Supporting Information Available

"Thioredoxin reductase, an emerging target for anticancer metallodrugs. Enzyme inhibition by cytotoxic gold(III) compounds studied with combined mass spectrometry and biochemical assays"

Experimental section

Materials. The various gold(III) complexes were synthesized as previously described (see the respective refs). *Auranofin* and *aurothiomalate* were purchased at Vinci-Biochem; $Na[AuCl_4]$ and $[AuCl(PEt_3)]$ were from Sigma-Aldrich. Highly purified cytosolic thioredoxin reductase (TrxR1) was prepared according to Luthman and Holmgren (M. Luthman, A. Holmgren A, Biochemistry 1982, 21, 6628) starting from rat liver. Mitochondrial thioredoxin reductase (TrxR2) was purified from liver following the procedure of Rigobello et al. (M.P. Rigobello, M.T. Callegaro, E. Barzon, M. Benetti, A. Bindoli, *Free Radic. Biol. Med.* **1998**, *24*, 370.) Proteins of the purified isoforms of thioredoxin reductases were assayed with the Lowry et al. procedure (O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **1951**, *193*, 265).

Enzyme activity. Enzyme activity was determined by measuring the ability of thioredoxin reductases to directly reduce DTNB in the presence of NADPH (M. Luthman, A. Holmgren A, *Biochemistry* **1982**, *21*, 6628). To monitor the effect of redox conditions on the inhibition of TrxR by gold complexes, after 5 min of pre-incubation, the indicated 2 μM gold(I/III) compounds were added and the reaction lasted for further 10 min. At the end of incubation, the various samples were diluted with 30 μl of the same buffer and immediately applied to a Micro Bio-Spin (Bio-Rad) desalting column and centrifuged at 1,000 g for 6 min. TrxR1 activity was measured in the recovered filtrate as indicated above and reported as percentage of the control pre-incubated in the absence of NADPH and gold compounds.

BIAM assay. TrxR1 (0.120 mg/ml) was pre-reduced with 130 μM NADPH in 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA. Afterwards, increasing concentrations of gold(I/III) complexes were added in a final volume of 15 μl and the reaction continued for 1 hour at room temperature. Aliquots of 3 μl of each sample were treated with 100 μM BIAM in a final volume of 20 μl of 0.2 M Hepes-Tris at either pH 6.0 and pH 8.5 for 30 minutes at 37 °C. The reaction was stopped with 10 μl of loading buffer containing 0.1 M DTT and heated at 100 °C for 10 minutes. Samples were then subjected to SDS-PAGE (7.5 %) and subsequently transferred to a nitrocellulose membrane. The biotinyl carboxamido methyl labeled proteins were detected by HRP-conjugated streptavidine followed by ECL.

MALDI-ToF analysis. TrxR1 (10 μM) was reduced with 50 eq of freshly prepared NADPH solution and then incubated with different amounts of gold complexes (ranging from 1 to 10 eq) for 1 h at room temperature in 50 mM Tris-HCl, 1 mM EDTA pH 7.5. Unbound complex was removed by ultafiltration using Centricon YM-3 columns (Amicon). Each sample was prepared in triplicate and then analysed by MALDI ToF MS on an Ultraflex ToF/ToF III (Bruker Daltonics, Bremen, Germany). 1 μl of gold treated TrxR1 samples were mixed with 1 μl of matrix solution (sinapinic acid 20 mg/ml in acetonitrile:TFA 0.1% 2:1) and loaded on MALDI target. Protein spectra were acquired using the mass spectrometer in linear mode, setting the accelerating voltage at 25 kV, the IS2 at 23.15 kV, the delay time at 180 ns.

Gold content quantification by ICP AES analysis

The determination of Au concentrations in samples of *Aubipy*:TrxR1 3:1 (Au:protein ratio) incubated for 1 h at r.t and prepared as described above for MS analysis was performed in triplicate by a Varian 720-ES Inductively Coupled Plasma Atomic Emission Spectrometer.

MALDI ToF-MS of digests.

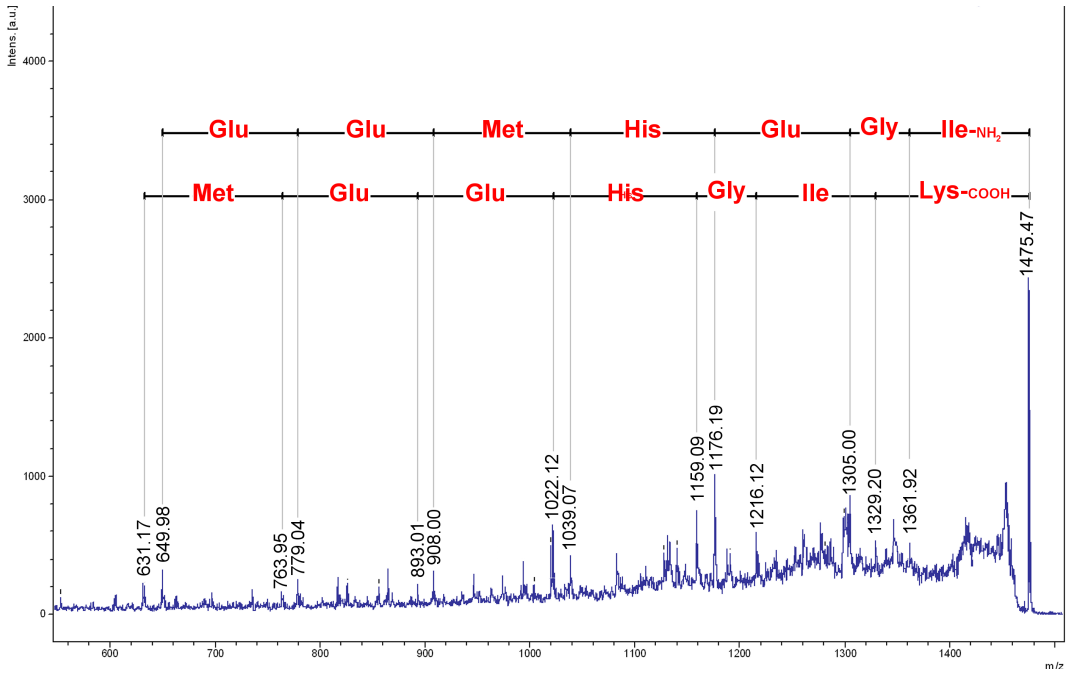
For peptide mass analysis, 30 μl of the TrxR1-gold treated samples were mixed with 1 μl of 1 M ammonium bicarbonate and trypsin (Sigma, St. Louis, MO, USA) was added to an enzyme/protein ratio of 1:20 (w/w). The mixture was incubated at 37 °C for 16 h; after this time 1 μl of the peptide mixture was mixed with 1 μl of matrix solution (20 mg/ml α-cyano-4-hydroxy cinnamic acid in acetonitrile: TFA 0.1% 2:1) and loaded on MALDI target as described before. Peptide spectra were acquired using the mass spectrometer in reflector mode, setting the accelerating voltage at 25 kV, the IS2 at 21.9 kV and the delay time at 20 ns.

Table S1. Inhibitory effects (IC_{50} [nM]) of gold(I/III) complexes on cytosolic (TrxR1) and mitochondrial (TrxR2) thioredoxin reductases.

	IC_{50} [nM]	
	TrxR1	TrxR2
Auranofin	0. 7 ± 0.1	4.3 ± 0.6
Aurothiomalate	5.0 ± 0.5	29.0 ± 0.5
Audien	2.9 ± 0.1	44.6 ± 1.0
Aupy	14.8 ± 0.3	494.0 ± 3.6
Aubipy	4.3 ± 0.1	28.1 ± 1.1
Auxyl	4.1 ± 0.3	17.0 ± 1.3

Aliquots of cytosolic (8 nM) and mitochondrial (15 nM) thioredoxin reductase were incubated as described in the Experimental section.

Figure S1. MALDI MS/MS spectrum of gold-bound ^{236}IGEHMEEHGIK^{246} tryptic peptide.
Almost complete /b/- and /y-/ion series (downer and upper lines, respectively) confirm the sequence of the peptide.



The isotopic pattern of the ion at 1475.43 shows that it corresponds to a single charged ion. The observed m/z can only be explained with the binding of an Au(I) (Mw = 197 Da) ion to the neutral *IGEHMEEHGIK* peptide (Mw=1278.6 Da). The hypothetical binding of an Au(III) ion to the *IGEHMEEHGIK*$^{2-}$ peptide, after loss of two H^+ from the glutamate residues, would be registered at

1473.43 m/z value. The binding of an Au(III) ion to the neutral *IGEHMEEHGIK* peptide would have a charge state 3+ and would be registered at 491.9 m/z.

The picture highlights the aminoacidic losses from the parent ion. Given that the Au(I) ion bound to the peptide is carrying the charge, all the shown b and y fragments maintain the Au(I) ion; in fact, loss of the Au(I) ion would result in the formation of a neutral fragment that could not be detected.

We proposed that the binding site for the Au ion can occur at one or the other histidine residues, since the b and y fragments containing no His (NH_2-IGE or GIK-COOH) are not detected. Clearly, loss of the amino acid carrying the charge results in the formation of a neutral, not detectable, fragment. In any case, the results cannot exclude that the ion at 1475.47 m/z is actually a mixture of the *IGEHMEEHGIK* peptide carrying the Au(I) ion either on His239 or His243.

Figure S2. Structures of the Ru(III) complexes used in this study.

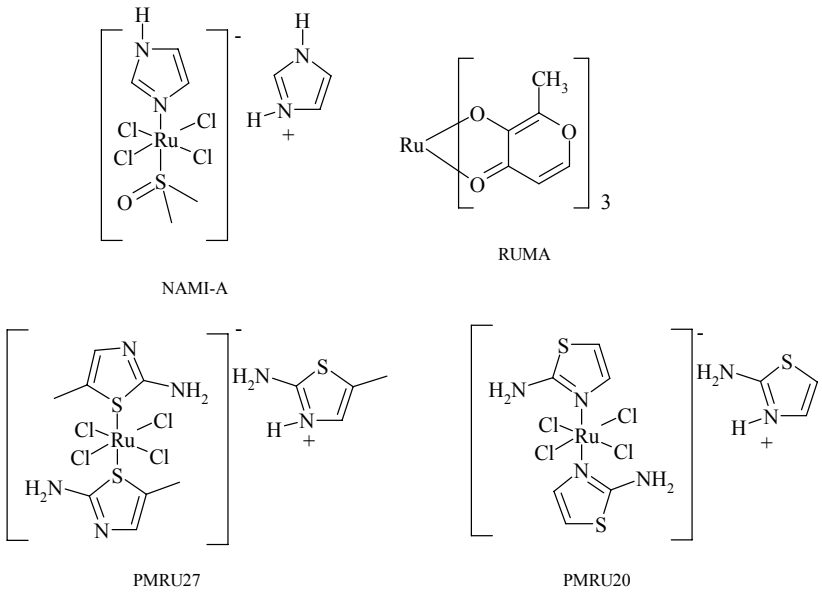


Figure S3. BIAM of rat TrxR1 after treatment with Ru(III) complexes 100 μM with respect to a control.