*-bis(4-methoxyphenylamino)ethane (1.4 g, 5 mmol) in dry THF (40 ml) was added 1,1'-thiocarbonyl diimidazole (1.1 g, 6 mmol) at 25 °C. The mixture was stirred overnight and subsequently heated for 2 h under reflux. After addition of water and ethyl acetate, the organic layer was washed with dilute HCl and brine, dried and concentrated. The pure product was obtained through recrystallization from 95% EtOH. Yield: 1.1 g (70%). ^{1}H

NMR (400 MHz, $CDCl_3$): δ 7.42 (d, J = 8.96, 4H), 6.95 (d, J = 8.96, 4H), 4.10 (s, 4H), 3.82 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 182.6, 158.6, 134.3, 127.6, 114.6, 55.9, 50.3. FAB-MS: m/z 315 $[M + H]^+$.

Synthesis of complexes 1-3

$[Au^I(TU)_2]Cl$ (1). To a solution of TU (0.31 g, 1 mmol) in CH_2Cl_2 (5 ml) was added [Au(THT)Cl] (0.16 g, 0.5 mmol) in distilled MeOH (5 ml) under an argon atmosphere. The mixture was stirred at room temperature overnight and subsequently filtered. The filtrate was left standing overnight, leading to the formation of colourless crystals, which were collected and dried *in vacuo*. Yield: 76%. FAB-MS: m/z 826 $[M]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.38 (d, J = 8.33, 8H), 7.02 (d, J = 8.35, 8H), 4.21 (s, 8H), 3.77 (s, 12H). IR (KBr, cm^{-1}): 2960 (w), 2929 (w), 2835 (w), 1606 (m), 1515 (s), 1283 (m), 1252 (s), 1162 (m), 1029 (m), 836 (s), 554 (m). Anal. Calcd for $AuC_{34}H_{36}N_4O_4S_2Cl$: C, 47.42; H, 4.21; N, 6.51; Found: C, 47.12; H, 4.00; N, 6.53.

$[Ag^I(TU)_2]OTf$ (2). TU (0.31 g, 1 mmol) was dissolved in EtOH (10 ml), and AgOTf (0.13 g, 0.5 mmol) was added under an argon atmosphere. The mixture was stirred at room temperature for 3 h, and subsequently filtered to remove the unreacted AgOTf. The filtrate was left standing overnight to give colourless crystals, which were collected and dried *in vacuo*. Yield: 82%. FAB-MS: m/z 736 $[M]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.33 (d, J = 8.94, 8H), 6.93 (d, J = 8.91, 8H), 4.21 (s, 8H), 3.71 (s, 12H). IR (KBr, cm^{-1}): 2961 (w), 2930 (w), 2839 (w), 1605 (w), 1512 (s), 1275 (s), 1250 (s), 1165 (m), 1032 (m), 831 (s), 555 (m). Anal. Calcd for $AgC_{35}H_{36}N_4O_7S_3F_3$: C, 47.46; H, 4.10; N, 6.33; Found: C, 47.01; H, 4.15; N, 6.36.

$[Cu^I(TU)_2]PF_6$ (3). TU (0.31 g, 1 mmol) was dissolved in CH_2Cl_2 (10 ml), and $[Cu(CH_3CN)_4]PF_6$ (0.19 g, 0.5 mmol) in distilled MeOH (10 ml) was added under an argon atmosphere. The mixture was stirred at room temperature for 2 h. The resulting white solid was filtered and washed with MeOH, Et_2O and dried *in vacuo*. Yield: 80%. FAB-MS: m/z 692 $[M]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.46 (d, J = 8.97, 8H), 6.96 (d, J = 8.90, 8H), 4.10 (s, 8H), 3.76 (s, 12H). IR (KBr, cm^{-1}): 2969 (w), 2934 (w), 2838 (w), 1606 (m), 1514 (s), 1287 (m), 1252 (s), 1166 (m), 1030 (m), 838 (s), 556 (m). Anal. Calcd for $CuC_{34}H_{36}N_4O_4S_2PF_6$: C, 48.77; H, 4.33; N, 6.69; Found: C, 48.12; H, 4.00; N, 6.32.

Cytotoxicity and apoptosis assays

Cell viability assays. Cells were seeded in a 96-well flat-bottomed microplate at 2 x 10^4 cells/well in 100 μl of MEM medium containing 10% fetal bovine serum and incubated for 24 h. Complexes **1-3** and cisplatin were dissolved in DMSO just before use. Serial dilution of each complex was added to each well with final concentration of DMSO ≤ 1%. The cells were exposed with the complexes for 72 h. 10 μl of (3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) was added to each well and incubation was continued for 4 h. 100 μl of solubilization solution (10% SDS in 0.01 M HCl) was added to each well. The $O.D._{550\ nm}$ was measured with a microplate reader.

Staining of apoptotic cells. HeLa cells were seeded in a 12-well microplate at 2×10^5 cells/well. After incubation overnight, the cells were treated with complexes **1-3** (10 μM) for 24 h, and were then stained with cell permeable DNA intercalating fluorescent dye Hoechst 33342 (10 μg/ml) and examined under fluorescence microscope.

Metal uptake by cells

HeLa cells were seeded in 12-well plate at 2×10^5 cells/well and incubated for 24 h. Complex **1**, **2** or $AgNO_3$ was then added at 10 μM. After incubation for 2 h, the medium was removed and the cell monolayer was washed three times with phosphate buffered saline (PBS). The cells were then lysed in water and digested in 70% HNO_3 at 80 °C for 2 h. The digests were diluted with water to 10 ml for ICP-MS analysis.

Cellular activities of thiol-dependent redox enzymes

Preparation of cellular extracts. Cells were seeded at 2×10^5/well in 6-well plates and incubated for 24 h. Complexes **1-3** (10^{-9} to 10^{-4} M) were serially diluted and added to the cells (final DMSO concentrations ≤ 1%). After incubation for 1 h, the cells were washed three times with PBS, and 100 μl of ice-cold lysis buffer (50 mM phosphate buffer, pH 7.4, 1 mM EDTA, 0.1% Triton-X 100) were added to the cell layer. Cell lysis was carried on ice for 5 min and the cell lysates were collected and stored at -80 ^oC or assayed immediately.

Thioredoxin reductase (TrxR). Cell lysates (10 μg proteins) were added to a buffer (100 μl) containing 100 mM potassium phosphate, pH 7.4, 1 mM EDTA and 0.2 mM NADPH. Reaction was initiated by adding 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, 3 mM final) and the TrxR activities were determined as increases in $O.D._{412\ nm}$ in 10 min.

Glutathione peroxidase (GPx). Cell lysates (10 μg proteins) were added to a buffer (100 μl) containing 2 mM GSH, 1 U glutathione reductase, and 0.2 mM NADPH. Reaction was initiated by adding *tert*-butyl hydroperoxide (300 μM) and the NADPH oxidation was measured as decreases in $O.D._{340\ nm}$ in 10 min. GPx activities were determined by subtracting the spontaneous NADPH oxidation in the absence of *tert*-butyl hydroperoxide.

Glutathione reductase (GR). Cell lysates (10 μg proteins) were added to a mixture (100 μl) containing 100 mM phosphate, pH 7.4, 1 mM EDTA, 1 mM GSSG and 0.2 mM NADPH. Immediately, reaction was initiated by adding DTNB (3 mM final) and the increases in $O.D._{412\ nm}$ were measured for 10 min. GR activities were determined by subtracting the increases in $O.D._{412\ nm}$ in the absence of GSSG.

Kinetic analysis of tight-binding inhibition of TrxR [5]

Determination of inhibitory constant (K_i) derived from residual activities of pre-formed enzyme-inhibitor complexes.[6,7] Recombinant rat TrxR1 (ICMO Corp, Sweden; 1 nM) was reduced with NADPH (0.2 mM) and then incubated with **1** (0.3-10 nM) for 30 min in a 100 mM potassium phosphate buffer, pH 7.4 and 1 mM EDTA. The residual activities (initial rates of increases in $O.D._{412\ nm}$) were measured using 0.75, 1.5 or 3 mM DTNB. The data were fit into Eq. 1 using GraphPad Prism 3.0 software.

$$v_s/v_o = (E_t\text{-}K_i\text{-}I_t + ((I_t + K_i' - E_t)^2 + (4K_i E_t))^{1/2})/(2E_t) \quad (1)$$

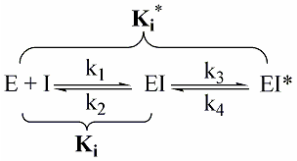
Eq. 1 describes the rate law of tight-binding inhibition in which case the inhibitor concentration is substantially depleted owing to formation of enzyme-inhibitor complex. In this equation, v_o is the observed velocity in the absence of inhibitor, v_s is the steady-state velocity in the presence of inhibitor, E_t is the total enzyme concentration, and I is the inhibitor concentration. The apparent inhibitory constant (K_i) so obtained was 0.67 nM. Average inhibitory constant (K_i^*) was calculated to be 36 pM using Eq. 2, which takes into account of competitive inhibition of the enzyme with the substrate and a predetermined K_m of 0.2 mM.

$$K_i = K_i^*(1+S/K_m) \quad (2)$$

Determination of K_i by progress curve analysis.[8,9] Complex **1** (3-100 nM) was added to a mixture containing NADPH (0.2 mM), TrxR1 (1 nM), DTNB (3 mM), 100 mM phosphate buffer, pH 7.4 and 1 mM EDTA. The progress curves were each fit into Eq. 3 using GraphPad Prism 3.0 software,

$$P = v_f t + ((v_i - v_f)/k_{app})(1\text{-}e^{-kappt}) \quad (3)$$

where P is the product concentration (increases in $O.D._{412\ nm}$), v_i and v_f are the initial and final steady-state velocities, respectively, and k_{app} is the apparent first-order rate constant for establishment of the final steady-state inhibition. A plot of the obtained k_{app} against the inhibitor concentrations followed a hyperbolic function, indicative of a two-step, tight-binding inhibition mechanism:



where EI is the initial collision complex, which subsequently undergoes isomerization to the final slow dissociating enzyme-inhibitor complex (EI*), k_3 is the forward

isomerization rate, and k_4 is the reverse isomerization rate. The k_3, k_4 and the dissociation constant of the initial collision complex EI (Ki) were obtained by fitting the data to Eq. 4.

$$k_{app} = k_4 + k_3 I_t (I_t + K_i(1+S/K_m)) \quad (4)$$

where I_t is the inhibitor concentration, S is the substrate (DTNB) concentration, and K_m is the Michaelis-Menten constant for reduction of DTNB by TrxR. Accordingly, k_3 = 0.011 s^{-1}, k_4 = 0.00014 s^{-1}, and K_i = 1.39 nM. The overall inhibitory constant K_i* was determined to be 18 pM using Eq. 5.

$$K_i^* = K_i(k_4/(k_3 + k_4)) \quad (5)$$

Effects of NADPH reduction of TrxR inhibition by complexes 1 and 2. TrxR1 (1 nM) was incubated with or without NADPH (0.2 mM) in reaction buffer (100 mM phosphate, pH 7.4, 1 mM EDTA) for 5 min. Complexes **1** or **2** (1-100 nM) was added and allowed to incubate for 30 min. DTNB (3 mM) and NADPH (0.2 mM) were then added. The TrxR activities were determined as the increases in $O.D._{412\ nm}$ over 10 min.

Probing the cysteine and selenocysteine residues of TrxR [10-12]

NADPH-reduced TrxR1 (0.1 μM) was treated with 1 (4 μM) or DMSO vehicle (2 %) in reaction buffer (100 mM phosphate buffer, pH 7.4, 1 mM EDTA) at room temperature for 1 h. 1 μl of the reaction mixture was taken out and added to new tubes containing 19 μl of 100 μM BIAM (Invitrogen) buffered with 200 mM Tris-HCl, pH 6.5 and 8.5, respectively). The incubation was carried out at 37 ^oC for 30 min to alkylate the remaining free –SeH and –SH groups of the enzyme. 20 μl of the reaction mixtures were mixed with loading buffer and subjected to SDS-PAGE on a 7.5% gel. The separated proteins were transferred to nitrocellulose membrane and the BIAM labelled proteins were detected with horseradish peroxidase conjugated streptavidin and enhanced chemiluminescence detection.

Size exclusion chromatography and ICP-MS analysis (SEC-ICP-MS) of TrxR

NADPH-reduced TrxR1 (0.18 μM) was treated with **1** (4 μM) or DMSO vehicle (2%) in reaction buffer (100 mM phosphate buffer, pH 7.4, 1 mM EDTA) at room temperature for 1 h and then subjected to SEC-ICP-MS analysis. For tryspin digestion, the reaction mixtures were passed through ultrafiltration membrane filter with molecular weight cut-off of 10 kDa (PALL Nanosep) and washed two times with milli-Q H_2O. The clean-up samples were reconstituted in 25 mM ammonium bicarbonate and digested with sequencing grade trypsin (Promega) (TrxR: trypsin = 10:1, 37 ^oC, overnight) before SEC-ICP-MS analysis. (*SEC-ICP-MS analysis was conducted by Prof. Ryszard Lobinski and Dr. Katarzyna Birela, Laboratory of Bio-Inorganic Analytical and Environmental Chemistry, CNRS, Pau, France)*

Tumour implantation in nude mice and *in vivo* drug treatment

The *in vivo* experiment was conducted in Pearl Materia Medica Development (Shenzhen) Limited and performed with approval from the Committee on the Use of Live Animals for Teaching and Research, The University of Hong Kong. SPF grade four-week-old female BALB/c AnN-nu mice (nude mice, 16–18 g) were used. Tumour cells (5×10^6) resuspended in RPMI medium were implanted by subcutaneous injection on the right flank of the mice. When tumours were approximately 50 mm^3 in size, animals were randomly separated into 3 groups to receive treatment of twice-a-week intraperitoneal injection of 10% PET vehicle control (where 10% PET = 6% polyethylene glycol 400, 3% ethanol, 1% Tween 80 and 90% PBS), complex **1** (100 mg/kg) or cyclophosphamide (30 mg/kg) for 8 times. After 28 days, the mice were sacrificed and the tumours were isolated and the tumour volume determined.

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Table S1 Crystallographic data and structure refinement for $[Au^{I}(TU)_2]Cl$ (**1**) and $[Ag^{I}(TU)_2]OTf$ (**2**)

	1	**2**
Empirical formula	$C_{136}H_{144}Au_4Cl_4N_{16}O_{16}S_8$	$C_{36}H_{38}AgCl_2F_3N_4O_7S_3$
Fw	3428.0	970.65
T/K	301(2)	294(2)
λ/Å	0.71073	0.71073
Crystal system	Monoclinic	Triclinic
Space group	*C* 2/*c*	*P*-1
a/Å	24.501(5)	12.0856(19)
b/Å	5.665(1)	13.665(2)
c/Å	24.806(5)	14.212(2)
α/°	90	72.566(3)
β/°	95.36(3)	83.781(3)
γ/°	90	66.621(3)
V/Å^3	3428.0(12)	2055.3(6)
Z	1	2
ρ_c/g cm^{-3}	1.669	1.568
μ/mm^{-1}	4.535	0.838
F(000)	1712	988
Crystal size/mm	0.4 × 0.15 × 0.1	0.28 × 0.22 × 0.14
$2\theta_{max}$/°	51.26	55.16
Index ranges	-29<=h<=28	-15<=h<=15
	-6<=k<=6	-17<=k<=17
	-30<=l<=30	-18<=l<=17
Reflections collected	11239	19439
Independent reflections	5310	9439
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data/restraints/parameters	3010/1/179	9439/9/518
GOF (on F^2)	1.159	1.01
Final R indices ($I > 2\sigma(I)$)	R_1 = 0.056, wR_2 = 0.149	R_1 = 0.051, wR_2 = 0.103
Largest diff. peak and hole/e Å^{-3}	0.566/-2.277	0.559/-0.418

Table S2 Tumour implantation in nude mice and *in vivo* drug treatment (no. of mice: 5)

	Tumor volume/mm^3		
	Vehicle control	Positive control (cyclophosphamide) (Dose: 30 $mg \cdot kg^{-1}$)	$[Au^{I}(TU)_2]Cl$ (**1**) (Dose: 100 $mg \cdot kg^{-1}$)
Day 9	318±309	-	209±95
Day 13	1421±625	3251±170	624±233
Day 17	2336±787	639±422	1232±229
Day 21	2802±1304	1032±594	1835±215
Day 25	3650±1722	1474±900	2389±595
Day 29	4437±222	1439±846	2742±805

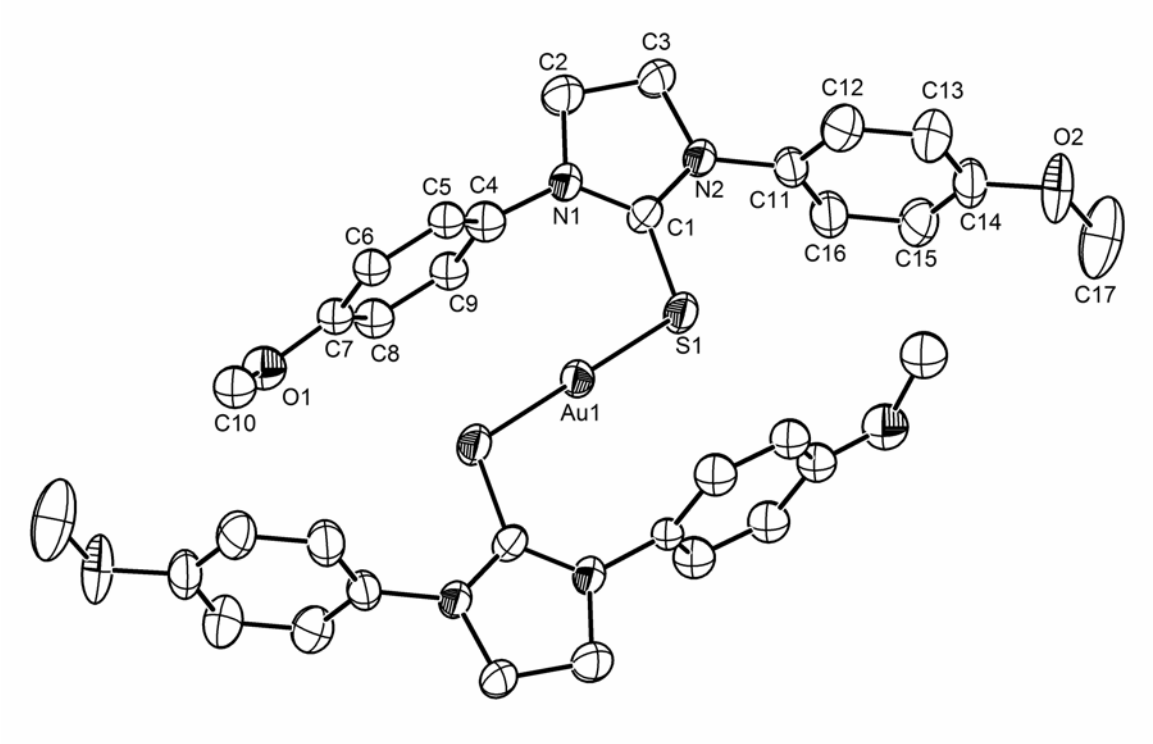

Fig. S1 ORTEP drawing of $[Au^{I}(TU)_2]Cl$ (**1**). Cl^- ion and hydrogen atoms were omitted for clarity. Selected bond distance (Å): Au1-S1 2.287(3). Selected bond angles (°): S1-Au1-S1' 180.0(1), C1-S1-Au1 110.3(3).

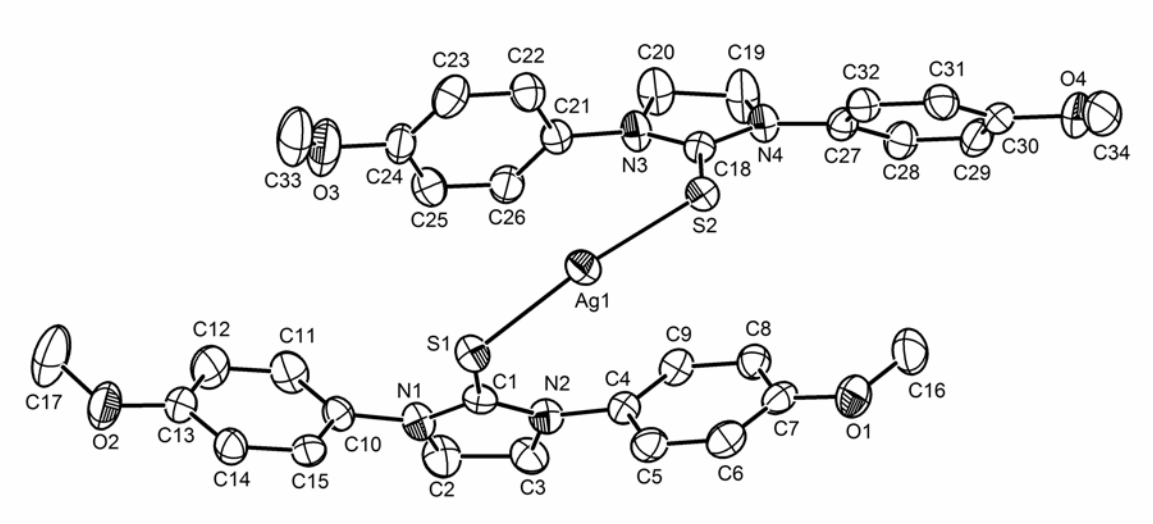

Fig. S2 ORTEP drawing of $[Ag^{I}(TU)_2]OTf$ (**2**). Triflate ion and hydrogen atoms were omitted for clarity. Selected bond distances (Å): Ag1-S1 2.4067(11), Ag1-S2 2.4075(10), Ag1···Ag1' 3.2894(7). Selected bond angles (°): S1-Ag1-S2 172.77(4), C1-S1-Ag1 104.11(12), C18-S2-Ag1 104.88(12).

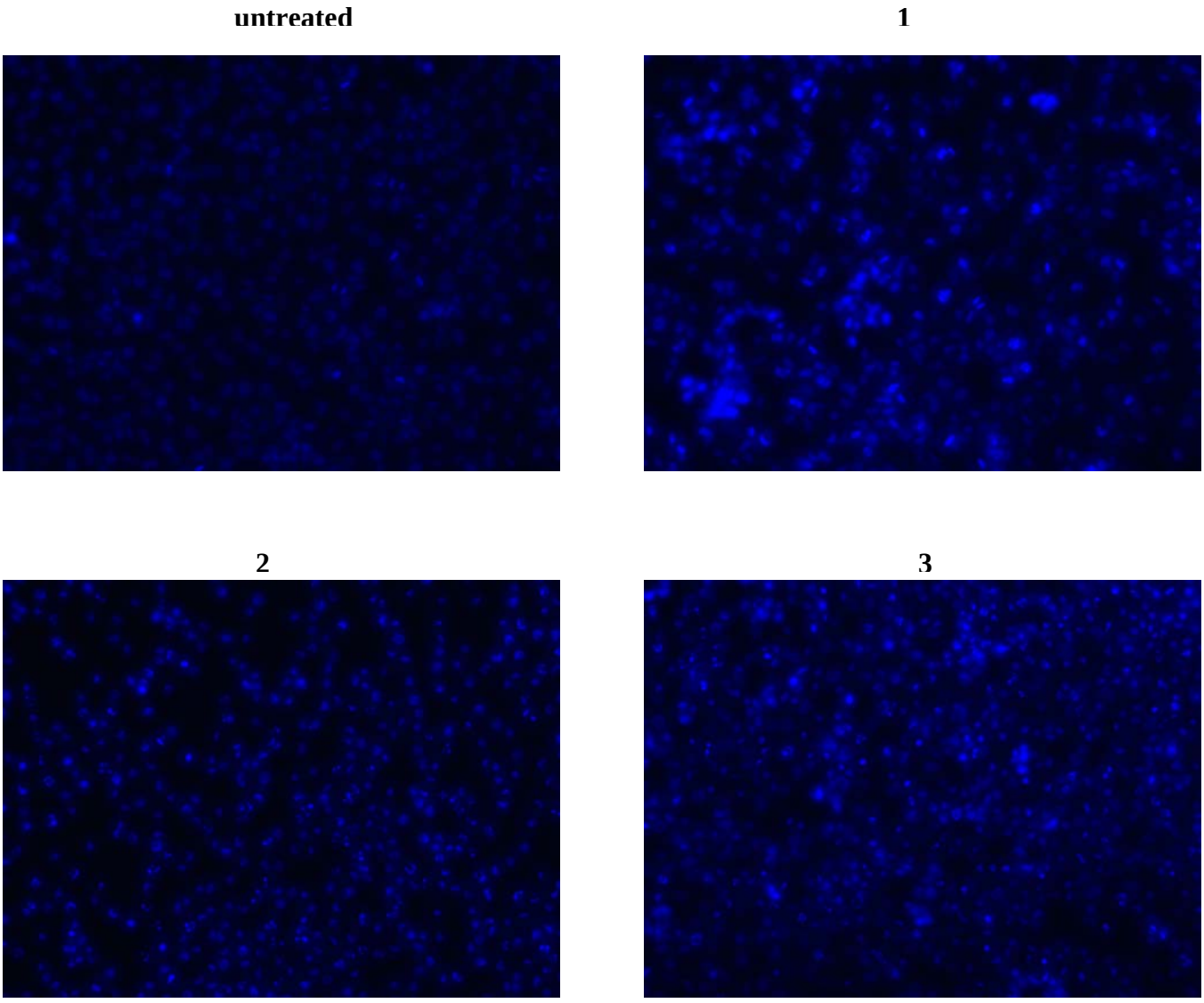

Fig. S3 Staining of apoptotic cells. In untreated cells, nuclear DNA showed round and homogenous blue staining. In cells treated with $[Au^{I}(TU)_2]Cl$ (**1**), $[Ag^{I}(TU)_2]OTf$ (**2**) and $[Cu^{I}(TU)_2]PF_6$ (**3**), the nuclear DNA appeared as bright blue condensed bodies or beads inside the nucleus, indicative of nuclear shrinkage and fragmentation associated with apoptosis.

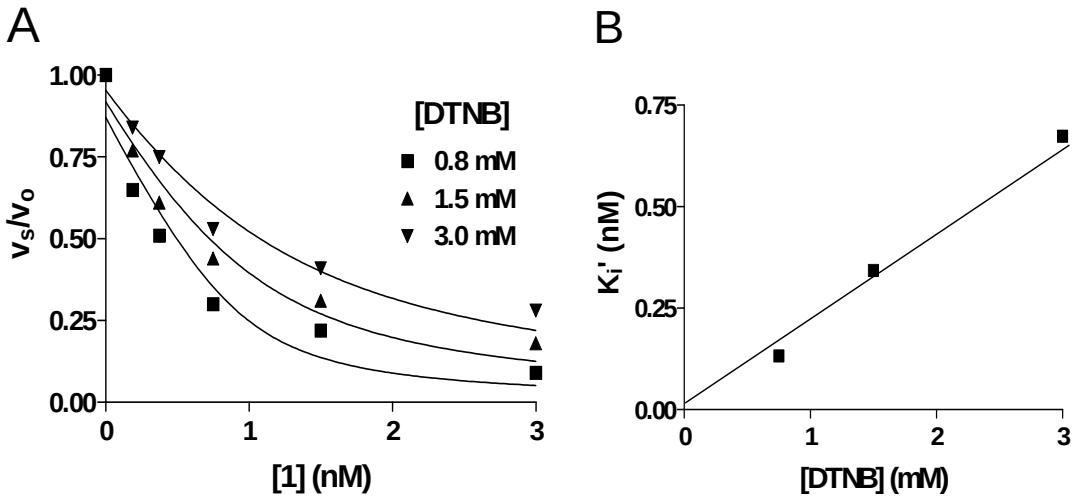

Fig. S4 Kinetic analysis of inhibition of TrxR pre-incubated with $[Au^{I}(TU)_2]Cl$ (**1**). A: Plot of relative steady state velocities against concentrations of **1**. Data were fit to Eq. 1 to obtain K_i'. B: Plot of K_i' against concentrations of DNTB substrate. Data were fit to Eq. 2 to obtain K_i.

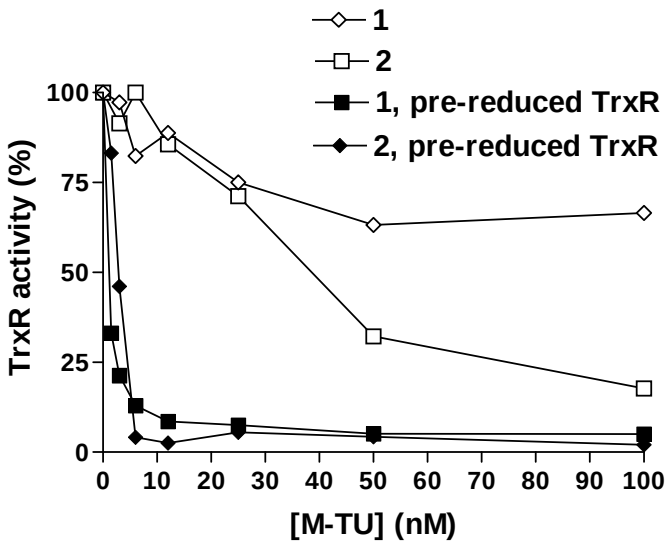

Fig. S5 Effects of pre-reduction of TrxR by NADPH on the enzyme inhibition by $[Au^{I}(TU)_2]Cl$ (**1**) and $[Ag^{I}(TU)_2]OTf$ (**2**).

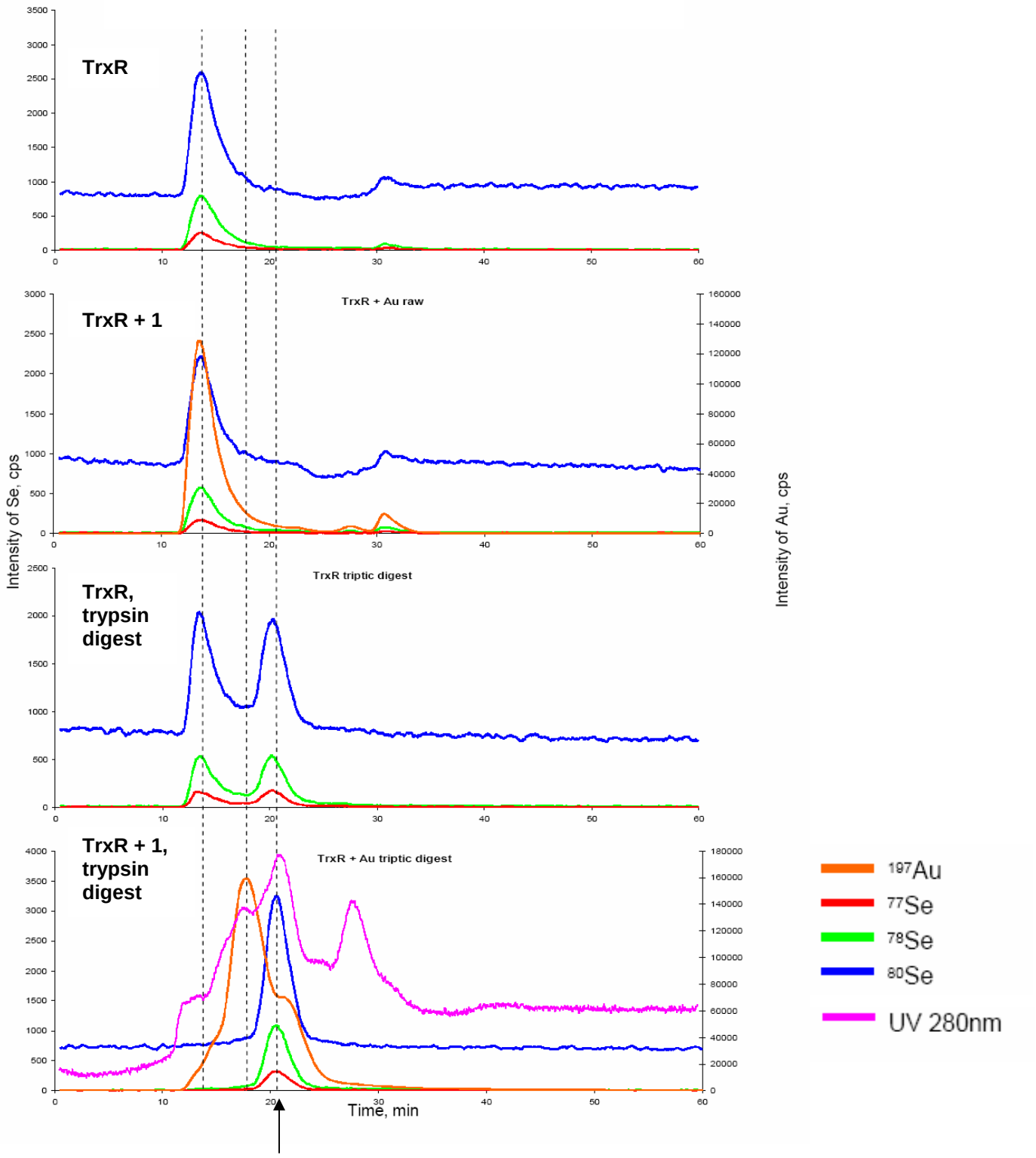

Fig. S6 SEC-ICP-MS analysis of TrxR treated with $[Au^{I}(TU)_2]Cl$ (**1**). Arrow indicates the peptide fraction co-eluted with Se and Au.