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Gold(I) Carbene Complexes Causing Thioredoxin 1 and Thioredoxin 2 Oxidation as Potential Anticancer Agents

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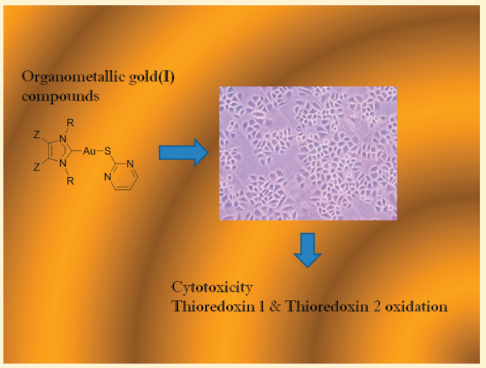
S *Supporting Information*

ABSTRACT: Gold(I) complexes with 1,3-substituted imidazole-2-ylidene and benzimidazole-2-ylidene ligands of the type NHC-Au-L (NHC = *N*-heterocyclic carbene L = Cl or 2-mercapto-pyrimidine) have been synthesized and structurally characterized. The compounds were evaluated for their antiproliferative properties in human ovarian cancer cells sensitive and resistant to cisplatin (A2780S/R), as well in the nontumorigenic human embryonic kidney cell line (HEK-293T), showing in some cases important cytotoxic effects. Some of the complexes were comparatively tested as thioredoxin reductase (TrxR) and glutathione reductase (GR) inhibitors, directly against the purified proteins or in cell extracts. The compounds showed potent and selective TrxR inhibition properties in particular in cancer cell lines. Remarkably, the most effective TrxR inhibitors induced extensive oxidation of thioredoxins (Trxs), which was more relevant in the cancerous cells than in HEK-293T cells. Additional biochemical assays on glutathione systems and reactive oxygen species formation evidenced important differences with respect to the classical cytotoxic Au(I)-phosphine compound auranofin.



■ INTRODUCTION

Gold compounds have been used in medicine for many years, the earliest use dating back to ancient China. In more modern times, gold complexes have been investigated for their activity against tuberculosis, and several gold containing antiarthritis drugs (Ridaura, Myocrisin, and Solganol) have subsequently entered the market and remain in clinical use today.[1] During the past decade, several research groups have developed an ensemble of structurally diverse Au compounds with anticancer properties.[2−4] In particular, gold(I) phosphine complexes have been tested against a variety of human tumor cell lines.[5] Among them, the antirheumatic drug auranofin displayed significant activity against HeLa cells *in vitro* and P388 leukemia cells *in vivo*, which led to the screening of a variety of triphenylphosphine gold(I) complexes.[6] In recent years, *N*-heterocyclic carbene (NHC) metal complexes have evolved from laboratory curiosities to compounds with unique properties for applications in catalysis and medicine.[7] Thus, the apparent similarity between NHC ligands and phosphines was the inspiration to examine the biological activity of silver, ruthenium, rhodium, platinum, and gold complexes with NHC ligands.[8]

In general, much effort has been directed to the understanding of the cytotoxic activity and related mode of action of gold-based cytotoxic agents, as well as to the identification of their preferential cellular targets.[9] In fact, although some gold(I) phosphine complexes were documented to interact with DNA or DNA polymerases, several subsequent studies strongly suggested that mitochondria and pathways of oxidative phosphorylation are the primary intracellular targets.[10] Within this frame, inhibition of the seleno-enzyme thioredoxin reductase (TrxR) seems to be a common mechanistic trait to explain (at least partially) the cytotoxic actions of Au-phosphine complexes, as strong TrxR inhibition may eventually lead to apoptosis through a mitochondrial pathway.[11] Both cytosolic (TrxR1) and mitochondrial (TrxR2) thioredoxin reductase forms are known and reduce the 12 kDa disulfide protein thioredoxin (Trx) to the corresponding dithiolic form.[12] Two distinct isoforms of Trx have been identified, the cytosolic isoform (Trx1) and the mitochondrial one (Trx2). Trx1 is an electron donor for ribonucleotide reductase, reduces methionine sulfoxide reductase, as well as thioredoxin peroxidase, and is involved in cell signaling.[13−15] Instead, the role of mitochondrial Trx2 in cancer cells is not completely clarified.[16] Notably, both TrxR and Trx systems play a major role in the

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regulation of the cellular redox state and thereby in health and disease.

Recent studies including the screening of some thiolato complexes of Au and Pt containing water-soluble phosphines[17−19] showed good correlation between cytotoxic activity and TrxR inhibition properties. In this respect, neutral NHC gold(I) halide complexes,[20] cationic bis(carbene) gold(I) salts,[21,22] and dinuclear gold(I) NHC complexes[23] have been also proved to be antimitochondrial antitumor agents.[8] Baker et al. successfully synthesized a series of NHC gold(I) analogues of auranofin and reported on their biological activity.[21] Au(I) N heterocyclic carbene complexes have also been designed as mitochondria-targeted antitumor agents that combine both selective mitochondrial accumulation and selective thioredoxin reductase inhibition properties within a single molecule.[24] A recent study by Rubbiani et al. reported a series of Au(I) complexes with benzimidazole derived NHC ligands that exerted selective inhibition of TrxR and significant antiproliferative effects.[25]

Within this frame, we report here on the synthesis and structural characterization of a family of gold(I) NHC complexes containing 1,3-substituted imidazole-2-ylidene or benzimidazole-2-ylidene and chloro or 2-pyrimidinethiolato ligands (Figure 1). The cytotoxic effects of the compounds

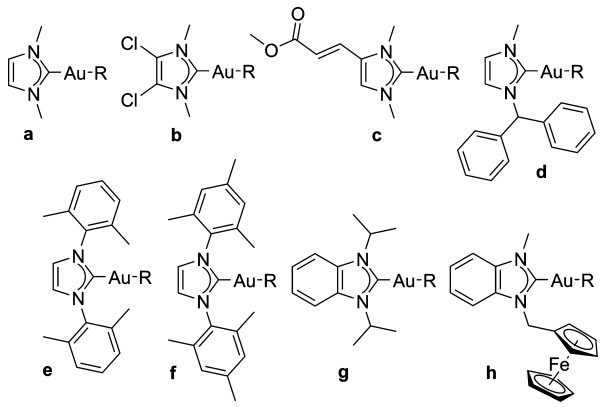


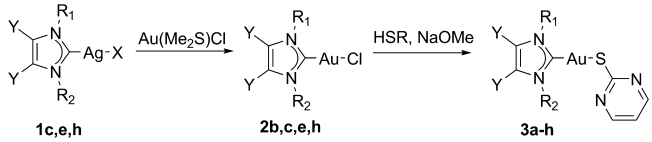
Figure 1. Structures of the NHC Au(I) complexes used in this study. R = Cl or *S*-pyrimidine. (a) IMe, (b) ^{Cl}IMe, (c) $^{MeOC(O)CHCH}$IMe, (d) IMeCHPh$_2$, (e) IXyl, (f) IMes, (g) *i*Pr-benzimiazole-2-ylidene, and (h) Me-(CH$_2$-fc)-benzimidazole-2-ylidene.

were investigated *in vitro* on different cell lines, cancerous and nontumorigenic. Afterward, we evaluated the TrxR inhibition properties of the Au(I) NHC complexes directly on the purified proteins or on cell extracts, in comparison to their ability to inhibit the enzyme glutathione reductase (GR), a pyridine-disulfide oxido-reductase which maintains glutathione in its reduced state. The effects of some of the new complexes on the oxidation state of Trx were also investigated by Western blot analysis. Finally, estimation of the glutathione content and reactive oxygen species (ROS) production was performed in gold-treated cells.

RESULTS AND DISCUSSION

Synthesis and Structural Characterization. Synthesis of the gold(I) NHC chloro complexes was achieved via the known route of transmetalation of the corresponding Ag(I) NHC complexes with [AuCl(SMe$_2$)] (Scheme 1). We found that, even in the case of the poorly soluble Ag(I) NHC complexes (**1c,h**) transmetalation to gold can be achieved simply by

Scheme 1. Synthesis of the NHC Gold Thiolate Complexes (3a−h)[a]



[a]X = Cl, Br or I. For the structures of the NHC backbones refer to Figure 1.

stirring the suspension containing [AuCl(SMe$_2$)] in dichloromethane for 12 h. In all cases, the desired gold derivatives were isolated in pure form in yields of 82−92%. The thiolato complexes were prepared by the reaction of the Au(I) NHC chloro complexes with the *in situ* generated sodium salt of 2-mercaptopyrimidine (Scheme 1). The resulting Au(I) NHC 2-pyrimidinethiolato complexes (Figure 1) were obtained in good yields as colorless, yellow, or orange solids, which are soluble in halogenated solvents, acetone, and methanol. The new compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy and elemental analysis. In several cases, the solid-state structures were determined by X-ray diffraction.

The most diagnostic feature in the ^{13}C NMR spectra of the gold(I) NHC compounds is the chemical shift of the carbene carbon atom. In the halide complexes, the signals are observed at around 170 ppm, while those of the 2-pyrimidinethiolato compounds are observed at approximately 180 ppm. The identity of compounds **2b**, **3b**, and **3e** was confirmed by X-ray diffraction analysis, whose molecular structures are shown in Figure 2.

Colorless needles of the chloro-substituted NHC gold(I) chloride complex **2b** were analyzed by X-ray diffraction and

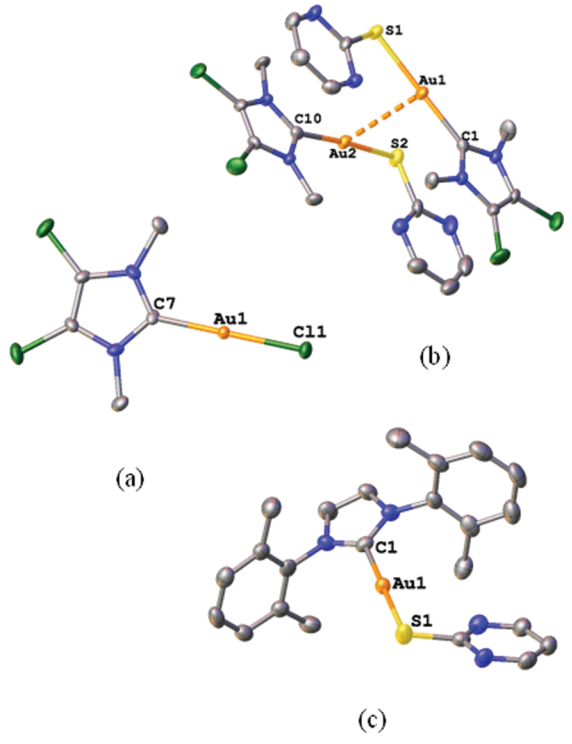


Figure 2. Molecular structures of (a) **2b** (^{Cl}IMeAuCl), (b) **3b** (^{Cl}IMeAuS-pyrimidine), and (c) **3e** (IMesAuS-pyrimidine). Hydrogen atoms have been omitted for clarity.

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found to consist of a C-bound NHC ligand and a chloride ligand linearly (180.0°) coordinated to the gold atom (Figure 2a). The Au−C and Au−Cl bond lengths of 1.993 Å and 2.291 Å, respectively, are typical for NHC−Au−Cl complexes. The structure of the 2-pyrimidinethiolato derivative **3b** was also solved (Figure 2b) and was found to be very similar to **2b**: the angle about the metal center is also close to linearity (176.8°), and the Au−C bond length is slightly longer than in the parent chloro complex. Notably, while the chloro derivative is monomeric, the thiolato derivative forms dimers through aurophilic interactions (Au···Au = 3.1934 Å), a feature which is often observed in the solid-state molecular structures of gold(I) complexes. The structure of **3e** (Figure 2c) contains two independent molecules in the unit cell. The angle about the metal center is with 179.4° and 179.3° almost linear and greater than in **2b**, the Au−C and the Au−S bond lengths of 1.990/1.986 Å and 2.288/2.293 Å, respectively are shorter than in the structure of **2b**, but typical for this type of complexes. Because of the bulky carbene ligand, there are no aurophilic interactions between molecules, but they are packed in an alternating arrangement of carbene ligands facing each other in one layer and two thiolato ligands facing each other in the second layer. The two layers are rotated away from each other by an angle of 43.9°.

Biological Studies. The gold(I) NHC compounds (**2a−2h** and **3a−3h**) were screened for their cytotoxic properties against human ovarian cancer cell lines sensitive (A2780S) and cisplatin resistant (A2780R), respectively (Table S2, Supporting Information), in comparison to cisplatin and auranofin. A dose-dependent inhibition of cell growth was observed in both cell lines with IC_{50} values ranging from ca. 2 to 30 μM after 72 h incubation. Interestingly, in some cases the gold complexes are more cytotoxic toward the resistant cell line indicating that these complexes are able to overcome the resistance to cisplatin. The most effective compounds resulted to be **3b**, **3f**, and **3g** (IC_{50} 1−4 μM), followed by **2f** (IC_{50} ca. 5 μM) on both cell lines. Notably, the chloro-substituted NHC Au(I) complexes are in general less effective than the corresponding 2-pyrimidinethiolato derivatives. As metal-NHC-bonds are generally stable, as well as Au-thiolate moieties, the chloride group could be expected to be the more labile ligand at the gold center, thus making the chloride derivatives more reactive and prone to be deactivated by different cellular components.

Afterward, the triggering of antiproliferative effects by the four most effective Au(I) NHC compounds out of the 72 h screening (**3b**, **3e**, **3f**, and **3g**) was also evaluated at shorter incubation times (24 h) on the same cancer cell lines, as well as on the nontumorigenic human embryonic kidney cell line (HEK-293T), in order to directly correlate the cytotoxicity data with the enzyme inhibition studies described below. The obtained results, summarized in Table 1, showed that the compounds behave similarly toward the various cell lines, with compounds **3b**, **3e**, and **3f** manifesting the tendency to a more pronounced cytotoxicity toward the cancerous cell lines with respect to the HEK-293T cells.

Since TrxR is a potential target for gold complexes, *in vitro* inhibition of purified rat TrxR by the four NHC Au(I) compounds was studied using an established protocol as described in the Experimental Section. All compounds inhibit both cytosolic (TrxR1) and mitochondrial (TrxR2) thioredoxin reductases, (Figure 3). In Table S3 (Supporting Information), are reported the IC_{50} values, compared with auranofin as the benchmark inhibitor. The most effective compounds on TrxR1

Table 1. Cell Viability IC_{50} Values of Selected NHC Au(I) Complexes against Human Ovarian Carcinoma Cell Lines Sensitive (A2780S) or Resistant to Cisplatin (A2780R) and against Human Embryonic Kidney Cell Line (HEK-293T) after 24 h of Incubation[a]

	IC_{50} (μM)		
compd	A2780S	A2780R	HEK-293T
3b	3.2 ± 0.7	4.9 ± 1.5	5.3 ± 1.2
3e	6.7 ± 0.5	8.1 ± 1.0	11.9 ± 2.3
3f	9.3 ± 1.6	9.9 ± 1.6	16.2 ± 1.9
3g	11.5 ± 2.6	12.7 ± 3.1	11.8 ± 1.9

[a]The reported values are the mean ± SD of at least three determinations.

are **3b** and **3g** with IC_{50} values of 4.9 ± 0.5 and 18.4 ± 2.3 nM, respectively. Compounds **3e** and **3f** are more moderate inhibitors of TrxR1 with IC_{50} values between 40 and 60 nM, but active in the nanomolar range. In general, the results correlate well with the high cytotoxic potency of the complexes. When tested against TrxR2, the four compounds showed a markedly reduced effect (IC_{50} in the 100−700 nM range), with compounds **3b** and **3g** again as the most effective.

Further studies demonstrated that the selected gold(I) NHC complexes are also able to inhibit the TrxR related but Se-free enzyme glutathione reductase (GR) (Figure 3), albeit at much higher concentrations (≥5 μM). The most effective compounds are **3f** and **3g** causing a reduction of GR activity up to 40% with respect to the controls. The poor inhibition of GR by Au(I) NHC complexes might be due to the high affinity of gold(I) compounds for the thiol−selenol C terminal residue present in TrxRs but not in GR[26] and acting as a potential Au(I) binding site.[27]

Additional complementary information on the enzyme metalation process and possible binding sites was obtained through the application of a specific biochemical assay relying on the thiol-tagging reagent BIAM (biotin-conjugate iodoacetamide[28]). BIAM selectively alkylates TrxR in a pH dependent manner; at pH 6.0 only selenocysteines (and low pK_a cysteines) are alkylated, whereas at pH 8.5 both cysteines (Cys) and selenocysteines (Sec) are modified. In our experiments, TrxR1 was treated with each gold complex (10 μM), and afterward, sample aliquots were treated with BIAM, either at pH 6.0 or 8.5, and the mixtures analyzed by SDS−PAGE. Proteins labeled with BIAM were detected with horseradish peroxidase-conjugated streptavidin (see Figure 4 and Experimental Section for further details), and iodoacetamide was used as the positive control. The immunoblotting indicates that all the gold NHC complexes are able to preferentially target the Sec at the redox active motif of TrxR1 (Figure 4, compare panel A and B), in good accordance with the concomitant poor inhibition of GR lacking the Sec residues. Interestingly, the observed effects for Au(I) NHC complexes markedly differ from those observed in the case of Au(III) complexes which, endowed with marked oxidizing properties, caused extensive oxidation of thiol groups at pH 8.5.[29]

To establish whether TrxR inhibition by the gold compounds could contribute to the observed antiproliferative effects on cells, enzyme activity was also evaluated on protein extracts obtained from A2780S and A2780R cancer cells, as well as from nontumorigenic HEK-293T cells, pretreated with each compound (8 μM) for 24 h. As described in Figure 5, only compound **3b** shows a weak inhibition of TrxR in the HEK-

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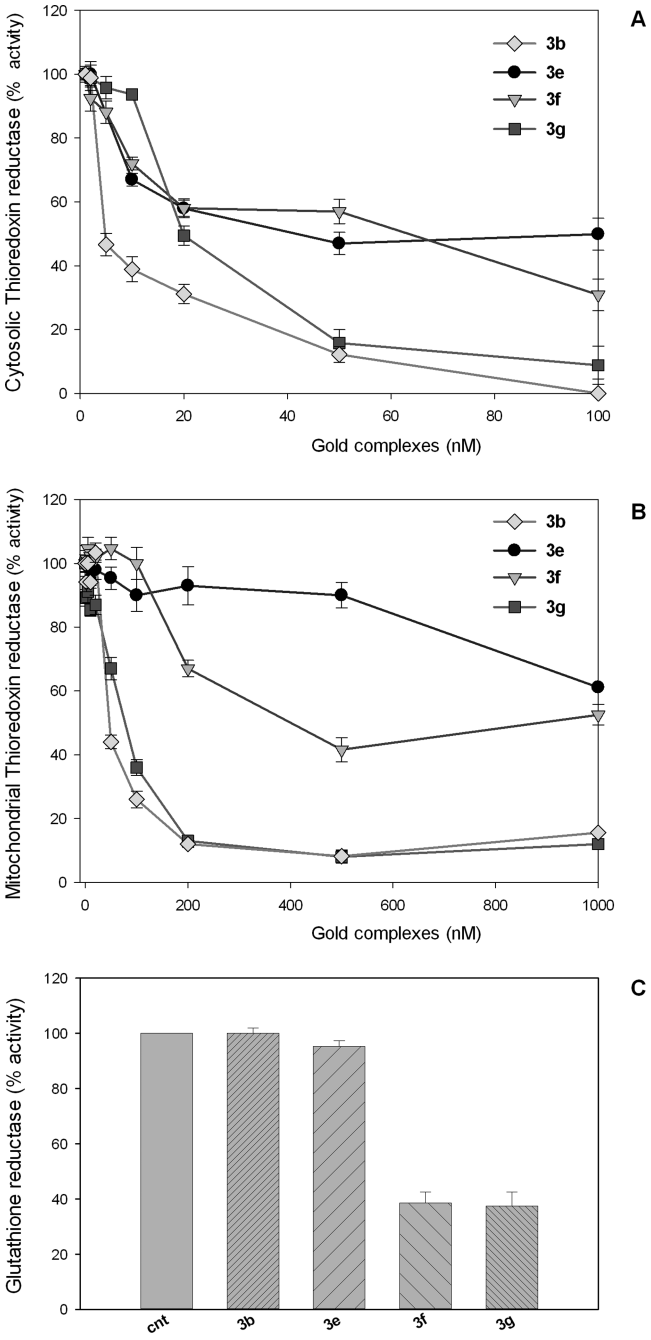

Figure 3. Effect of gold(I) carbene on cytosolic and mitochondrial thioredoxin reductase and glutathione reductase activities. TrxR1 (60 nM) and TrxR2 (130 nM) were incubated as described in the Experimental Section. DTNB reduction was estimated spectrophotometrically at 412 nm. GR (15nM) was tested in the presence of 5 μM gold complexes. NADPH oxidation was followed at 340 nm.

293T nontumoral cell line, while all the other Au(I) NHC compounds have almost no effect on thioredoxin reductase on this cell line. Conversely, TrxR inhibition is evident in tumor cell line lysates, the A2780S cells being the most sensitive, in particular in the presence of **3b** and **3e**. Compound **3b** is also the most active in the A2780R cell line, while **3e** is practically ineffective. Interestingly, auranofin causes potent enzyme inhibition in all cases without manifesting selectivity in dependence of the cell line. When GR activity was tested in the same cell lysates, no significant changes in both

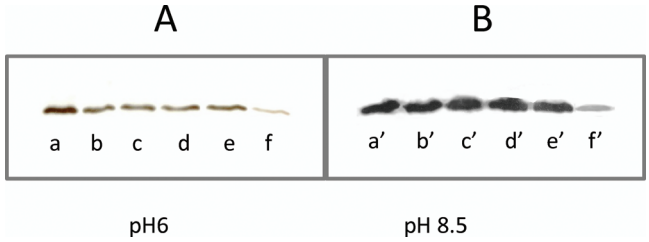

Figure 4. Alkylation with BIAM of rat liver TrxR1 treated in the presence of gold complexes or iodoacetamide. TrxR1 was incubated with 10 μM gold(I) NHC complexes or 100 μM iodoacetamide as indicated in the Experimental Section. Afterward, the enzyme was alkylated with BIAM at either pH 6.0 (A) (a, control; b, **3e**; c, **3f**; d, **3g**; e, **3b**; f, iodoacetamide) or pH 8.5 (B) (a′, control; b′, **3e**; c′, **3f**; d′, **3g**; e′, **3b**; f′, iodoacetamide).

nontumorigenic (HEK-293T) and cancerous cell lines (A2780S/R) were detected (see Supporting Information available Figure S1).

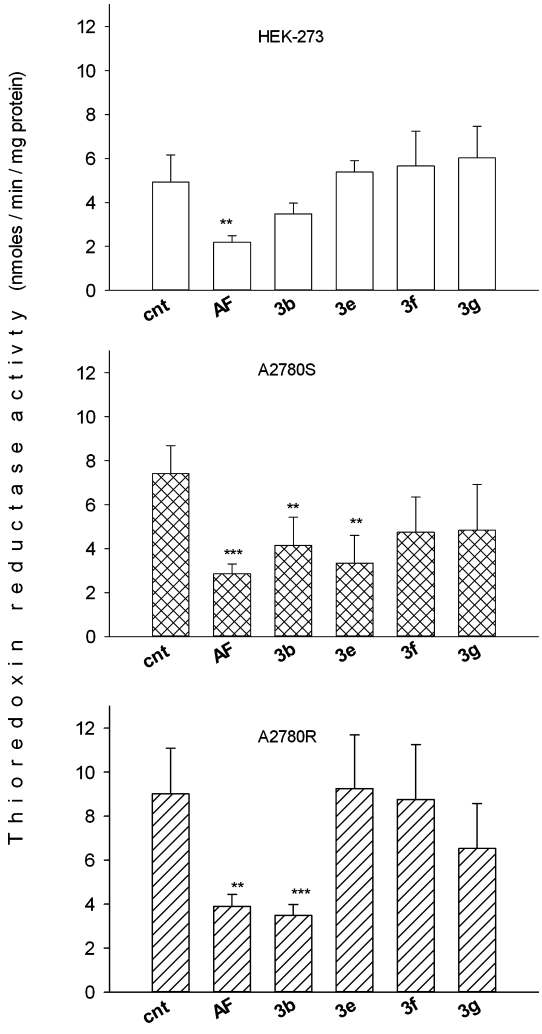

Figure 5. Effects of gold(I) NHC complexes and auranofin (AF) on TrxR in cell extracts. HEK-293T cells and A2780 cancer cells sensitive (S) and resistant (R) to cisplatin were incubated for 24 h with 8 μM of tested compounds; 2 mM auranofin (AF) was used as the benchmark. Subsequently, cells were washed twice with PBS and lysed. TrxR1 activity was assayed by measuring NADPH-dependent reduction of DTNB at 412 nm (***$p < 0.001$; ** $p < 0.01$).

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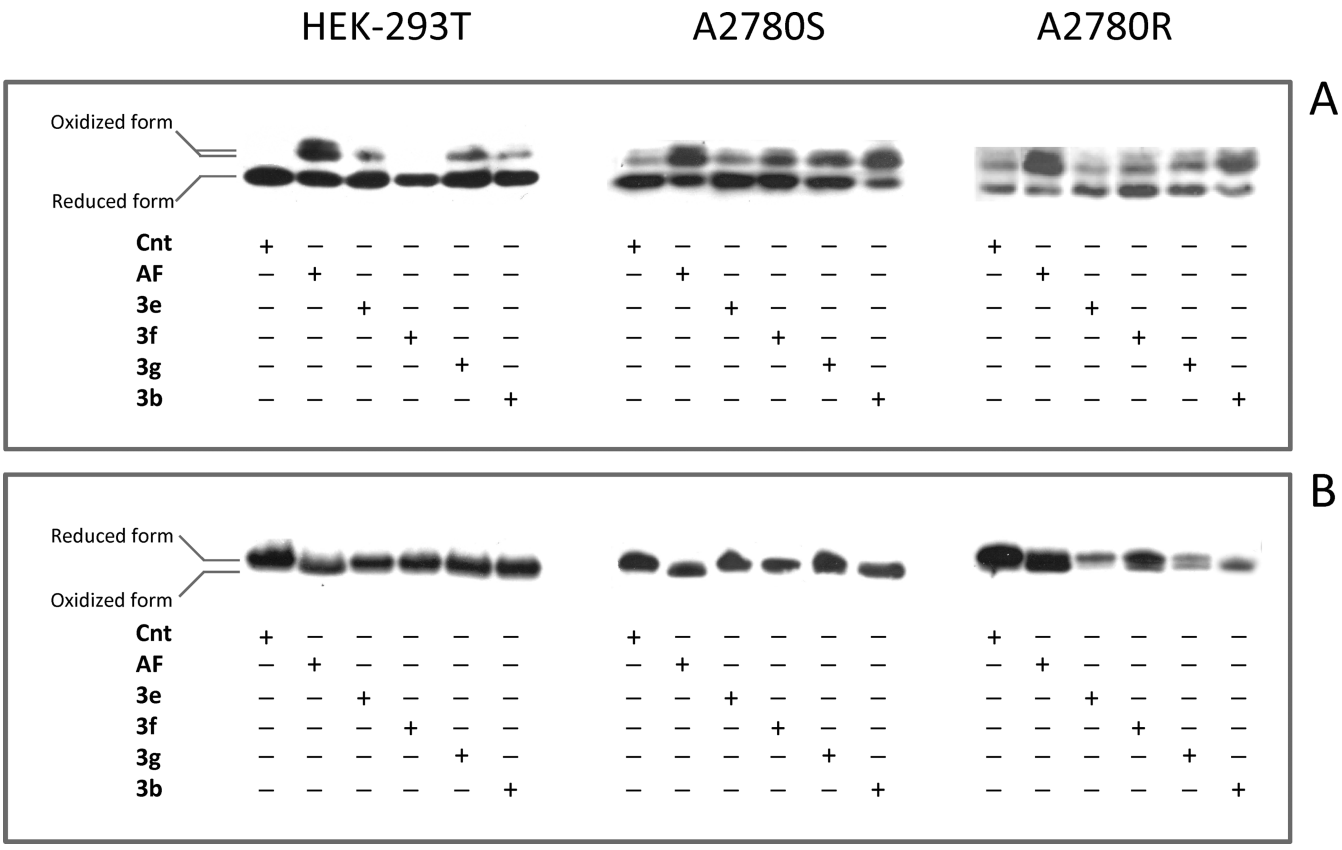

Figure 6. Trx 1 and Trx 2 redox states in the presence of gold complexes. Redox Western blot of thioredoxin 1 (A) and thioredoxin 2 (B). A2780 cells sensitive (S) and resistant (R) to cisplatin and HEK-293T cells were incubated with 6 μM gold(I) NHC complexes or auranofin (AF) for 3 h. Cells were treated as indicated in the Experimental Section and rapidly derivatized with IAA for Trx 1 and with AIS for Trx2, respectively.

In general, the inhibition of thioredoxin reductase by hindering the transfer of reducing equivalents leads to a large oxidation of thioredoxin (Trx). Indeed, previous studies have demonstrated that the redox conditions of Trx or of other components of such redox pathways can be profoundly altered by specific TrxR inhibitors or oxidants.[30,31] Therefore, we examined the redox state of cytosolic (Trx1) and mitochondrial (Trx2) thioredoxins directly in cells after treatment with gold complexes. Such a quantification requires different thiol reagents depending on the Trx isoform. In the case of Trx1, thiols were derivatized with iodoacetic acid (IAA), which adds one additional negative charge for each modified thiol. Derivatized samples were subjected to native, nonreducing PAGE where oxidized thioredoxin moves slower than its reduced form, due to the presence of additional negative charges in the latter and hence allowing the differential detection of the two redox forms. Briefly, cultured cells were incubated with 6 μM Au(I) NHC complexes or auranofin as control and then treated with IAA (50 mM) as reported in the Experimental Section. In almost all cases, treatment with gold compounds leads to a dramatic oxidation of Trx1 in the three cell lines examined. However, while auranofin induces oxidation of Trx1 both in HEK-293T cells and in A2780S/R cancer cells, gold(I) NHC complexes oxidize thioredoxin preferentially in the tumor cell lines, and the oxidation is further pronounced in the cisplatin-resistant cell line (Figure 6, panel A). Among the various compounds, **3b** appears to be among the most efficient in driving a marked oxidation of Trx1 comparable to that induced by auranofin treatment.

A similar nonreducing PAGE analysis was performed to determine the oxidation of mitochondrial Trx2. The oxidized form of Trx2 is detectable as a shift in the band at the lower molecular weight, using 4-acetamido-4′-((iodoacetyl)amino)-stilbene-2,2′-disulfonic acid (AIS) as the derivatizing agent. Trx2 contains two Cys residues, and consequently, the thiol-reactive agent AIS added to the protein lysates increases the thioredoxin 2 protein size of 13 kDa (624 Da for each cysteine). The obtained results are shown in Figure 6, panel B. As can be observed, auranofin leads again to a rapid oxidation of Trx2 in all the three cell lines, including tumor and nontumor cells. The Au(I) NHC complexes show also an increase in Trx2 oxidation in all cell lines, with again **3b** among the most effective compounds.

It is worth noting that oxidation of Trx proteins by metal compounds such as arsenic, cadmium, and mercury has already been reported.[32] In the case of Cr(VI) compounds (e.g., chromates), it has been demonstrated that their direct inhibition of TrxRs can indirectly induce the oxidation of Trxs.[33] However, such a mechanism has never been described previously for Au(I) NHC complexes.

The glutathione redox pair (GSH/GSSG) is another fundamental component of the cell redox regulation in cisplatin resistant cell.[34] Therefore, our study was oriented to the analysis of total glutathione in cisplatin-sensitive and -resistant cancer cells and in normal cells, after treatment with gold compounds. In addition, we examined the GSH/GSSG ratio. Cells were treated with 6 μM gold complexes, and the obtained samples were estimated both for total (reduced + oxidized) and oxidized glutathione (GSSG) contents as described in the

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Experimental Section. As shown in Figure 7, the total glutathione content is not dramatically affected by gold

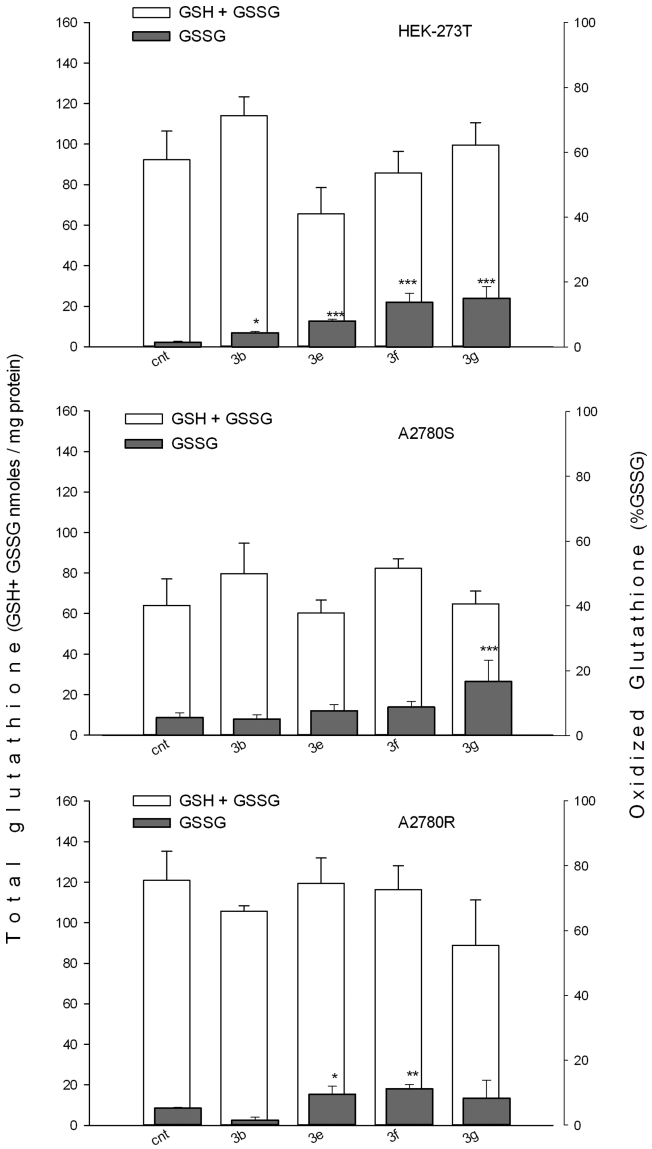

Figure 7. GSH and GSSG levels in the presence of gold complexes. Levels of total glutathione and oxidized glutathione were determined in HEK-293T, A2780S, and A2780 R cells, after incubation with gold(I)carbene complexes (6 μM) (***$p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

compound treatment in any of the three cell lines examined, but the amount of GSSG slightly increases in the presence of **3f** and **3g**, in accordance with the compounds' above-mentioned inhibition effects of glutathione reductase. Interestingly, **3b** does not show any glutathione oxidation.

Reactive oxygen species (ROS) are products of the physiological mitochondrial cell metabolism and are involved in cellular redox homeostasis. Their induction indicates a perturbation of the cellular antioxidant defense system. In particular, mitochondria can generate hydrogen peroxide, thus playing a crucial role in the apoptotic process. Moreover, it has been previously shown that TrxR inhibition alters cell conditions causing increases of hydrogen peroxide concentration, as well as an imbalance in cell redox state leading to mitochondrial membrane permeabilization and swelling.[35,36] On the grounds of these considerations, we evaluated the alterations of basal H_2O_2 production in cancer (A2780S and R) and noncancerous cells (HEK-293T) upon gold treatment. Thus, gold(I) NHC complexes (2 μM) were administered to cells in presence of the peroxide-sensitive fluorescent probe CM-DCFDA. All the compounds were able to increase to a relevant level the basal production of hydrogen peroxide only in the A2780R cells. In the HEK-293T and in the cisplatin-sensitive A2780S cells, no significant increase in ROS was detected (Figure 8). This is different from what has already

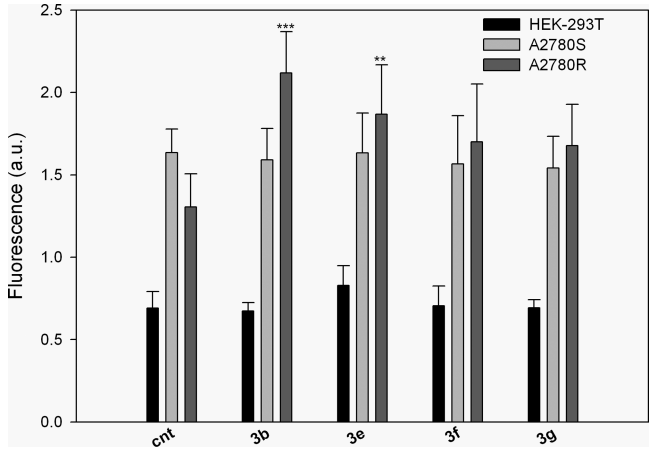

Figure 8. Effect of gold(I) compounds on ROS formation in cancer and normal cells. A2780S/R and HEK-293T cells were preincubated in PBS/10 mM glucose medium for 20 min at 37 °C in the presence of 10 μM CM-DCFDA and then treated with IC_{50} concentrations of gold(I) NHC compounds (***$p < 0.001$; ** $p < 0.01$).

been shown, for example, in the case of Au(III) and Au(I) NHC complexes, although caution has to be applied in comparing different experimental protocols.[37,38]

CONCLUSIONS

We report here on the synthesis and chemical characterization of a series of Au(I) complexes of the 1,3-substituted imidazole-2-ylidene and benzimidazole-2-ylidene gold with chloro and 2-pyrimidinethiolato ligands. X-ray structures for complexes **2b**, **3b**, and **3e** have also been obtained and described.

The compounds were screened for their antiproliferative effects on human ovarian cancer cell lines and were found to be potent cytotoxic agents. Four among the most cytotoxic Au(I) NHC complexes were screened for their TrxR inhibition properties both on the purified enzyme and on cell extracts *in vitro*. The compounds inhibited cytosolic TrxR1 better than mitochondrial TrxR2 and even to a much lesser extent GR. Interestingly, unlike auranofin, TrxR inhibition by Au(I) NHC appears to be relevant in cancerous cells, while the enzyme was almost unaffected in the nontumorigenic HEK-293T cell line; the A2780S cells were the most sensitive, in particular in the presence of **3b** (^{Cl}IMeAu-S-pyrim) and **3e** (IXylAu-S-pyrim).

The proposed mechanism of TrxR inhibition involves direct coordination of the gold 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, as well as by the BIAM assay results. The BIAM assay also suggests that the compounds are not markedly oxidative agents as other cytotoxic gold(III) complexes.

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An interesting aspect of our studies concerns the observation of Trx oxidation upon treatment of cell extracts with Au(I) NHC compounds, which is more relevant in cancerous cell lines with respect to nontumorigenic cells in accordance with the TrxR inhibition trend. In particular, compound **3b**, similarly to auranofin, produces marked effects on both cytosolic Trx1 and mitochondrial Trx2 implying that the complex is able to reach both cell compartments. Notably, **3b** is also one of the most cytotoxic agents in our series. Trx oxidation might be induced either by inhibition of TrxR by gold(I) NHC complexes or by a massive increment of H_2O_2. However, the latter has not been detected at least in the A2780S and HEK-293T cells. This behavior is different with respect to what has been already reported for auranofin and gold(I) phosphine derivatives.[36]

It is worth mentioning that very recently gold(I) *N*-heterocyclic carbene complexes were described as potent thioredoxin reductase inhibitors and antimitochondrial anticancer agents.[37] However, as far as we are aware, our study is the first report of Au(I) NHC influence on Trx redox state. Finally, the Au(I) NHC complexes did not affect the total GSH content in any of the three cell lines examined, although **3f** (IMesAu-S-pyrim) and **3g** (*i*Pr-benzimidazole-2-ylideneAu-S-pyrm) induced an increase of oxidized glutathione levels in accordance with the compounds' inhibition effects of GR.

Discrimination between the oxidation of the GSH/GSSG system and the Trx system can be very informative in terms of mechanisms of toxicity since different cellular pathways are controlled by GSH and Trx. For example, while the GSH/GSSG system controls the activation and nuclear translocation of the transcription factor Nrf2, Trx1 influences its DNA binding.[39] Moreover, the poor influence of the compounds on the total GSH content suggests that the latter does not affect the compounds' cytotoxic potency, as for cisplatin in the case of certain resistant cancer cells.

In conclusion, although the obtained results clearly indicate a correlation between cytotoxicity and Trx oxidation via TrxR inhibition in cancerous cells, further studies are necessary to validate our mechanistic hypothesis and to exclude other possible intracellular targets. For example, other studies are in progress in our laboratories to assess the role of zinc-finger proteins in the anticancer effects of Au-based compounds.[40,41] Nevertheless, the herein described Au(I) NHC complexes may constitute promising anticancer drug leads able to induce Trx oxidation via direct inhibition of TrxR in cancer cells.

EXPERIMENTAL SECTION

Chemistry. Unless otherwise stated, all manipulations were carried out without taking precautions to exclude air and moisture. All chemicals and solvents (HPLC grade or better) were sourced commercially and used as received. The silver complexes **1a**,[42] **1b**,[43] **1d**,[44] **1f**,[45] **1g**[46] as well as the gold complexes **2a**,[47] **2d**,[44] **2f**,[48] **2g**,[49] and the imidazolium salts 1,3-dimethyl-4,5-dichloroimidazolium iodide,[50] 1,3-dimethyl-4-acrylic acid methyl ester imidazolium iodide,[50] and 1,3-bis-(2,6-dimethylphenyl)imidazolium chloride[51] were prepared as described in the literature. The purity of the Au compounds was confirmed by elemental analysis, and all of them showed purity greater than 95%.

Instrumentation. *NMR Spectroscopy.* ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 400 or Bruker Avance III 600 MHz spectrometers and are referenced to external TMS.

IR Spectroscopy. IR spectra were run as KBr disks on a Bruker Tensor 27 instrument.

Elemental Analyses. Elemental analyses were performed by staff of the microanalytical laboratory of the University of Wuppertal.

X- ray Crystallography. Diffraction data were collected at 150 K using an Oxford Diffraction Gemini E Ultra diffractometer, equipped with an EOS CCD area detector and a four-circle kappa goniometer. For the data collection, the Mo source emitting graphite-monochromated Mo−Kα radiation (λ = 0.71073 Å) was used. Data integration, scaling, and empirical absorption correction were carried out using the CrysAlis Pro program package.[52] The structures were solved using Direct Methods or Patterson Methods and refined by Full-Matrix-Least-Squares against F^2. The non-hydrogen atoms were refined anisotropically, and hydrogen atoms were placed at idealized positions and refined using the riding model. All calculations were carried out using the program Olex2.[53] Full crystallographic and refinement parameters as well as tables with bond lengths and angles are included in the Supporting Information.

Synthesis. *General Procedure for the Synthesis of [(NHC)AuCl].* [AuCl(SMe$_2$)] (1 equiv.) was added to a solution or suspension of the NHC Ag compound (1equiv.) in CH_2Cl_2 (50 mL), and the mixture was stirred for 6 h at room temperature. The suspension was filtered over Celite, and the solvent was concentrated in vacuum. The product was precipitated by the addition of hexane, isolated by filtration, and washed with hexane.

*1,3-Dimethyl-4,5-dichloroimidazole-2-ylidene-gold(I)-chloride (**2b**).* Greyish solid; yield, 77%. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 3.86 (s, 6H, NMe) ppm. ^{13}C NMR (151 MHz, $CDCl_3$): δ = 171.9 (C2), 117.6 (C−Cl), 37.1 (N−C) ppm. Anal. Calcd. for $C_5H_6AuCl_3N_2 \cdot 0.1C_5H_6AuCl_3I_2N_2$: C, 14.28; H, 1.44; N, 6.66; found, C, 14.25; H, 1.19; N, 6.67%.

*1,3-Dimethyl-4-acrylic Acid Methyl Ester Imidazole-2-ylidene-gold(I)-chloride (**2c**).* Colorless solid; yield, 82%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.44 (d, J = 16.1 Hz, 1H, OC−CH=CH), 7.31 (s, 1H, NCH), 6.32 (d, J = 16.1 Hz, 1H, CH=CH), 3.93 (s, 3H, NMe), 3.87 (s, 3H, N-Me), 3.83 (s, 3H, O-Me) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ = 174.6 (C2), 166.0 (C=O), 130.0 (C-NMe), 127.8 (OCCH=CH), 121.8 (CH-NMe), 120.9 (CH=CH), 52.2 (O-Me), 38.6 (N-Me), 36.4 (N-Me) ppm. Anal. Calcd. for $C_9H_{12}AuClN_2O_2 \cdot 0.5CH_2Cl_2$: C, 25.07; H, 2.88; N, 6.16; found, C, 25.13; H, 2.55; N, 6.16%.

*1,3-(2,6-Dimethylphenyl)imidazole-2-ylidene-gold(I)-chloride (**2e**).* Pale brown solid; yield, 95%. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.35 (t, J = 7.6, 2H, p-CH), 7.22 (d, J = 7.6, 4H, m-CH), 7.16 (s, 2H, NCHCHN), 2.18 (s, 12H, Me) ppm. ^{13}C NMR (151 MHz, $CDCl_3$): δ = 173.1 (C2), 137.1 (C_{ipso}), 135.1 (CMe), 130.0 (p-CH), 128.9 (m-CH), 122.1 (NCHCHN), 17.9 (Me) ppm. Anal. Calcd. for $C_{19}H_{20}AuClN_2 \cdot 0.25$Hex: C, 46.45; H, 4.44; N, 5.29; found, C, 46.42; H, 4.69; N, 5.62%.

*1-Methyl-3-(ferrocenylmethyl)imidazole-2-ylidene-gold(I)-chloride (**2h**).* Orange solid; yield, 92%. ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 7.93 (m, 1H, CH), 7.74 (m, 1H, CH), 7.47 (m, 2H, CH), 5.47 (s, 2H, NCH_2), 4.55 (t, J = 1.8 Hz, 2H, Cp_{sub}), 4.29 (s, 5H, Cp_{unsub}), 4.15 (t, J = 1.8 Hz, 2H, Cp_{sub}), 3.98 (s, 3H, N-Me) ppm. ^{13}C NMR (101 MHz, [D$_6$]DMSO): δ = 176.6 (C2), 133.2, 132.0 (C_{BnImid}), 124.3, 112.1 (CH_{BnImid}), 82.1 (C_{Cp}), 69.2 (Cp_{sub}), 68.7 (Cp_{unsub}), 68.2 (Cp_{sub}), 47.5 (NCH_2), 35.0 (N-Me) ppm. Anal. Calcd. for $C_{19}H_{18}AuClFeN_2$: C, 40.65; H, 3.22; N, 4.98; found, C, 40.29; H, 2.84; N, 5.28%.

General Procedure for the Synthesis of [Au(NHC)(S-pyrim)]. 2-Mercaptopyrimidine (1.5 equiv.) and NaOMe (2 equiv.) was dissolved in MeOH (15 mL) and stirred for 5 min. The NHC−Au-Cl complex (1 equiv.) was added, and the mixture was stirred at room temperature for 6 h. The solvent was concentrated in vacuum, and hexane was added until appearance of a precipitate. The solid was isolated by filtration and washed with 0.2 M KOH in MeOH and water and subsequently dried in air.

*1,3-Dimethylimidazole-2-ylidene-gold(I)-pyrimidine-2-thiolate (**3a**).* Yellow solid; yield, 79%. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.36 (d, J = 4.8 Hz, 2H, m-CH), 6.95 (s, 2H, NCHCHN), 6.79 (t, J = 4.8 Hz, 1H, p-CH), 3.92 (s, 6H, Me) ppm. ^{13}C NMR (151 MHz, $CDCl_3$): δ = 182.5 (C−Au), 181.2 (C−S), 156.5 (m-CH), 121.7 (NCHCHN),

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115.0 (p-CH), 38.0 (Me) ppm. Anal. Calcd. for $C_9H_{11}AuN_4S$: C, 26.74; H, 2.74; N, 13.86; S, 7.93; found, C, 26.31; H, 2.75; N, 13.65; S, 7.56%.

*1,3-Dimethyl-4,5-dichloroimidazole-2-ylidene-gold(I)-pyrimidine-2-thiolate (**3b**).* Orange solid; yield, 71%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.35 (d, J = 4.8 Hz, 2H, m-CH), 6.81(t, J = 4.8 Hz, 1H, p-CH), 3.91 (s, 6H, Me) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ = 182.7 (C−Au), 180.9 (C−S), 156.4 (m-CH), 117.4 (C−Cl), 115.1 (p-CH), 36.8 (Me) ppm. Anal. Calcd. for $C_9H_9AuCl_2N_4S{\cdot}1CH_2Cl_2$: C, 21.58; H, 1.98; N, 10.07; S, 5.76; found, C, 21.15; H, 2.14; N, 10.86; S, 5.53%.

*1,3-Dimethyl-4-acrylic Acid Methyl Ester Imidazole-2-ylidene-gold(I)-pyrimidine-2-thiolate (**3c**).* Pale gray solid; yield, 83%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.32 (d, J = 4.8 Hz, 2H, m-CH), 7.43 (d, J = 16.1 Hz, 1H, OC−CH═CH), 7.33 (s, 1H, NCHCN), 6.77 (t, J = 4.8 Hz, 1H, p-CH), 6.30 (d, J = 16.1 Hz, 1H, CH═CH), 3.97 (s, 3H, N-Me), 3.91 (s, 3H, N-Me), 3.80 (s, 3H, O-Me) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ = 185.2 (C−Au), 181.0 (C−S), 166.1 (C═O), 156.4 (m-CH), 129.9 (NCCHN), 128.0 (OCCH═CH), 121.9 (NCHCN), 120.4 (CH═CH), 115.0 (p-CH), 52.0 (O-Me), 38.3 (N-Me), 36.1 (N-Me) ppm. Anal. Calcd. for $C_{13}H_{15}AuN_4O_2S{\cdot}0.25CH_2Cl_2$: C, 31.24; H, 3.05; N, 11.00; S, 6.29; found, C, 31.01; H, 2.84; N, 11.28; S, 5.76%.

*1-Methyl-3-(diphenylmethyl)imidazole-2-ylidene-gold(I)-pyrimidine-2-thiolate (**3d**).* Colorless solid; yield, 83%. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.31 (d, J = 4.8 Hz, 2H, m-CH), 7.58 (s, 1H, $CHPh_2$),7.38 (m, 6H, m,p-CH_{Ph}), 7.25 (dd, J = 1.6 Hz, J = 7.2 Hz, 4H, o-CH_{Ph}), 6.96 (d, J = 1.9 Hz, 1H, NCHCHN), 6.91 (d, J = 1.9 Hz, 1H, NCHCHN), 6.78 (t, J = 4.8 Hz, 1H, p-CH), 3.94 (s, 3H, Me) ppm. ^{13}C NMR (151 MHz, $CDCl_3$): δ = 183.0 (C−Au), 181.3 (C−S), 156.4 (m-CH), 138.6 (C_{ipso}-Ph), 128.9 (CH_{Ph}), 128.4 (CH_{Ph}), 128.4 (CH_{Ph}), 121.9 (NCHCHN), 119.5 (NCHCHN), 115.0 (p-CH), 68.1 (CH-Ph_2), 38.2 (Me) ppm. Anal. Calcd. for $C_{21}H_{19}AuN_4S$: C, 45.33; H, 3.44; N, 10.07; S, 5.76; found, C, 45.09; H, 3.67; N, 9.97; S, 5.37%.

*1,3-(2,6-Dimethylphenyl)imidazole-2-ylidene-gold(I)-pyrimidine-2-thiolate (**3e**).* Colorless solid; yield, 92%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.04 (d, J = 4.8 Hz, 2H, m-CH), 7.30 (t, J = 7.6 Hz, 2H, p-CH_{Ar}), 7.18 (d, J = 7.5 Hz, 4H, m-CH_{Ar}), 7.15 (s, 2H, NCHCHN), 6.61 (t, J = 4.8 Hz, 1H, m-CH), 2.21 (s, 12H, Me) ppm. ^{13}C NMR (101 MHz, $CDCl_3$): δ = 183.8 (C−Au), 181.0 (C−S), 155.8 (m-CH), 137.3 (C_{ipso}-Ar), 135.4 (C-Me), 129.5 (p-CH_{Ar}), 128.7 (m-CH_{Ar}), 121.9 (NCHCHN), 114.6 (p-CH), 18.0 (Me) ppm. Anal. Calcd. for $C_{23}H_{23}AuN_4S$: C, 47.26; H, 3.97; N, 9.59; S, 5.49; found, C, 47.33; H, 4.20; N, 9.50, S, 5.26%.

*1,3-(2,4,6-Trimethylphenyl)imidazole-2-ylidene-gold(I)-pyrimidine-2-thiolate (**3f**).* Colorless solid; yield, 87%. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.07 (d, J = 4.8 Hz, 2H, m-CH), 7.13 (s, 2H, NCHCHN), 7.00 (s, 4H, m-CH_{Ar}), 6.64 (t, J = 4.8 Hz, 1H, p-CH), 2.36 (s, 6H, p-Me), 2.19 (s, 12H, o-Me) ppm. ^{13}C NMR (151 MHz, $CDCl_3$): δ = 184.1 (C−Au), 181.1 (C−S), 155.7 (m-CH), 139.2 (C_{ipso}), 134.9 (C_{ipso}), 129.2 (CH_{Ar}), 121.9 (NCHCHN), 114.5 (p-CH), 21.1 (p-Me), 17.8 (o-Me) ppm. Anal. Calcd. for $C_{25}H_{27}AuN_4S$: C, 49.02; H, 4.44; N, 9.15; S, 5.23; found, C, 49.01; H, 4.90; N, 9.10, S, 5.04%.

*1,3-Diisopropylbenzimidazole-2-ylidene-gold(I)-pyrimidine-2-thiolate (**3g**).* Yellow solid; yield, 74%. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.36 (d, J = 4.8 Hz, 2H, m-CH), 7.66 (dd, J = 3.2 Hz, J = 6.2 Hz, 2H, CH_{Ar}), 7.39 (dd, J = 3.1 Hz, J = 6.2 Hz, 2H, CH_{Ar}), 6.81 (t, J = 4.8 Hz, 1H, p-CH), 5.60 (sept, J = 7.0 Hz, 2H, $CHMe_2$), 1.81 (d, J = 7.0 Hz, 12H, Me) ppm. ^{13}C NMR (151 MHz, $CDCl_3$): δ = 186.5 (C−Au), 181.1 (C−S), 156.4 (m-CH), 132.6 (C_{ipso}), 123.6 (CH_{Ar}), 115.0 (p-CH), 112.9 (CH_{Ar}), 53.7 ($CHMe_2$), 21.8 (Me) ppm. Anal. Calcd. for $C_{17}H_{21}AuN_4S$: C, 40.00; H, 4.15; N, 10.98; S, 6.28; found, C, 39.55; H, 4.53; N, 10.53; S, 5.68%.

*1-Methyl-3-(ferrocenylmethyl)benzimidazole-2-ylidene-gold(I)-pyrimidine-2-thiolate (**3h**).* Orange solid; yield, 67%. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.44 (d, J = 4.8 Hz, 2H, m-CH), 7.50 (m, 4H, CH_{Ar}), 6.87 (t, J = 4.8 Hz, 1H, p-CH), 5.54 (s, 2H, NCH_2), 4.67 (m, 2H, Cp_{sub}), 4.23 (s, 5H, Cp_{unsub}), 4.15 (m, 2H, Cp_{sub}), 4.08 (s, 3H, Me) ppm. ^{13}C NMR (151 MHz, $CDCl_3$): δ = 186.0 (C−Au), 182.2 (C−S), 156.6 (m-CH), 134.0 (C_{ipso}), 124.3, 124.2 (CH_{Ar}), 115.2 (p-CH), 111.5, 111.2 (CH_{Ar}), 81.7 (C_{Cp}), 69.7 (Cp), 68.9 (Cp), 68.8 (Cp), 48.5 (NCH_2), 35.0 (Me) ppm. Anal. Calcd. for $C_{23}H_{21}AuFeN_4S{\cdot}1MeOH$: C, 42.98; H, 3.73; N, 8.36; S, 4.78; found, C, 42.58; H, 3.50; N, 8.54; S, 4.60%.

Preparation of Thioredoxin Reductases. Highly purified cytosolic thioredoxin reductase (TrxR1) was prepared according to Luthman and Holmgren starting from rat liver.[54] Mitochondrial thioredoxin reductase (TrxR2) was purified from isolated liver mitochondria following the procedure of Rigobello et al.[55]Yeast glutathione reductase was obtained from Sigma (St Louis Mo, USA) and used without further purification. Protein contents were assayed using the procedure by Lowry et al.[56]

Estimation of Enzyme Activities Inhibition *in Vitro*. Thioredoxin reductases activity was determined by measuring the ability of the enzyme to directly reduce DTNB in the presence of NADPH.[54] Aliquots of highly purified TrxR1 (60 nM) and TrxR2 (130 nM) in 0.2 M Na,K-phosphate buffer (pH 7.4), 5 mM EDTA, and 0.25 mM NADPH were preincubated for 5 min with the gold complexes. Afterward, the reaction was started with 1 mM DTNB and monitored spectrophotometrically at 412 nm for about 10 min.[57] Glutathione reductase activity was measured in 0.2 M Tris HCl buffer (pH 8.1), 1 mM EDTA, and 0.25 mM NADPH after 5 min of preincubation with the various gold complexes. The assay was initiated by the addition of 1 mM GSSG and followed spectrophotometrically at 340 nm.

BIAM Assay. TrxR (1 μM) prereduced in the presence of NADPH was incubated with 10 μM of gold complexes for 30 min at room temperature, in 20 mM Tris-HCl buffer (pH 7.4) containing 200 μM NADPH and 1 mM EDTA. After incubation, 8 μL of the reaction mixture was removed and added to 50 μM biotinylated iodoacetamide (BIAM) in 0.1 M Tris-HCl at pH 8.5 and/or 0.1 M Hepes-Tris pH 6.0.[28] Samples were incubated at room temperature for an additional 30 min to alkylate the remaining −SH groups in the enzyme. Then, BIAM-modified enzyme was mixed with loading buffer, the mixture was subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on a 7.5% gel, and the separated proteins were transferred to a nitrocellulose membrane. Proteins labeled with BIAM were detected with horseradish peroxidase-conjugated streptavidin and enhanced chemiluminescence detection.

Cell Culture. Human ovarian carcinoma cell line A2780S (sensitive), A2780R (cisplatin resistant), and HEK-293T (human embryonic kidney) were used. A2780S/R cells were grown at 37 °C in 5% carbon dioxide atmosphere using RPMI 1640 medium, containing 10% fetal calf serum, and supplemented with 2 mM L-glutamine. HEK-293T cells were grown in DMEM (high glucose) with 10% fetal calf serum, supplemented with 2 mM L-glutamine.

MTT Assay. Cell viability was assayed with the MTT reduction assay. A2780S, A2780R, and HEK-293T cells (10×10^3) were treated with increasing concentrations of gold complexes at the indicated time. At the end of incubation, cells were treated for 3 h at 37 °C with 0.5 mg/mL MTT dissolved in PBS. Afterward, MTT was removed and 100 μL of stop solution (90% isopropanol and 10% DMSO) were added to each well. After 15 min of incubation at 37 °C, samples were estimated in a plate reader (Multiskan EX, Labsystems, Finland) at 540−690 nm.

TrxR and GR Activity Determinations in Cell Lysates. A2780S, A2780R, and HEK-293T cells (1×10^6) were incubated for 24 h with the indicated concentrations of gold complexes. After incubation, cells were harvested and washed with PBS. Each sample was lysed with a modified RIPA buffer: 150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 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 of incubation at 0 °C, lysates were centrifuged at 14,000g for 5 min. The obtained supernatants were tested for enzyme activities. Aliquots (50 μg) of lysates were subjected to thioredoxin reductase determination in a final volume of 250 μL of 0.2 M Na,K-phosphate buffer (pH 7.4) with 5 mM EDTA, containing 2 mM DTNB. After 2 min, the reaction was started with 0.300 mM NADPH. Glutathione

reductase activity was estimated at 25 °C on 80 μg protein/mL as reported above.

Redox Western Blot Analysis of Thioredoxin 1 and Thioredoxin 2. To assess the redox state of thioredoxins, we used the method described by Hansen et al.[32] with modifications. Trx1 redox state was measured by derivatizing thiols with 50 mM iodoacetic acid (IAA). Cells (2×10^5) were plated (12-wells plate) and incubated with 6 μM gold complexes for 3 h in RPMI or DMEM complete depending on the cell line. Then, cells were centrifuged in a rotor plate at 500*g* for 5 min. Medium was removed, and cells were washed with cold PBS. Samples were added to 60 μL of a solution containing 6 M guanidine, 50 mM Tris-HCl buffer, pH 8.3, 3 mM EDTA, 0.5% Triton X-100, and 50 mM IAA. Cells were rapidly scraped and maintained at 37 °C for 50 min. After incubation, samples were applied to MicrospinTM G-25 Columns (GE Healthcare, Little Chalfont, UK) to remove the excess of IAA. Proteins of eluted samples were measured with the Lowry et al. procedure.[56] The samples to be subjected to electrophoresis were treated with 5× sample buffer (0.5 M Tris-HCl, pH 6.8, 50% glycerol (v/v), bromophenol blue 0.05% (w/v)), loaded onto a native gel (15%) and subjected to Western blotting, using a polyclonal primary antibody anti Trx1 (FL-105) (Santa Cruz Biotechnology, Santa Cruz, CA. USA).

For the determination of Trx2 redox state, after incubation time with gold complexes, cells were centrifuged at 500*g*, in a rotor plate, for 5 min, washed with cold PBS, and then treated with 1 mL of 10% trichloro-acetic acid. The sample were scraped, transferred to Eppendorf tubes, and kept at 4 °C for 30 min, and then centrifuged for 10 min at 10000*g* at 4 °C. The obtained pellet was resuspended in 0.5 mL of ice-cold 100% acetone and maintained at 4 °C for 10 min, then centrifuged again at 10000*g* for 10 min at room temperature. After removing the excess of acetone, the pellet was dissolved in 62.5 mM Tris HCl at pH 8, 1% SDS containing 8 mM AIS (4-acetamido-4′-((iodoacetyl) amino)stilbene-2,2′-disulfonic acid) (Invitrogen). Derivatization lasted 1 h and 30 min, at room temperature, followed by further 30 min at 37 °C. Samples were loaded, without reducing agents, onto Bis-Tris Gel NUPAGE (12%) and blotted. To asses the redox state of thioredoxin **2** a polyclonal antibody H-75 (Santa Cruz Biotechnology) was used.

Glutathione Content and Redox State Determination. A2780S, A2780R, and HEK-293T cells (3×10^5) were plated in 6-wells plate and treated with 6 μM gold complexes for 3 h. After incubation, the medium was rapidly removed, cells were washed with PBS, and then deproteinized in each well with 6% *meta*-phosphoric acid and scraped. After 10 min at 4 °C, the deproteinized samples were centrifuged, and the supernatant was neutralized with 15% of Na_3PO_4. Aliquots of neutralized samples were tested for total glutathione,[58] and 300 μL was derivatized with 6 μL of 2-vinylpyridine in order to remove the reduced glutathione to determine the oxidized glutathione.[59] In addition, the obtained pellets after deproteinization were washed with 1 mL of ice-cold acetone, centrifuged at 11000*g*, taken to dryness, and then dissolved in 62.5 mM Tris HCl buffer (pH 8.1) containing 1% SDS and utilized for protein determination.

Evaluation of ROS Production. The generation of ROS was assessed by the fluorogenic probe CM-DCFH2-DA (Molecular Probes, Invitrogen) in A2780S, A2780R, and HEK-293T cells. Cells (2×10^4) were seeded in 96-wells plate and after 24 h washed in PBS/10 mM glucose and loaded with 10 μM CM-DCFH2-DA for 20 min in the dark at 37 °C. Afterward, cells were washed with the same medium and incubated with gold complexes (2 μM). Fluorescence increase was estimated in a plate reader (Fluoroskan Ascent FL, Labsystem, Finland) at 485 nm (excitation) and 527 nm (emission) for 2 h.

Statistical Analysis. All the values are the means ± SD of not less than five measurements. Multiple comparisons were made by one-way analysis of variance followed by the Tukey−Kramer multiple comparison test.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthesis of the NHC Ag halides 1,3-dimethyl-4-acrylic acid methyl ester imidazole-2-ylidene-silver(I)-iodide (**1c**), 1,3-(2,6-dimethylphenyl)imidazole-2-ylidene-silver(I)-chloride (**1e**), and 1-methyl-3-(ferrocenylmethyl)benzimidazole-2-ylidene-silver(I)-iodide (**1h**) and crystallographic details and additional material for the biological studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AIS, (4-acetamido-4′-((iodoacetyl) amino)stilbene-2,2′-disulfonic acid; AF, auranofin; BIAM, biotinylated iodoacetamide; GSH, glutathione; GR, glutathione reductase; IAA, iodoacetic acid; IMe, 1,3-dimethylimidazole-2-ylidene; ^{Cl}IMe, 4,5-dichloro-1,3-dimethylimidazole-2-ylidene; $^{MeOC(O)CHCH}$IMe, 4-acrylic acid methyl ester-1,3-dimethylimidazol-2-ylidene; IMeCHPh$_2$, 1-methyl-3-diphenylmethylimidazole-2-ylidene; IXyl, 1,3-(2,6-dimethylphenyl)imidazole-2-ylidene; IMes, 1,3-(2,4,6-trimethylphenyl)imidazole-2-ylidene; fc, ferrocenyl; NHC, *N*-heterocyclic carbene; ROS, reactive oxygen species; Trx, thioredoxin; TrxR, thioredoxin reductase

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