Characterization of hydrophilic gold(I) N-heterocyclic carbene (NHC) complexes as potent TrxR inhibitors using biochemical and mass spectrometric approaches

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ABSTRACT

We report here on the synthesis of a series of mono- and dinuclear gold(I) complexes exhibiting sulfonated bis(NHC) ligands and novel hydroxylated mono(NHC) Au(I) compounds, which were also examined for their biological activities. Initial cell viability assays show strong antiproliferative activities of the hydroxylated mono(NHC) gold compounds (**8** > **9** > **10**) against 2008 human ovarian cancer cells even after 1 h incubation. In order to gain insight into the mechanism of biological action of the gold compounds, their effect on the pivotal cellular target seleno-enzyme thioredoxin reductase (TrxR), involved in the maintenance of the intracellular redox balance, was investigated in-depth. The compounds' inhibitory effects on TrxR and glutathione reductase (GR) were studied comparatively, using either the pure proteins or cancer cell extracts. The results show strong and selective inhibitory effect of TrxR, specifically for the hydroxyl-functionalized NHC gold(I) complexes (**8** - **10**). Valuable information on the gold compounds' molecular reactivity with TRxR was gained using the BIAM (biotin-conjugated iodoacetamide) assay and performing competition experiments by mass spectrometry (MS). In good agreement, both techniques suggest binding affinity of the mono-NHC Au(I) complexes towards selenols and thiols. Notably, for the first time, bis-carbene formation from mono-carbenes in buffered solution could be observed by MS, which may provide new insights into the speciation mechanisms of bioactive Au(I) NCH complexes. Furthermore, the compounds' interactions with another relevant *in cellulo* target, namely telomeric G-quadruplex DNA - a higher-order DNA structure playing key roles in telomere function - was investigated by means of FRET melting assays. The lack of interactions with this type of nucleic acid secondary structure support the idea of selective targeting of the hydrophilic Au(I) NHC compounds towards proteins such as TrxR.

INTRODUCTION

Triggered by the success of cisplatin, metal-based anticancer therapy has immensely progressed in the scientific and clinical community over the last decades.[1,2] Early observations that cisplatin treatment may lead to drug-resistance[3] and is often associated with severe side effects (e.g. nephrotoxicity, neurotoxicity, ototoxicity)[4,5] fostered the development of different cytotoxic Pt(II) and Pt(IV) derivatives (e.g. carboplatin, oxaliplatin, satraplatin),[6–8] as well as of numerous antiproliferative agents based on other metals such as Ru[9], Rh[10], Cu[11] and Ag[12]. As a consequence, over the last years structure-activity relationships (SAR) of a plethora of metal-based compounds have been established, offering a useful perspective on the rational design of future metallodrugs.[13–18]

Notably, gold-based compounds have been successfully tested for their anticancer effects *in vitro* and *in vivo*.[19–22] Among them, auranofin ([2,3,4,6-tetra-*o*-acetyl-1-thio-*β*-ᴅ-glucopyranosato-S-(triethyl-phosphine)Au(I)]), Ridaura®) - a linear mixed thiolato/phosphine Au(I) drug (Figure 1) originally utilized to treat rheumatoid arthritis - was recently repurposed for its anticancer properties and is currently undergoing evaluation in three distinct clinical trials in US.[23,24] To a lesser extent, gold compounds have also been evaluated as antimicrobial[25], anti-malarial[26] and anti-HIV[27] agents. Despite their apparently favorable biological properties, coordination Au(I) complexes are subjected to extensive reactivity in physiological conditions, including ligand exchange reactions and reduction to colloidal gold. In this context, organometallic chemistry offers a convenient way for the stabilization of the Au(I) center and "fine-tuning" of the complexes' physiochemical properties *via* the establishment of direct metal-carbon bonds.

Within this framework, in the last decade, N-heterocyclic carbene ligands (NHCs), with chemical properties that resemble phosphine ligands,[28] became of interest for medicinal applications, and since then, the research on organometallic Au(I) NHC complexes as potential antiproliferative agents increased strongly. [29,30]

Concerning the possible mechanisms of biological action, a considerable amount of studies outline gold-mediated apoptosis related to the inhibition of specific intracellular proteins and enzymes.[19,20,22,31,32] Among these, a prominent representative is the zinc-finger protein poly(adenosine diphosphate (ADP)-ribose) polymerase 1 (PARP-1) which can be efficiently inhibited by certain Au(III) and Au(I) compounds.[33,34] Moreover, since their discovery as antiproliferative agents, various experiments on cancer cells revealed a variety of effects of Au(I) NHCs on cellular metabolism, including high increase of ROS formation and reduced mitochondrial activity, finally resulting in apoptotic cell death. Notably, most of these effects could be attributed to the strong and selective inhibition of the seleno-enzyme thioredoxin reductase (TrxR) by Au(I) NHC complexes.[33,35]

Thioredoxin reductases are homodimeric flavoproteins catalysing the NADPH-dependent reduction of thioredoxin (Trx), which is the major protein disulfide reductase in cells.[36] Mammalian TrxRs possess a conserved -Cys-Val-Asn-Val-Gly-Cys- catalytic site, which is also found in human glutathione reductase (GR), located in the FAD-binding domain of the enzyme, and a NADPH binding site. In addition, TrxRs contain a selenocysteine (Sec) residue at the C-terminal active site that is crucial for catalysis[37] and that distinguishes them from GR. The thioredoxin system regulates crucial cell functions such as viability and proliferation.[38] Indeed, both TrxR and Trx appear overexpressed in certain cancer types,[39,40] leading to the assumption that the thioredoxin system exerts a crucial role in tumor onset and progression, and that could be used as a pharmacological target.[41,42]

It is also worth mentioning that recently the biscarbene cationic complex [Au(9-methylcaffein-8-ylidene)$_2$]$^+$ (AuTMX$_2$, Figure 1) was reported to be a selective binder of telomeric G-quadruplexes (G4) by FRET DNA melting assays[43], and the two caffeine ligands (guanine analogues) stabilizing the Au(I) centre were shown to associate to guanine moieties in G4 via π-stacking interactions[44]. G4s are peculiar nucleic acid architectures adopted by guanine-rich DNA and RNA sequences, whose stability originates in the stacking of contiguous G-quartets

(a planar and cyclic K$^+$-promoted association of four guanines in a Hoogsteen hydrogen-bonding arrangement). Currently, G-quadruplexes are intensively studied, because they are suspected to play important roles in key cellular events: being found in eukaryotic telomeres and in promoter regions of identified oncogenes. Their stabilization by selective small molecules is thus currently investigated as a mean to control key cellular events and as a novel anticancer strategy.[45]

In the past few years, the number of studies on water-soluble and hydrophilic metal complexes has markedly increased to achieve novel anticancer agents. Although certain lipophilicity is crucial to cross (cancer) cell membranes, the hydrophilic character of a species is significant for *in vitro* investigations and efficient *in vivo* administrations correlating with enhanced bioavailability under physiological conditions. Therefore, studies on different metals, including Ru(II)[9,46–49], Pt(II)[50,51], Ag(I)[52] and Au(I, III)[50,53–58], coordinated to water-soluble phosphine ligands, *e.g.* 1,3,5-triaza-7-phosphaadamantane (PTA), 3,7-diacetyl-1,3,7-triaza-5-phosphabicyclo[3.3.1]nonane (DAPTA) and 1,4,7-triaza-9-phosphatricyclo[5.3.2.1]tridecane (CAP), or attached to appropriately functionalized N-heterocyclic carbene ligands, have shown promising cytotoxic activity. In this context Santini *et al.* have recently reported the synthesis of NHC ligands functionalized with hydrophilic groups such as esters or amides, which show moderate antiproliferative effects along with TrxR inhibition in the nanomolar range.[59]

In this work, the synthesis and characterization of a series of hydrophilic N-heterocyclic carbene ligands functionalized with sulfonate and hydroxyl groups and their corresponding mononuclear Ag(I) and Au(I) complexes (Figure 1) is presented. In order to investigate the effect of multinuclearity on the biological activity, a dinuclear bis(NHC) Au(I) complex was added to the series. The Au(I) NHC compounds were tested as potential anticancer agents against ovarian cancer cell line 2008 and thoroughly investigated for their binding behaviour towards TrxR by various biochemical assays and mass spectrometry. Furthermore, the

compounds' ability to induce ROS formation, as well as their potential to target G-quadruplex-DNA was investigated.
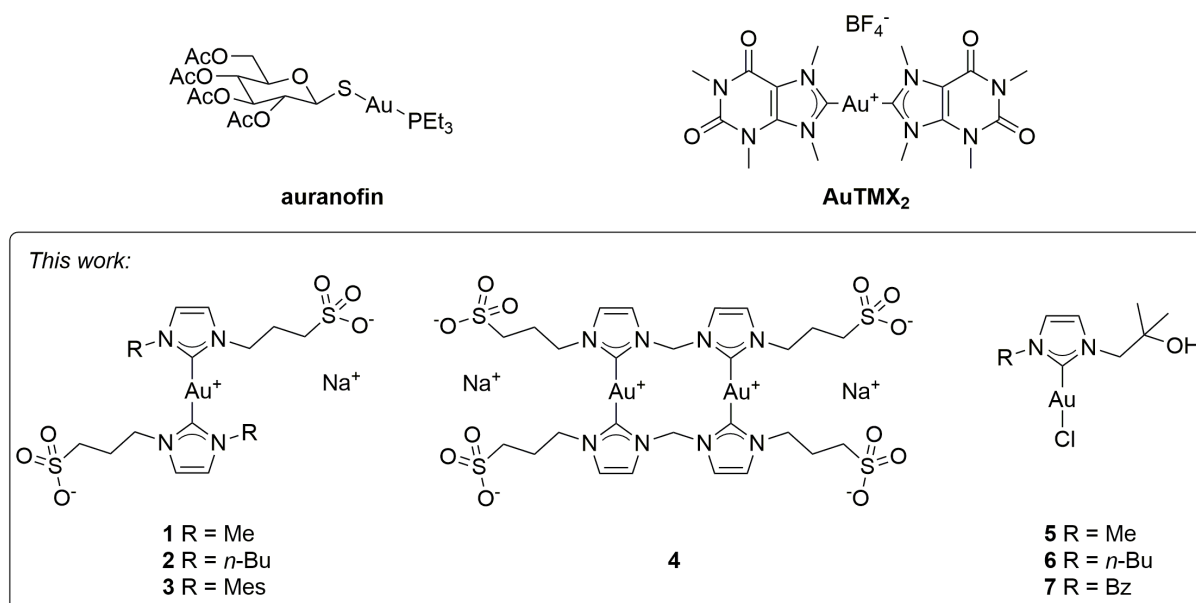

Figure 1. Structures of auranofin, G4-targeting AuTMX$_2$ and of the hydrophilic NHC Au(I) complexes studied in this paper. Mes = 2,4,6-trimethylphenyl.

RESULTS AND DISCUSSION

Synthesis and Characterization.

Starting from the sulfonated imidazolium salts **a-d**, three mono- (**1-3**) and a novel dinuclear (**4**) biscarbene Au(I) complexes have been synthesized (Scheme 1). The target compounds were obtained from an established two-step procedure consisting of the formation of silver carbene intermediates followed by the transmetalation to the Au(I) analogue.[60] Ligands **a-d** and the respective complexes **1** and **2** were prepared based on published procedures.[61–65] The synthesis of the Ag(I) precursors of **3** and **4** has been reported previously.[65–68] However, to date no direct synthetic pathway for **3** has been published. In this study compound **3** was prepared by the straightforward reaction of the respective silver biscarbene complex and equimolar amounts of the transmetalation agent Au(SMe$_2$)Cl. Complexes **3** and **4** were characterized by ^{1}H and ^{13}C NMR spectroscopy, ESI-MS and elemental analysis. As expected for this class of compound,[63] the Au-C$_{carbene}$ signals clearly appear around 180 ppm in the ^{13}C NMR spectra (see Fig. 2, 4 in

SI). Compounds **1-4** are highly soluble in water (> 300 mg/mL) and exhibit high stability in air in solid state and dissolved in D_2O for at least a month (no significant changes in the ^1H NMR spectra were observed).
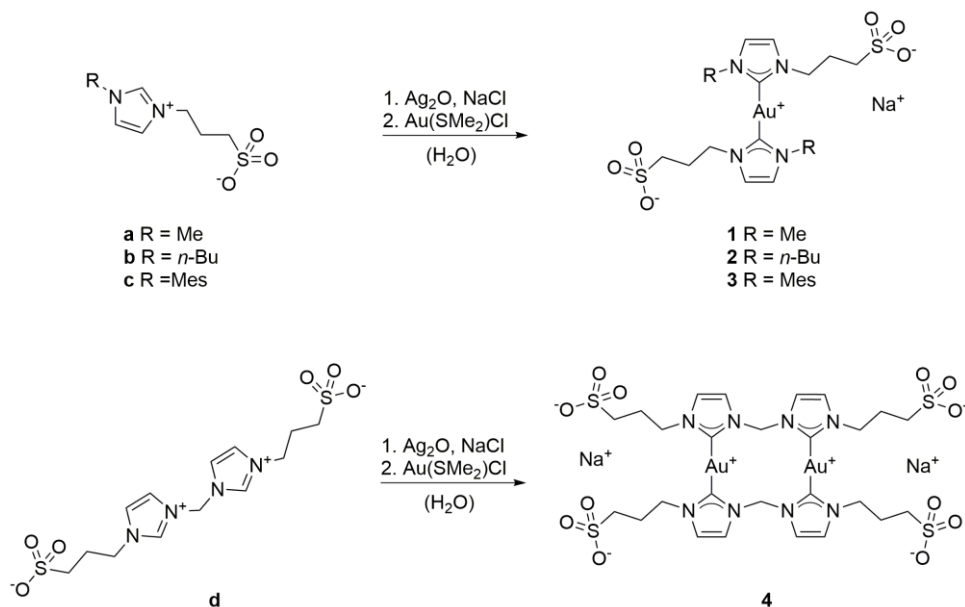
Scheme 1. Synthetic pathways of sulfonated mono- and dinuclear Au(I) NHC complexes **1-4**.

In addition, three novel monocarbene gold(I) complexes **8-10** featuring hydroxyl substituents were prepared (Scheme 2). The ligands **e-g** were synthesized in a two-step procedure starting from the neat reaction of 1*H*-imidazole and 1,2-epoxy-2-methylpropane. In contrast to the literature-known procedure by Yus *et al.* an excess of the epoxide was necessary at elevated temperature to yield 75% of 1-(1*H*-Imidazol-1-yl)-2-methylpropan-2-ol.[69] In the next step the latter was quaternized by iodomethane, 1-butylchloride and benzyl bromide, to obtain the respective imidazolium salts **e-g**, while **f** was firstly published by Martin-Matute *et al.*[70]

Compounds **e-g** were then deprotonated in the presence of a slight excess of Ag_2O under the formation of silver carbene complexes **5-7**. The reactions were performed at room temperature in dry dichloromethane except for **5**, where little acetonitrile is needed for complete dissolution. After short reaction times (< 2 h) the ligand systems are fully converted to the silver complexes. The transmetalation reaction was performed by using equimolar amounts of $Au(SMe_2)Cl$,

yielding the respective monocarbene Au(I) complexes **8-10** after 24 h at r.t. Compounds **8-10** readily dissolve in solvents such as MeCN, CH_2Cl_2, acetone and DMSO but are only slightly soluble in water (< 10 mg/mL). They are stable in air both as a solid and in solutions of acetonitrile and DMSO (no significant changes in the ^{1}H NMR spectra were observed) for at least one month.

The signals in the ^{1}H and ^{13}C NMR spectra of ligands **e-g** appear as expected. Typically, upon quaternization of the nitrogen atom of ligand precursor 1-(1*H*-Imidazol-1-yl)-2-methylpropan-2-ol the acidic proton signals at the C2 position are shifted downfield from 7.45 ppm to 8.73 ppm, 9.30 ppm and 9.10 ppm, respectively. In addition, the hydroxyl proton signals of **e-g** give sharp singlets in the range of 3.60 ppm – 5.30 ppm. The assignment of these signals can be confirmed by the addition of one drop D_2O to each sample in CD_3CN which leads to their complete disappearance due to rapid hydroxyl proton exchange with deuterium.

The ^{1}H NMR spectra of silver complexes **5-7** display similar chemical shifts but, as expected, vary significantly in the absence of the acidic proton signal in the C2 position at 8.73 ppm (**e**), 9.31 ppm (**f**) and 9.78 ppm (**g**), respectively, for the silver species. In the ^{13}C NMR spectra the Ag-C$_{carbene}$ signals emerge in a narrow range from 184.51 ppm (**5**) to 181.36 (**6**). Due to the temperature-dependent fluxional behavior of Ag(I) NHC complexes in solution, the carbene carbon signals appear each as sharp singlets instead of doublet of doublets (based on the coupling constants of the silver isotopes [107]Ag and [109]Ag) at room temperature.[71]

The Au(I) NHC compounds **8-10** have also been characterized by elemental analysis, ^{1}H and ^{13}C NMR and ESI mass spectrometry. By means of ^{1}H NMR spectroscopy the hydroxyl protons can be observed as sharp singlets at 2.93 ppm (**8**), 2.96 (**9**) and 2.99 (**10**), respectively (see Fig. 11, 13, 15 in SI). These signals can be easily identified by proton exchange experiments with D_2O (which lead to the disappearance of the singlets). Moreover, the formation of the Au(I) complexes is confirmed through ^{13}C NMR spectroscopy, since the carbenes adjacent to the

more electron rich gold atom in **8**-**10** display upfield shifted signals (~10 ppm) compared to the respective silver species.
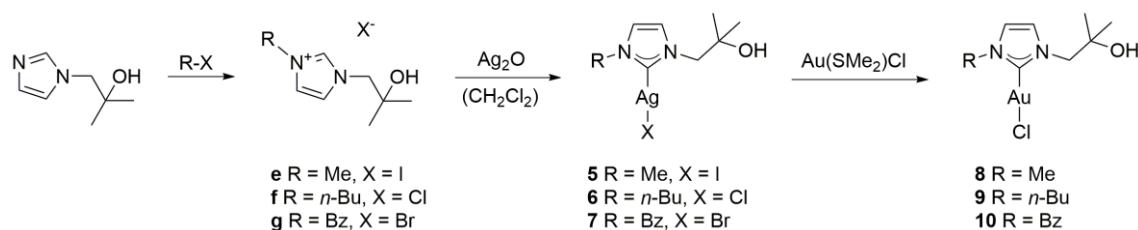

Scheme 2. Synthesis of targeted hydroxylated gold(I) NHC complexes **8**-**10** *via* transmetalation of the respective silver compounds **5**-**7**.

Studies of stability in solution.

Initially, the stability of the mono and bis(NHC) Au(I) complexes was investigated for **4** and **8**. For this purpose, the compounds were incubated at 37 °C in aqueous solution and aliquots for TOF-MS analysis were taken after 30 min and 24 h, respectively. In solution, complex **8** rapidly hydrolyses and the parent signal vanishes within a few minutes. Instead, a single mass signal was observed at m/z 505.1992, suggesting the formation of the biscarbene of the type $[(L^1)_2Au]^+$ (m_{theor} = 505.1878, Table S1, supplementary material), where L^1 is the ^{i}PrOH-NHC ligand derived from **e** (Scheme 3, right). This mass signal is not observed in buffered solution (20 mM NH_4CO_3, pH 7.4) where the hydrolysis product of **8** seems to be stabilized by forming an ammine complex with the buffer component corresponding to $[L^1Au(NH_3)]^+$ found at m/z 368.1270 (m_{theor} = 368.1037, Scheme 3, left). Thus, the complex moiety $[Au(NHC)]^+$ remains unaltered at 37 °C for at least 24 h and, as expected, the mono-carbenic Au(I) complex is reactive towards nucleophiles (as in NH_3) and is able to undergo chloride exchange reactions, which is promising for further enzyme inhibition assays. In contrast, the bis(NHC) complex **4** turned out to be exceptionally stable in aqueous solution and the parent mass signals ($[(L^2)_2Au_2]^{2-}$ and $[(L^2)_2Au_2 + Na]^-$ with L^2 being the deprotonated ligand **d**) are the only

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detected signals from this compound in both aqueous and buffered solution for over 24 h (Table S1, Figure 5C).
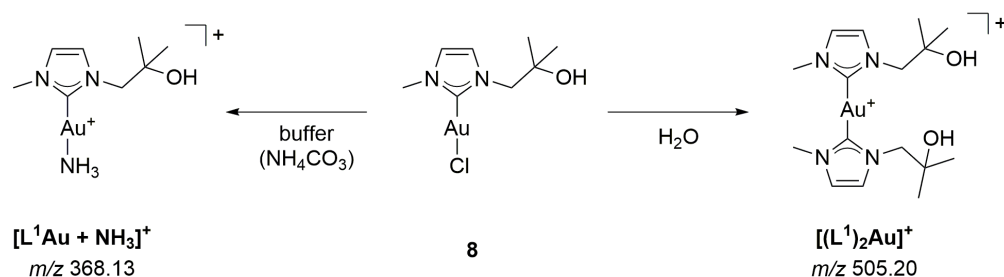
Scheme 3. Chemical behaviour of **8** in aqueous and buffer (20 mM NH_4CO_3, pH 7.4) solution as observed in MS studies.

Inhibition of isolated TrxR and GR.

In order to elucidate the reactivity towards the putative biological target, all Au(I) NHC complexes were initially *in vitro* evaluated for their inhibitory potential towards TrxR. The gold(I) compounds **1**-**4** and **8**-**10** were tested against both purified cytosolic (TrxR1) and mitochondrial (TrxR2) thioredoxin reductase according to established protocols as described in the experimental section. In addition, the inhibition of glutathione reductase (GR), which exhibits a closely related structure to TrxR but lacks selenocysteine residues was investigated to determine the selectivity of enzyme binding. In Table 1 the half maximal inhibitory concentrations (IC_{50}) of the NHC Au(I) complexes for enzyme inhibition are reported in comparison to bench-mark gold(I) compound auranofin. Most strikingly, the monometallic bis(NHC) complexes **1**-**3** were less efficient and could inhibit cytosolic TrxR at much higher concentrations (4.8-23 µM) than the monocarbenic compounds **8**-**10** (0.018-0.073 µM). These results are in line with previously reported studies on other Au(I) NHC systems.[72–75] This finding correlates to the results from the stability investigations where a biscarbenic compound proves to be less prone to nucleophilic attack than a monocarbenic complex. Due to the higher stability of the two organometallic Au-C bonds the gold complexes **1**-**3** are most probably impeded to undergo facile ligand exchange reactions with Cys and/or Sec residues in order to bind to TrxR. Interestingly, the bimetallic bis(NHC) gold complex **4** is slightly more potent at

lower concentrations than its monometallic congeners **1**-**3**, demonstrating that TrxR could be more efficiently targeted by doubling the amount of reactive gold centres. In comparison, the activity of TrxR is less influenced in the presence of the respective ligand **d** (4.8 µM) demonstrating the necessity of the gold centres for inhibition of the seleno-enzyme. Remarkably, the activity of cytosolic TrxR is efficiently inhibited by all three monocarbenic Au(I) complexes displaying IC_{50} values in the nanomolar range, comparable to auranofin, following the order of activity **9** > **10** > **8**. Regarding the inhibition of mitochondrial thioredoxin reductase (TrxR2) the order of activity is slightly changed to **9** > **8** > **10**. As expected, the inhibitory effect on both TrxR isoforms drops significantly by a 10-100-fold of magnitude, approximately, when treating with the metal-free ligand **g** instead of the respective complex **10**. In comparison, in all cases the activity of GR is inhibited at substantially higher concentrations (> 10 µM), indicating a preferential binding mode especially of complexes **4** and **8**-**10** for purified TrxR.

Table 1. Inhibition of cytosolic (TrxR1) and mitochondrial (TrxR2) thioredoxin reductase, in comparison to glutathione reductase (GR).

Compound	TrxR1	TrxR2	GR
	IC_{50} (µM)[a]		
d	4.8 ± 0.1	n.d.	> 10
g	> 2	> 10	> 10
1	5.1 ± 0.2	n.d.	> 10
2	23.0 ± 2.0	n.d.	> 10
3	3.66 ± 0.09	n.d.	> 10
4	0.41 ± 0.05	3.3 ± 0.09	> 10
8	0.073 ± 0.002	0.31 ± 0.04	> 10
9	0.018 ± 0.001	0.27 ± 0.02	> 10
10	0.025 ± 0.003	0.5 ± 0.1	> 10
auranofin	0.007 ± 0.001	0.004 ± 0.001	> 10

Antiproliferative effects.

The compounds which show the strongest inhibitory activity towards TrxR (**8-10**) were preliminarily evaluated for their antiproliferative effects in the human ovarian cancer cell line 2008. In addition, the dinuclear complex **4** was included due to its stronger TrxR inhibition compared to the other bis(NHC) compounds. For this purpose, the cells were treated with the respective gold compounds for different times over 24 h and the final cell viabilities were quantified using the MTT technique as detailed in the experimental section. Remarkably, whereas dinuclear biscarbenic **4** is not cytotoxic at a concentration of 60 µM, all three tested mono(NHC) Au(I) complexes are remarkably potent even after only 1 h of incubation time, displaying IC_{50} values in the low µM range (13.2 ± 3.5 µM, 17.5 ± 1.5 µM and 24.5 ± 4.2 µM) following the order of cytotoxicity **8** > **9** > **10** (Figure 2). At 24 h, the compounds cytotoxicity did not display significant differences, suggesting the idea that either reduced accumulation or compounds' inactivation by extracellular components may be in place. It is worth mentioning that recently, Ott and coworkers reported on the negative effect of serum components of the cell culture media on the bioavailability of Au(I) NHC complexes, influencing their speciation.[76]
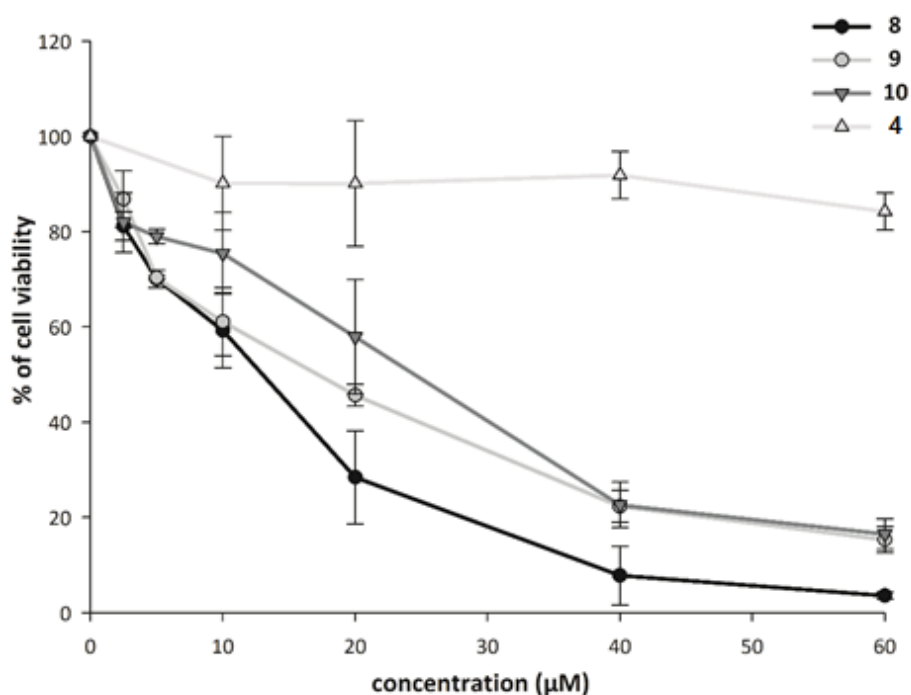
Figure 2. Dose-dependent viability of ovarian carcinoma cells 2008 after 1 h of incubation in the presence of selected NHC Au(I) compounds **4** and **8**-**10**.

Inhibitory effects on TrxR and GR activity in cell lysates.

In order to endorse a possible contribution of the TrxR inhibition of the compounds to their observed antiproliferative effects on cells, enzyme activity was also assessed in cell extracts from 2008 pre-treated with compounds **4** and **8**-**10** (20 µM, 40 µM and 60 µM) for 3 h (Figure 3). For the dinuclear biscarbene complex **4**, a negligible inhibition is obtained even at the highest examined concentration (60 µM), which correlates well with its observed non-cytotoxicity (Figure 2). Notably, at a concentration close to the IC_{50} value for the antiproliferative effects (20 µM) monocarbenic gold species **8**-**10** cause a substantial decrease of thioredoxin reductase activity (> 70%) following the same order of antiproliferative activity **8** > **9** > **10**. A further increase of the compound concentration leads to a significant decline in TrxR activity, reaching ca. 90% inhibition at 60 µM. By contrast, GR activity is clearly less affected with approximately 50% inhibition only at higher complex concentrations. In comparison, ligand **g** is completely ineffective on both TrxR and GR activities (data not shown).

Hence, these studies coincide well with the order of observed enzyme selectivity TrxR > GR (Table 1) as well as the determined cytotoxicity, which appears to correlate well with the TrxR inhibition by the gold compounds.
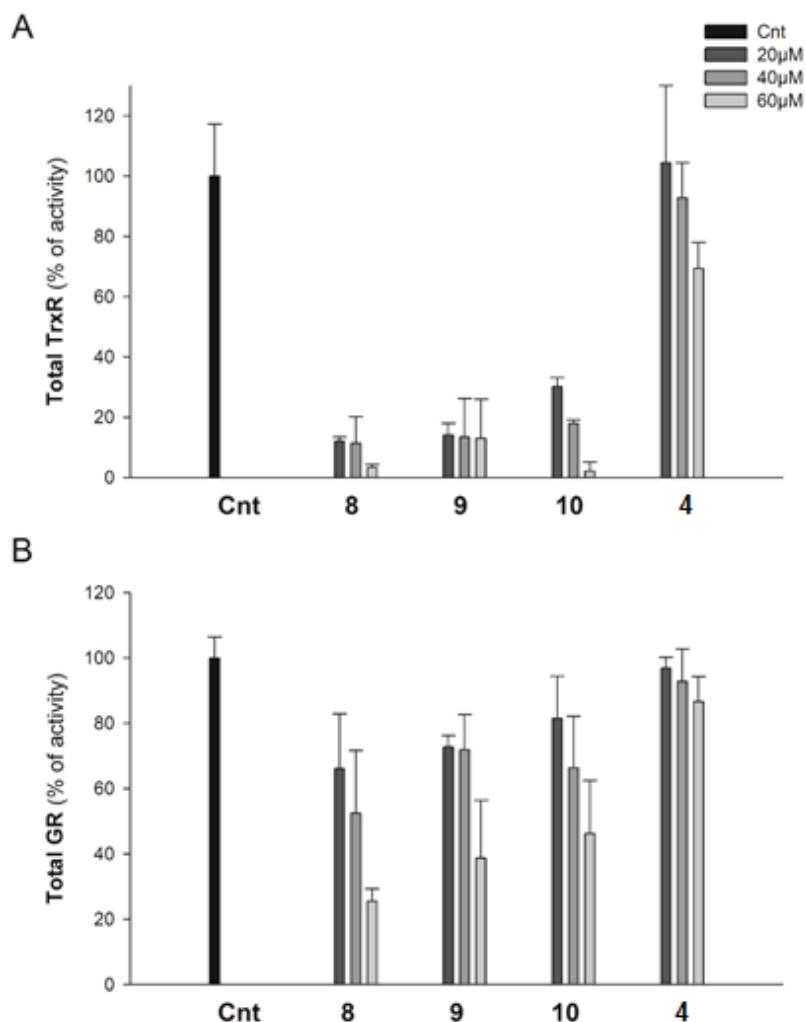
Figure 3. Total thioredoxin reductase (TrxR) and glutathione reductase (GR) activities in 2008 cell lysates. Cells (7×10^5) were treated with compounds **4** and **8**-**10** at the indicated concentrations in PBS/10 mM glucose medium as described in the experimental section. After 3 h, the cells were lysed and processed. (A) TrxR activity (B) GR activity.

BIAM assay.

It was of further interest to investigate possible binding sites and thus the status of the C-terminal redox active centre of reduced TrxR when treated with the mono(NHC) gold complexes **8**-**10** and, for comparative reasons, with bis(NHC) species **1**-**3**. For this purpose, the BIAM assay, a reliable biochemical method using BIAM (biotin-conjugated iodoacetamide)

which can alkylate TrxR in a pH-dependent manner, was applied. Based on the different pK_a values of Cys (8.5^{77}) and Sec (5.2^{78}) at pH 6.0 only selenocysteine (and low pK_a cysteines) are alkylated, whereas at pH 8.5 both Cys and Sec are modified. The labelled enzymes were detected with horseradish peroxidase-conjugated streptavidin, triggering chemiluminescence (see experimental section for details). However, pre-treatment of TrxR with thiol- and/or selenol-binding species (such as gold) blocks BIAM-binding and thus reduces the chemiluminescent effect. The obtained results are shown in Figure 4.

The blotting band intensities after the treatment of 100 µM of gold complexes **1**-**3** indicate a generally poor interaction with the enzyme although a slight preference for Sec is observed for **1** and **2**. In accordance with the TrxR inhibition (see results above), 100 µM of compounds **8** and **9**, respectively, completely derivatize the enzyme at pH 6 implying their strong binding to selenol groups, but also with Cys as it appears at pH 8.5. However, in contrast to **8**, a slight preference for Sec is observed for **9**. On the other hand, lower concentrations of compound **10** (*e.g.* 20 µM) are sufficient to significantly and to some extent selectively bind to selenol groups (Figure 4B: 42%, lane d *vs.* 58%, lane d'). The immunoblotting indicates a strong TrxR-binding of the three mono(NHC) Au(I) complexes, where a slight binding preference for Sec is observed for **9** and **10**.
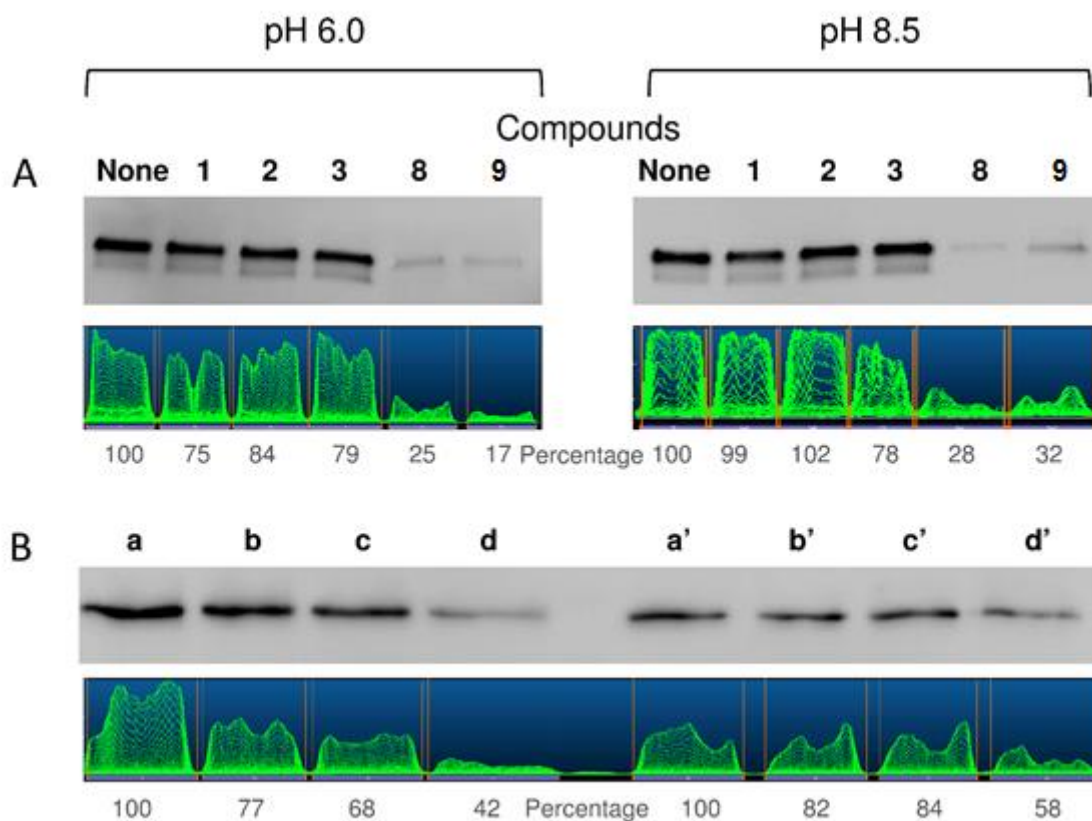
Figure 4. BIAM assay after treatment of TrxR with selected Au(I) NHC compounds. A: gold compounds (100 μM) were incubated with TrxR (1 μM). Then, aliquots (8 μL) of the reaction mixture were added to 50 μM biotinylated iodoacetamide (BIAM) in buffer either at pH 6 (0.1 M Hepes-Tris) or pH 8.5 (0.1 M Tris-HCl). The samples were subjected to SDS-PAGE as described in the experimental section. Volumes of densitometric analysis were shown in the lower panel. B: Interaction of compound **10** with TrxR was analyzed at different concentrations (a: none; b: 5 µM; c: 10 µM; d: 20 µM) and densitometric analysis is reported (Nine Alliance software).

MS studies on interactions with mixtures of amino acids and tripeptide GSH.

The mechanisms of action of metal-based therapeutic agents are at least partly dictated by the molecular reactivity of the respective agent. Thus, characterizing activation mechanisms and ligand exchange reactions especially under competitive conditions give insights into potential binding preferences in the cellular context.[79,80] Previously reported studies, showed that the binding preferences of several organometallic and coordination Au(III) compounds with selenocysteine (Sec) correlate well with the inhibitory effect on TrxR and their antiproliferative activity.[81]

The reported approach was further extended to two representative Au(I) NHC compounds, namely to bimetallic bis(NHC) **4** and the most cytotoxic complex **8**. In order to investigate the binding preferences towards differently functionalized amino acids in a competitive manner, *e.g.* as in proteins, the complexes were treated with mixtures of Glu (carboxylic acid), His (imine), Met (thioether) and Cys (thiol). The amino acids were chosen based on previous literature providing indications for their gold-binding character.[20,82] In order to obtain additional information on the selectivity towards the selenol residue at the C-terminal site of TrxR, the mixture was extended by Sec. Furthermore, the interaction with tripeptide glutathione (GSH), which acts *e.g.* in the protection of cellular components against ROS, was tested. For this purpose, the reactivity of the compounds was separately investigated over 24 h in the presence of a Glu : His : Met : Cys (1:1:1:1) mixture, a mixture of Glu : His : Met : Cys : Sec (1:1:1:1:1) and GSH alone, respectively. The reactions were carried out in purely aqueous solution and in buffered solution using ammonium carbonate (20 mM, pH 7.4), respectively.

Studies on the binding character of the bimetallic Au(I) complex **4** display only parent mass signals in the presence of GSH and the amino acid mixtures, respectively, in both aqueous and buffered solution over 24 h (see Figure 5C). This observed exceptional stability towards nucleophiles is in accordance with the weak enzyme inhibition in 2008 cell lysates (Fig. 3) and could substantiate the non-cytotoxic effect as described above (Fig. 2).

The reaction between **8** and substrate GSH under both aqueous and buffered conditions yields reaction prodrugs corresponding to $[Au + GSH - 2H]^-$, $[L^1Au + GSH - 2H]^-$ and $[2(L^1Au) + GSH - H]^+$ (Figure 5A left, Table S1). Less intense mass signals can be observed in buffered solution suggesting that ammine coordination (see Scheme 3) may be responsible for the reduced reaction kinetics compared to purely aqueous solution.

Interestingly, when exposing mono(NHC) Au(I) compound **8** to a mixture of amino acids containing Glu : His : Met : Cys in buffer and aqueous solution, cysteine adducts corresponding to $[L^1Au + Cys - 2H]^-$ ($m/z = 470.0845$, $m_{theor} = 470.0813$, Table S1) and $[2(L^1Au) + Cys - H]^+$

(m/z = 822.1796, m_{theor} = 822.1901, Table S1) (Figures 5A right, 6A) are exclusively formed

and no reaction with Glu, His and Met is observed after 24h. Similar adducts are also observed

in the presence of both Cys and Sec when incubated with the mixture containing

Glu : His : Met : Cys : Sec in aqueous solution (Figure 6B). Notably, additional mass signals of

gold adducts with selenocysteine as in $[L^1Au + Sec – 2H]^-$ (m/z = 518.0275, m_{theor} = 518.0284,

Table S1) (Figure 5B) and $[2(L^1Au) + Sec - H]^+$ (m/z = 870.1276, m_{theor} = 870.1372, Table S1)

(Fig. 6B) are also observed. Therefore, it is suggested that complex **8** hydrolyses the chlorido

ligand and interacts with both selenocysteines and cysteine, which is in accordance with the

observed BIAM results.
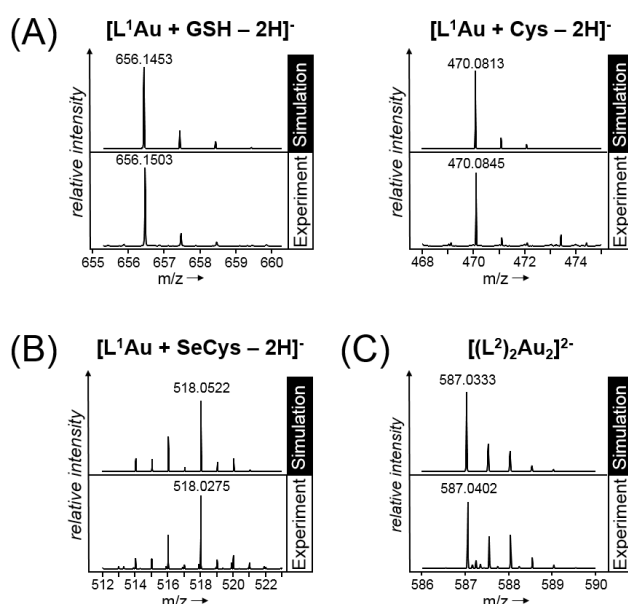
Figure 5. Experimental and simulated mass spectra of several identified Au reaction products: (**A**) The reaction between **8** and GSH alone (left); and between **8** and Glu : His : Met : Cys yielded L^1Au-GSH and L^1Au-Cys adducts (right) under buffered conditions. (**B**) In purely aqueous conditions, the reaction between **8** and Glu : His : Met : Cys : Sec yielded an L^1Au-Sec adduct in addition to the Cys adduct. (**C**) Compound **4** is stable towards amino acids and GSH and the parent mass signals are the only observed species.
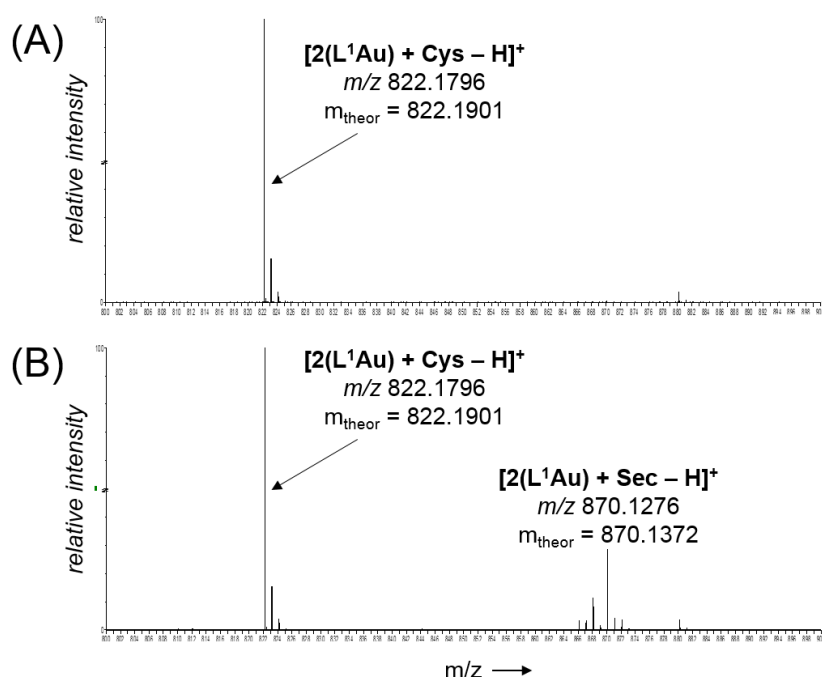
Figure 6. Compound **8** was incubated with an equimolar mixture of Glu : His : Met : Cys (**A**) and Glu : His : Met : Cys : Sec (**B**) in aqueous solution for 24 h.

ROS production in the presence of gold compounds.

Reactive oxygen species (ROS) are products of the physiological mitochondrial cell metabolism and are involved in cellular redox homeostasis. Their formation may perturb the cellular antioxidant defence system. It has been shown previously that TrxR inhibition alters cell conditions causing an increase of H_2O_2 concentration, as well as an imbalance of the cellular redox state leading to mitochondrial membrane permeabilization (MMP), cell swelling and thus apoptosis. In this study, the effect of gold compounds **8**-**10** (10 µM – 30 µM) on the ROS production was evaluated in 2008 ovarian cancer cells over a period of 2 h using the peroxide-sensitive fluorescent probe CM-H_2DCFDA (see Experimental section for details). In Figure 7 the results of gradual ROS formation detected in the presence of 20 µM compounds are reported. Overall, all of the three gold compounds stimulate ROS formation. Among these, in particular complex **8** induces the highest amount of ROS, which is in good agreement with the results from the cytotoxicity assay and the strongest inhibitory effect towards the redox-related enzyme TrxR.

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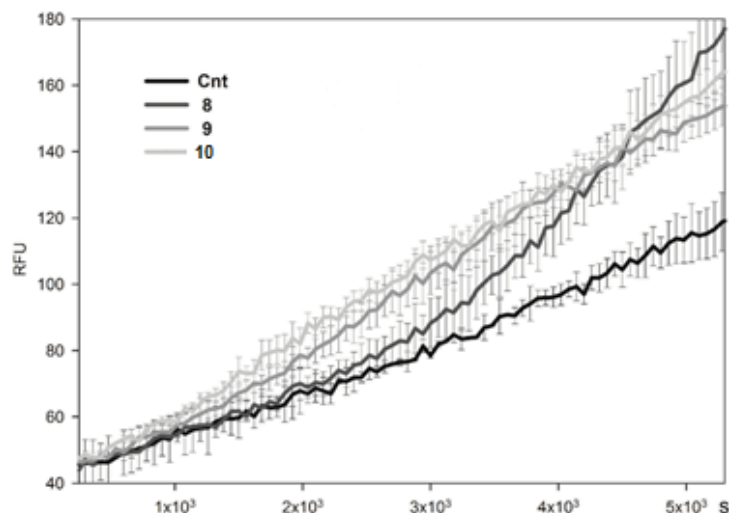
Figure 7. Cellular ROS estimation with CM-H$_2$DCFDA. Ovarian cancer cells 2008 (1 x 10^4) were treated in PBS/10 mM glucose with 20 µM gold compounds. Reaction was followed as fluorescence increase as described in Experimental details. Wavelengths: E$_x$ 485 nm, E$_m$ 527 nm.

Interactions with G-quadruplexes by FRET melting assay.

In order to gain further mechanistic insights and inspired by our recent results that highlighted the exquisite G4-quadruplex selectivity of the bis(caffeine) complex AuTMX$_2$ against telomeric G4-DNA, we subsequently investigated the properties of representative bis(NHC) complexes **2**-**4** and the most potent species **8** as G-quadruplex DNA stabilizers.[43] Experiments were performed with the most classically used G-quadruplex-forming oligonucleotide F21T, which mimics the human telomere sequence (FAM-d[^{5}'G$_3$(T$_2$AG$_3$)$_3$^{3}']-TAMRA). The stability imparted by a ligand (expressed as ΔT$_{1/2}$ values, in °C) is readily monitored through the modification of the FRET phenomenon (fluorescence resonance energy transfer) and enables an easy quantification of its apparent affinity for quadruplex-DNA. Thus, the compounds (1 µM) were incubated with fixed amounts of G4 for 10 min at room temperature as described in the experimental section. The AuTMX$_2$ complex was also screened for the purpose of comparison.

As it can be observed in Figure 8, none of the newly tested Au(I) NHC complexes is able to substantially induce the stabilization of the G4 structure, confirming their binding preferences to protein targets such as TrxR.
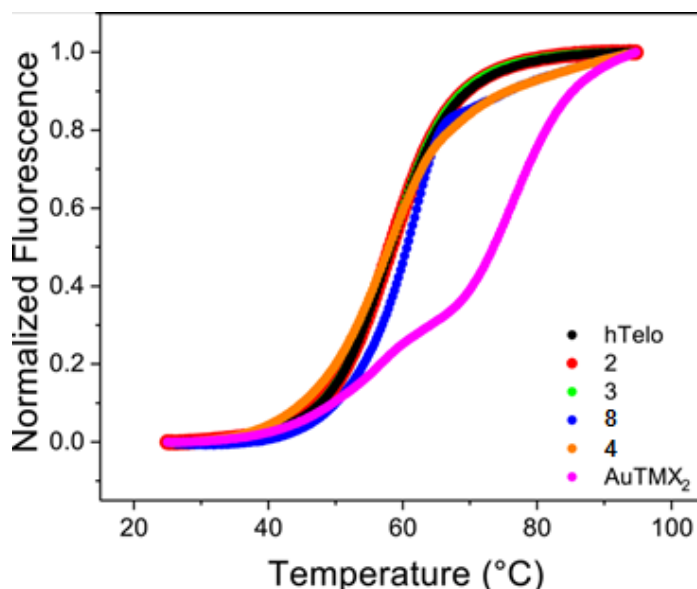
Figure 8. FRET melting results of experiments carried out with 0.2 μM DNA (FAM-d[$^{5'}$G$_3$(T$_2$AG$_3$)$_3$$^{3'}$]-TAMRA, hTelo) without (black curve) or with Au(I) NHC complexes (colored curves) in 60 mM potassium cacodylate buffer (pH 7.4).

CONCLUSION

A series of novel hydroxylated mono(NHC) Au(I) and mono- and dinuclear gold compounds featuring sulfonated bis(NHC) ligands is reported. The compounds have been comparatively screened for their inhibitory effects on purified TrxR and GR and on cell extracts *in vitro*. In contrast to the sulfonated bis(NHC) Au(I) complexes, all three monocarbenic compounds are considerably selective at targeting the seleno-enzyme TrxR over glutathione reductase. In fact, the most active inhibitors are the Au(I) monocarbene complexes **8**-**10** with similar IC$_{50}$ values as auranofin in the nanomolar range. The same compounds were also tested for their antiproliferative effects on the human ovarian cancer cell line 2008 and display promising activity (**8** > **9** > **10**) after only 1 h of incubation time. Notably, a similar order of activity is observed for the inhibition of TrxR in 2008 cell lysates, indicating a possible correlation between the protein binding and the cytotoxic effect.

The proposed mechanism of TrxR inhibition involves direct coordination of the Au(I) center to the Sec in the enzyme active site, as suggested by the limited inhibition of the enzyme glutathione reductase lacking the Sec-containing domain. However, two independent techniques, namely BIAM assays and mass spectrometric studies confirm possible binding of Sec and Cys. Notably, the selected compounds show high selectivity towards protein targets with respect to nucleic acids as determined *via* FRET melting assays with telomeric G-quadruplex DNA.

Thus, the obtained results clearly indicate a correlation between cytotoxicity and TrxR inhibition in cancerous cells; however, further studies are necessary to validate our mechanistic hypothesis and to exclude other possible intracellular targets. It should also be noted that the obtained MS results on the stability of the complexes in solution indicate bis-carbene formation from mono-carbenes in buffered aqueous solution. The latter type of reactivity has never been described before for similar systems. In general, the influence of buffer and cell culture media components (including serum proteins) on the metallodrug speciation should be always considered to avoid compound's deactivation as well as to determine the real pharmacologically active species. For example, the negative influence of serum proteins on Au(I) NHC complexes cellular accumulation has been demonstrated recently demonstrated.[76]

Finally, while the hydroxylated mono(NHC) gold(I) complexes **8**-**10** display promising anticancer activities and the selected scaffolds constitute attractive leads for further drug design, the quest for selectivity of such complexes towards cancer cells needs still to be addressed. In fact, recent *ex vivo* studies on mono- and bis-NHC Au(I) compounds, featuring a 1-butyl-3-methyl-imidazol-2-yilidene scaffold, demonstrated that the compounds display severe toxicity in healthy kidney tissue, even higher than cisplatin, while being potent anticancer agents *in vitro*.[83] We suggest that, in order to improve gold NHC complexes selectivity, the robust carbene scaffold may be derivatized to conjugate the metal complex to targeting moieties, such as peptides and antibodies.

EXPERIMENTAL DETAILS

General. All synthetic manipulations were performed under an argon atmosphere using standard Schlenk techniques. Solvents were obtained water-free from an Mbraun solvent purification system. Deionized water (type II quality) was obtained with a Millipore Elix 10 UV Water Purification System. All other reagents were purchased from commercial suppliers and were used without further purification. All reactions including silver carbene compounds as reactant or product were performed under exclusion of light.

Ligand precursor 1-(2,4,6-Trimethylbenzyl)-1*H*-imidazole[84] and ligands 3-(1-Methyl-1*H*-imidazol-3-ium-3-yl)propane-1-sulfonate (**a**)[61], 3-(1-Butyl-1*H*-imidazol-3-ium-3-yl)propane-1-sulfonate (**b**)[62], 3-(1-Mesityl-1*H*-imidazol-3-ium-3yl)propane-1-sulfonate (**c**)[66], zwitterionic ligand **d**[65] and 3-Butyl-1-(2-hydroxy-2-methylpropyl)-1*H*-imidazol-3-ium chloride (**f**)[70] were prepared according to the given literature. Gold(I) NHC complexes **1** and **2** were prepared based on published synthetic procedures.[63]

For the characterization of compounds NMR spectra were recorded on a Bruker Avance III 400. Chemical shifts are given in parts per million (ppm) and the spectra were referenced by using the residual solvent shifts as internal standards (^{1}H NMR: $CDCl_3$, δ 7.26; CD_3OD, δ 3.31; CD_3CN, δ 1.94; D_2O, δ 4.79; ^{13}C NMR: $CDCl_3$, δ 77.16; CD_3OD, δ 49.00; CD_3CN, δ 1.32). Electrospray ionization (ESI) mass spectrometry (MS) analyses were performed on a Thermo Scientific LCQ/Fleet spectrometer by Thermo Fisher Scientific. Elemental analysis was obtained from the microanalytical laboratory of TUM.

Synthetic Procedures. *1-(1H-Imidazol-1-yl)-2-methylpropan-2-ol.* The synthesis is performed according to a modified procedure by Yus *et al.*[69] 1*H*-Imidazole (3.0 g, 44 mmol, 1.0 equiv.) and 1,2-epoxy-2-methylpropane (5.1 mL, 57 mmol, 1.3 equiv.) are stirred at 60°C for 22 h under argon atmosphere. The reaction mixture is rinsed with Et_2O and then dried under vacuum. The product is obtained as colorless solid (4.6 g, 75%). ^{1}H NMR ($CDCl_3$): δ = 7.44 (s, 1H,

NC*H*N), 6.92 (s, 1H, NC*H*CHN), 6.90 (s, 1H, NCHC*H*N), 4.18 (s, 1H, O*H*), 3.84 (s, 2H, NC*H₂*), 1.19 (s, 6H, C(C*H₃*)₂). ^{1}H NMR (CD₃CN): δ = 7.45 (s, 1H, NC*H*N), 7.03 (s, 1H, NC*H*CHN), 6.88 (s, 1H, NCHC*H*N), 3.87 (s, 2H, NC*H₂*), 1.11 (s, 6H, C(C*H₃*)₂). ^{13}C NMR (CDCl₃): δ = 138.28, 128.07, 120.82, 69.75, 58.10, 27.10.

*1-(2-Hydroxy-2-methylproply)-3-methyl-1H-imidazol-3-ium iodide (**e**).* In a pressure tube a solution of 1-(1*H*-Imidazol-1-yl)-2-methylpropan-2-ol (3.06 g, 21.9 mmol, 1 equiv.) and methyl iodide (1.36 mL, 21.9 mmol, 1 equiv.) in acetonitrile (15 mL) is heated at 80°C for 2 h. The solvent is evaporated under vacuum at 40°C and the resulting oily raw product is rinsed with CHCl₃. Filtration and subsequent drying under vacuum affords a yellow solid (4.3 g, 69%). ^{1}H NMR (CD₃CN): δ = 8.73 (s, 1H, NC*H*N), 7.48 (t, 1H, NC*H*CHN), 7.37 (t, 1H, NC*H*CHN), 4.18 (s, 2H, NC*H₂*), 3.87 (s, 3H, NC*H₃*), 3.61 (s, 1H, O*H*), 1.19 (s, 6H, C(C*H₃*)₂). ^{13}C NMR (CD₃CN): δ = 137.74, 124.84, 123.65, 69.89, 59.96, 37.01, 26.89. MS-ESI (*m/z*): [5 - I]+ calc'd, 155.12; found, 155.04.

*3-Benzyl-1-(2-hydroxy-2-methylpropyl)-1H-imidazol-3-ium bromide (**g**).* In a pressure tube a solution of 1-(1*H*-Imidazol-1-yl)-2-methylpropan-2-ol (2.00 g, 14.3 mmol, 1.00 equiv.) and benzyl bromide (2.04 mL, 17.1 mmol, 1.20 equiv.) in toluene (20 mL) is heated at 100°C for 18 h. The resulting oil is separated from the residual solvent and washed with toluene (3 x 10 mL). After drying *via* rotary evaporator the product is obtained as brown oil (4.18 g, 94%). ^{1}H NMR (CD₃CN): δ = 9.10 (s, 1H, NC*H*N), 7.54 (t, 1H, NC*H*CHN), 7.46-7.41 (m, 5H, Ar*H*), 7.39 (t, 1H, NC*H*CHN), 5.40 (s, 2H, NC*H₂*(C₆H₅)), 5.01 (s, 1H, O*H*), 4.23 (s, 2H, NC*H₂*C(CH₃)₂OH), 1.17 (s, 6H, C(C*H₃*)₂). ^{13}C NMR (CD₃CN): δ = 137.72, 135.09, 130.16, 130.05, 129.49, 125.40, 122.30, 69.68, 59.99, 53.69, 26.95. MS-ESI (*m/z*): [7 - Br]+ calc'd, 231.32; found, 231.20.

Sodium[1-mesityl-3-(3-sulfonatopropyl)imidazole-2-ylidene]gold(I) (3). For the synthesis of the Ag(I) intermediate a modified procedure of a literature-known process was used.[66] Ligand **c** (260 mg, 0.84 mmol, 1.0 equiv.) is dissolved in deionized water (10 mL) and Ag_2O (195 mg, 0.84 mmol, 1.0 equiv.) is added. After the suspension has been stirred at 50°C for 22 h NaCl (49 mg, 0.84 mmol, 1.0 equiv.) is added and the mixture is stirred for 30 min at r.t. After filtration over Celite® $Au(SMe_2)Cl$ (124 mg, 0.42 mmol, 0.5 equiv.) is added to the clear solution and is stirred at r.t. overnight. The solvent is evaporated at 40°C under reduced pressure, re-dissolved with methanol and filtered three times over Celite®. The compound is obtained as colorless solid after drying in vacuo (300 mg, 85%). ^1H NMR (CD_3OD): δ = 7.58 (d, $^3J_{H\text{-}H}$ = 1.89 Hz, 2H, H^{Im}), 7.22 (d, $^3J_{H\text{-}H}$ = 1.87 Hz, 2H, H^{Im}), 7.05 (s, 4H, $m\text{-}H^{Mesityl}$), 4.04 (t, $^3J_{H\text{-}H}$ = 6.83 Hz, 4H, $NCH_2CH_2CH_2$), 2.57 (t, $^3J_{H\text{-}H}$ = 7.4 Hz, 4H, $NCH_2CH_2CH_2$), 2.38 (s, 6H, $p\text{-}CH_3^{Mesityl}$), 2.15-2.08 (m, 4H, $NCH_2CH_2CH_2$), 1.88 (s, 12H, $o\text{-}CH_3^{Mesityl}$). ^{13}C NMR (CD_3OD): δ = 185.56, 141.16, 136.40, 136.34, 130.41, 124.42, 123.55, 49.86, 48.92, 28.37, 21.30, 17.74. MS-ESI (*m/z*): [10-Na+2H]+ calc'd, 813.20; found, 813.45; [10+H]+ calc'd, 835.19; found, 835.38; [10+Na]+ calc'd, 857.17; found, 857.62. Elem. Anal. Calc'd for $C_{30}H_{38}AuN_4NaO_6S_2$·NaCl: C, 40.34; H, 4.29; N, 6.27; S, 7.18; Found: C, 40.68; H, 4.53; N, 6.22; S, 7.28.

Disodium[1,1'-methyl-bis(3-(sulfonatopropyl)imidazole-2-ylidene]gold(I)] (4). The synthesis of the respective silver(I) intermediate is performed according to previous literature.[68] Ligand **d** (209 mg, 0.53 mmol, 1.0 equiv.) is dissolved in deionized water (10 mL) and Ag_2O (123 mg, 0.53 mmol, 1.0 equiv.) is added. After the suspension is stirred at 50°C for 2 h, NaCl (34 mg, 0.58 mmol, 1.1 equiv.) is added and the mixture is stirred for 30 min at r.t. After filtration over Celite® $Au(SMe_2)Cl$ (157 mg, 0.53 mmol, 1.0 equiv.) is added to the clear solution and is stirred at r.t. overnight. The solvent is evaporated at 45°C under reduced pressure, re-dissolved with deionized water and filtered three times over Celite®. The compound is obtained as

colorless solid after drying in vacuo (238 mg, 73%). ^{1}H NMR (D$_2$O): δ = 7.50 (s, 4H, H^{Im}), 7.36 (d, 4H, H^{Im}), 6.96 (d, $^2J_{H\text{-}H}$ = 13.99 Hz, 2H, NCH_2N), 6.14 (d, $^2J_{H\text{-}H}$ = 13.99 Hz, 2H, NCH_2N), 4.33-4.28 (m, 8H, NCH_2CH$_2$CH$_2$), 2.86-2.80 (m, 8H, NCH$_2$CH_2CH$_2$), 2.27 (t, $^3J_{H\text{-}H}$ = 6.91 Hz, 8H, NCH$_2$CH$_2$CH_2). ^{13}C NMR (D$_2$O/CD$_3$OD): δ = 184.28, 123.95, 122.45, 64.08, 51.00, 48.68, 27.30. MS-ESI (m/z): [10-2Na+3H]+ calc'd, 1117.09; found, 1177.09; [10-Na+2H]+ calc'd, 1199.07; found, 1199.07; [10+H]+ calc'd, 1221.05; found, 1221.05; [10+3Na]+ calc'd, 1243.04; found, 1243.04. Elem. Anal. Calc'd for C$_{26}$H$_{36}$Au$_2$N$_8$Na$_2$O$_{12}$S$_4$: C, 25.58; H, 2.97; N, 9.18; Found: C, 25.31; H, 3.05; N, 9.01.

*[3-Methyl-1-(2-hydroxy-2-methylpropyl)imidazol-2-ylidene]silver(I) iodide (**5**).* To a solution of 1-(2-hydroxy-2-methylproply)-3-methyl-1H-imidazol-3-ium iodide (100 mg, 0.35 mmol, 1.00 equiv.) in dry CH$_2$Cl$_2$/MeCN = 6/1 (3.5 mL) Ag$_2$O (44.0 mg, 0.19 mmol, 0.53 equiv.) is added. This suspension is stirred for one hour at room temperature. The mixture is then filtered over Celite® and used for the subsequent transmetalation step. ^{1}H NMR (CD$_3$CN): δ = 7.20 (d, $^3J_{H\text{-}H}$ = 1.76 Hz, 1H, H^{Im}), 7.12 (d, $^3J_{H\text{-}H}$ = 1.73 Hz, 1H, H^{Im}), 4.12 (s, 2H, NCH_2), 3.84 (s, 3H, NCH_3), 1.17 (s, 6H, C(CH_3)$_2$). ^{13}C NMR (CD$_3$CN): δ = 184.54, 124.01, 122.81, 70.59, 62.29, 39.13, 27.63.

*[3-Butyl-1-(2-hydroxy-2-methylpropyl)imidazol-2-ylidene]silver(I) chloride (**6**).* The silver carbene intermediate is synthesized based on a previous literature.[70] Imidazolium salt **f** (225 mg, 0.97 mmol, 1.00 equiv.) is dissolved in dry CH$_2$Cl$_2$ (6 mL) and Ag$_2$O (119 mg, 0.51 mmol, 0.53 equiv.) is added subsequently. After the reaction mixture is stirred for two hours at room temperature, it is filtered over Celite® and used for the subsequent transmetalation step. ^{1}H NMR (CD$_3$CN): δ = 7.23 (d, $^3J_{H\text{-}H}$ = 1.82 Hz, 1H, H^{Im}), 7.15 (d, $^3J_{H\text{-}H}$ = 1.76 Hz, 1H, H^{Im}), 4.09-4.05 (m, 4H, NCH_2C(CH$_3$)$_2$OH, NCH_2(CH$_2$)$_2$CH$_3$), 1.79-1.71 (m, 2H, NCH$_2$CH_2CH$_2$CH$_3$),

1.33-1.23 (m, 2H, N(CH$_2$)$_2$CH_2CH$_3$), 1.28 (s, 6H, C(CH_3)$_2$), 0.90 (t, $^3J_{\text{H-H}}$ = 7.30 Hz, 3H, N(CH$_2$)$_3$CH_3). ^{13}C NMR (CD$_3$CN): δ = 181.43, 124.41, 121.46, 70.23, 62.80, 52.18, 34.34, 28.03, 20.40, 14.01.

*[3-Benzyl-1-(2-hydroxy-2-methylpropyl)imidazol-2-ylidene]silver(I) bromide (**7**).* Imidazolium salt **g** (300 mg, 0.96 mmol, 1.00 equiv.) is dissolved in CH$_2$Cl$_2$ (7 mL) and Ag$_2$O (134 mg, 0.58 mmol, 0.60 equiv.) is added. The suspension is stirred for 45 minutes at room temperature. The mixture is then filtered over Celite® and used for the subsequent transmetalation step. ^{1}H NMR (CD$_3$CN): δ = 7.32-7.17 (m, 6H, H^{Phenyl}, H^{Im}), 7.16 (d, $^3J_{\text{H-H}}$ = 1.80 Hz, 1H, H^{Im}), 5.28 (s, 2H, NCH_2(C$_6$H$_5$)), 4.08 (s, 2H, NCH_2C(CH$_3$)$_2$OH), 1.13 (s, 6H, C(CH_3)$_2$). ^{13}C NMR (CD$_3$CN): δ = 183.45, 138.28, 129.89, 129.13, 128.47, 124.75, 121.99, 121.87, 70.41, 62.59, 55.69, 49.74, 27.65.

*[3-Methyl-1-(2-hydroxy-2-methylpropyl)imidazol-2-ylidene]gold(I) chloride (**8**).* A mixture of Au(SMe$_2$)Cl (104 mg, 0.35 mmol, 1.0 equiv.) and the silver carbene mixture **5** (136 mg, 0.35 mmol, 0.10 M in CH$_2$Cl$_2$/MeCN) is stirred at room temperature overnight. The crude product is filtered over Celite® and dried under reduced pressure to obtain a pale yellow powder in 49% yield (66.3 mg). ^{1}H NMR (CD$_3$CN): δ = 7.21 (d, $^3J_{\text{H-H}}$ = 2.0 Hz, 1H, H^{Im}), 7.09 (d, $^3J_{\text{H-H}}$ = 1.92 Hz, 1H, H^{Im}), 4.12 (s, 2H, NCH_2), 3.78 (s, 3H, NCH_3), 2.93 (s, 1H, OH), 1.19 (s, 6H, N(CH_3)$_2$). ^{13}C NMR (CD$_3$CN): δ = 172.03, 123.64, 122.68, 70.61, 61.77, 38.62, 27.81. ^{1}H NMR (CDCl$_3$): δ = 7.24 (d, $^3J_{\text{H-H}}$ = 1.95 Hz, 1H, H^{Im}), 6.92 (d, $^3J_{\text{H-H}}$ = 1.98 Hz, 1H, H^{Im}), 4.19 (s, 2H, NCH_2), 3.84 (s, 3H, NCH_3), 2.00 (s, 1H, OH), 1.28 (s, 6H, C(CH_3)$_2$). ^{13}C NMR (CD$_3$Cl): δ = 172.04, 122.65, 122.70, 70.63, 61.77, 38.65, 27.83. MS-FAB (*m/z*): [11-Cl]+ calc'd, 351.08; found, 351.00. Elem. Anal. Calc'd for C$_8$H$_{14}$AuClN$_2$O*0.1MeCN: C, 25.21; H, 3.69; N, 7.53; Found: C, 25.34; H, 3.75; N, 7.26.

[3-Butyl-1-(2-hydroxy-2-methylpropyl)imidazol-2-ylidene]gold(I) chloride (9). Au(SMe$_2$)Cl (285 mg, 0.97 mmol, 1.00 equiv.) is added to the silver carbene mixture **6** (329 mg, 0.97 mmol, 0.16 M in MeCN) and the suspension is stirred at room temperature overnight. The crude product is filtered over Celite® and dried under reduced pressure. The product is yielded as an orange powder (86.0 mg, 43%). ^{1}H NMR (CDCl$_3$): δ = 7.23 (d, $^3J_{H-H}$ = 1.97 Hz, 1H, H^{Im}), 6.92 (d, $^3J_{H-H}$ = 1.98 Hz, 1H, H^{Im}), 4.21-4.16 (m, 4H, NCH_2C(CH$_3$)$_2$OH, NCH_2(CH$_2$)$_2$CH$_3$), 2.61 (s, 1H, OH), 1.86-1.79 (m, 2H, NCH$_2$CH_2CH$_2$CH$_3$), 1.40-1.32 (m, 2H, N(CH$_2$)$_2$CH_2CH$_3$), 1.28 (s, 6H, C(CH_3)$_2$), 0.95 (t, $^3J_{H-H}$ = 7.39 Hz, N(CH$_2$)$_3$CH_3). ^{13}C NMR (CDCl$_3$): δ = 171.41, 122.57, 120.00, 70.89, 61.08, 51.30, 33.20, 27.86, 19.79, 13.79. MS-ESI (*m/z*): [12-Cl+MeCN]+ calc'd, 434.15; found, 434.10. Elem. Anal. Calc'd for C$_{11}$H$_{20}$AuClN$_2$O: C, 30.82; H, 4.70; N, 6.53; Found: C, 31.13; H, 4.94; N, 6.35.

*[3-Benzyl-1-(2-hydroxy-2-methylpropyl)imidazol-2-ylidene]gold(I) chloride (**10**).* A mixture of Au(SMe$_2$)Cl (284 mg, 0.96 mmol, 1.0 equiv.) and the silver carbene mixture **7** (403 mg, 0.96 mmol, 0.14 M in CH$_2$Cl$_2$) is stirred at room temperature overnight. The crude product is filtered over Celite® twice and dried under reduced pressure. The resulting oil is rinsed with *n*-pentane to obtain a green solid in 54% yield (240 mg). ^{1}H NMR (CD$_3$CN): δ = 7.40-7.33 (m, 5H, ArH), 7.25 (d, $^3J_{H-H}$ = 2.0 Hz, 1H, H^{Im}), 7.14 (d, $^3J_{H-H}$ = 2.0 Hz, 1H, H^{Im}), 5.38 (s, 2H, NCH_2(C$_6$H$_5$)), 4.16 (s, 2H, NCH_2C(CH$_3$)$_2$OH), 3.28 (s, 1H, OH), 1.20 (s, 6H, N(CH_3)$_2$). ^{13}C NMR (CD$_3$CN): δ = 172.15, 137.74, 129.87, 129.23, 128.56, 124.36, 121.66, 70.63, 62.01, 55.24, 27.84. MS-ESI (*m/z*): [14-Cl+MeCN]+ calc'd, 468.13; found, 468.10. Elem. Anal. Calc'd for C$_{14}$H$_{18}$AuClN$_2$O: C, 36.34; H, 3.92; N, 6.05; Found: C, 36.38; H, 4.10; N, 5.84.

Enzymatic activity estimation of isolated TrxR1, TrxR2 and GR

Stock solutions of the complexes 10 mM in DMSO were prepared. Highly purified cytosolic (TrxR1) and mitochondrial (TrxR2) thioredoxin reductase were prepared from rat liver

according to what was previously reported by Luthman and Holmgren[85] and by Rigobello and Bindoli[86], respectively. The protein content of purified enzyme preparations was measured according to Lowry et al.[87]. Thioredoxin reductase activity was determined by estimating the DTNB-reducing property of the enzymes in the presence of NADPH. Aliquots of highly purified TrxR1 or TrxR2 in 0.2 M NaKPi buffer (pH 7.4), 5 mM EDTA and 0.25 mM NADPH were incubated with the various compounds. Afterward, the reaction was initiated with 1 mM DTNB and monitored spectrophotometrically at 412 nm.

GR activity (yeast glutathione reductase) was measured in 0.2 M Tris-HCl buffer (pH 8.1), 1 mM EDTA, and 0.25 mM NADPH in the presence of the various compounds. The assay was started by addition of 1 mM GSSG and followed spectrophotometrically at 340 nm.

BIAM assay

TrxR (1 μM) pre-reduced in the presence 60 μM NADPH, was incubated with gold compounds for 30 min at room temperature, in 50 mM Tris-HCl buffer (pH 7.4) containing 200 μM NADPH and 1 mM EDTA. After incubation, 8 μL of the reaction mixture was added to 8 μL of 100 μM biotinylated iodoacetamide (BIAM) in 0.1 M Tris-HCl at pH 8.5 or in 0.1 M Hepes-Tris pH 6.0.[88] Samples were incubated at room temperature for an additional 30 min to allow BIAM alkylation of free -SH/SeH groups of the enzyme. Then, BIAM-modified enzyme was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel, and transferred to a nitrocellulose membrane. BIAM labelled enzyme was detected with horseradish peroxidase-conjugated streptavidin and enhanced chemiluminescence detection.

MTT assay

Cell viability was determined with the 3-[4,.5-dimethylthiazol-2-yl]-2,.5- diphenyltetrazolium bromide (MTT) reduction assay. 2008 cells (1×10^4) were seeded in complete medium for 24 h.

Then, cells were washed and incubated in PBS/10 mM glucose for 1h in presence of increasing concentrations of gold compounds. At the end of the treatment, medium was removed and cells were washed and treated for 3 h at 37 °C with 0.5 mg/mL MTT dissolved in phosphate-buffered saline (PBS). Afterward, MTT solution was removed and 100 µL of stop solution (90% isopropanol, 10% DMSO) were added to each well. After 15 min, the absorbance at 595nm and 690 nm was estimated using a plate reader (Tecan Infinite® M200 PRO). For MTT analysis, the experiments are the mean with SD of, at least, three different experiments, in replicates.

Determination of TrxR and GR activities in cell lysates

The human ovary 2008 cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen) at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Cells ($7x10^5$) were seeded and after 24 h, washed twice and incubated for 3 h in the presence of gold complexes diluted in PBS/10mM glucose at increasing concentrations (20-60 µM). Then harvested and washed with PBS. Each sample was lysed with a modified RIPA buffer: 150 mM NaCl, 50 mM Tris–HCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% DOC, 1 mM NaF and an antiprotease cocktail ("Complete" Roche, Mannheim, Germany) containing 0.1 mM PMSF. After 40 min at 4 °C, the lysates were centrifuged at 12000g for 6 min. The obtained supernatants were tested for enzymatic activities. Aliquots (50 µg) of lysates were subjected to thioredoxin reductase determination in a final volume of 250 µL of 0.2 M NaKPi buffer (pH 7.4), 5 mM EDTA, and 2 mM DTNB. After 2 min the reaction was started with 0.4 mM NADPH. Glutathione reductase activity was estimated on 50 µg protein in 0.2 M Tris-HCl buffer (pH 8.1), 1 mM EDTA, and 0.25 mM NADPH. The measurement was started adding 1 mM GSSG and followed spectrophotometrically at 340 nm.

Estimation of ROS production

ROS generation in 2008 cell line was assessed using the fluorogenic probe CM-H2DCFDA (Molecular Probes, Invitrogen). Cells (1×10^4) were seeded in 96-wells plate, and, after 24 h, washed in PBS/10 mM glucose and loaded with 10 µM dye for 20 min in the dark at 37 °C. Afterwards, cells were washed with the same medium and incubated with gold complexes at 20 µM final concentration. Fluorescence increase was estimated on a plate reader (Tecan Infinite® M200 PRO, Männedorf, CH)) at 485 nm (excitation) and 527 nm (emission) for 1.5 h.

Mass spectrometry studies

Stock solutions of 10 mM were prepared of **4** in water and of **8** in DMSO and prior the experiments. The compounds were incubated at 37 °C alone or in the presence of GSH (1 equiv.) and aliquots were taken after 30 min and 24 h. The compounds were similarly incubated and sampled with a mixture of Glu : His : Met : Cys (1 : 1 : 1 : 1 : 1) and Glu : His : Met : Cys : SeCys (1 : 1 : 1 : 1 : 1 : 1), respectively. l-Selenocysteine (SeCys) was obtained by incubating seleno-l-cystine with three equivalents of DTT for 30 min at 37 °C. The final concentration of the gold compounds was 50 µM during incubations. The aliquots were diluted (1 + 9 v/v) with MeOH before injection into the mass spectrometer. The entire set of experiments was carried out in purely aqueous and in buffered solution (ammonium carbonate, 20 mM, pH 7.4).

Samples were analysed on a Synapt G2-Si time-of-flight (TOF) mass spectrometer (Waters) by direct infusion (DI) at an infusion rate of 3 µL·min⁻¹ and averaged over 0.5 min. Mass spectra were acquired and processed using MassLynx V4.1 (Waters). The instrumental parameters were as follows: 2.5–3.5 kV capillary voltage, 120 °C source temperature, 180 °C desolvation temperature, 90 L·h⁻¹ cone gas, 480–720 L·h⁻¹ desolvation gas and 6 bar nebulizer.

FRET melting assays

FRET experiment were performed in 96-well plates and run on an Applied Biosystems® QuantumStudio5 Real-Time PCR cycler equipped with a FAM filter (λ_{ex} = 492 nm; λ_{em} = 516 nm). Fluorolabelled 21-mer hTelo oligonucleotide, d[GGG(TTAGGG)$_3$], was purchased from Eurogentec (Belgium) in HPLC purity grade. The FRET probes used were FAM (6-carboxyfluorescein) and TAMRA (6-carboxy-tetramethylrhodamine). The lyophilized strands were firstly diluted in MilliQ water to obtain 100 μM stock solutions. Stock solutions were diluted to a concentration of 400 nM in 60 mM potassium cacodylate buffer (pH 7.4) and then annealed to form G4 structures by heating to 95 °C for 5 min, followed by slowly cooling to room temperature overnight.

Experiments were carried out in a 96 well plate with a total volume of 30 μl. Final concentration of the oligonucleotide was 200 nM. All gold compounds were previously dissolved in DMSO to give 1 mM stock solutions, which were further diluted using 60 mM potassium cacodylate, and added to the wells to obtain a final concentration of 1 μM (with a total percentage of DMSO ca. 0.1%). The machine was set to perform a stepwise increase of 0.3 °C every 30 s starting from 25 °C to reach 95 °C, and measurements were acquired after each step. To compare different sets of data, FAM emission was normalised (0 to 1). $T_{1/2}$ is defined as the temperature at which the normalised emission is 0.5. Measurements were made in triplicate.

ASSOCIATED CONTENT

Supporting Information

Experimental section for synthesis, MS, FRET and biochemical assays; NMR spectra

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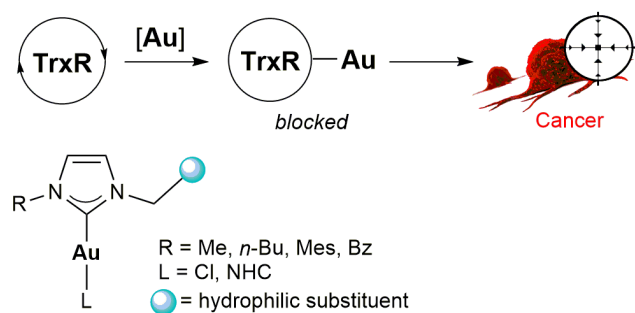*Table of Contents Graphic: For Table of Contents Only*

Table of Contents Synopsis.

The synthesis of novel hydrophilic gold(I) NHC complexes is reported. Selected compounds distinctly target the pivotal biological target thioredoxin reductase (TrxR) in the nanomolar range as benchmark anticancer drug auranofin, and exhibit remarkable antiproliferative effects against human ovarian cancer cells.