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Chemistry and Biology of Two Novel Gold(I) Carbene Complexes as Prospective Anticancer Agents

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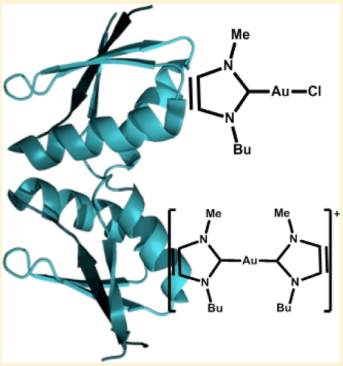
Supporting Information

ABSTRACT: Two novel gold carbene compounds, namely, chlorido (1-butyl-3-methyl-imidazole-2-ylidene) gold(I) (**1**) and bis(1-butyl-3-methyl-imidazole-2-ylidene) gold(I) (**2**), were prepared and characterized as prospective anticancer drug candidates. These compounds consist of a gold(I) center linearly coordinated either to one N-heterocyclic carbene (NHC) and one chloride ligand (**1**) or to two identical NHC ligands (**2**). Crystal structures were solved for both compounds, the resulting structural data being in good agreement with expectations. We wondered whether the presence of two tight carbene ligands in **2** might lead to biological properties distinct from those of the monocarbene complex **1**. Notably, in spite of their appreciable structural differences, these two compounds manifested similarly potent cytotoxic actions in vitro when challenged against A2780 human ovarian carcinoma cells. In addition, both were able to overcome resistance to cisplatin in the A2780R line. Solution studies revealed that these gold carbene complexes are highly stable in aqueous buffers at physiological pH. Their reactivity with proteins was explored: no adduct formation was detected even upon a long incubation with the model proteins cytochrome c and lysozyme; in contrast, both compounds were able to metalate, to a large extent, the copper chaperone Atox-1, bearing a characteristic CXXC motif. The precise nature of the resulting gold-Atox-1 adducts was elucidated through ESI-MS analysis. On the basis of these findings, it is proposed that the investigated gold(I) carbene compounds are promising antiproliferative agents warranting a wider pharmacological evaluation. Most likely these gold compounds produce their potent biological effects through selective metalation and impairment of a few crucial cellular proteins.



INTRODUCTION

Following the introduction of auranofin in the clinics for the oral treatment of rheumatoid arthritis (1985) and the discovery of its remarkable antiproliferative properties in vitro, gold compounds were increasingly considered and evaluated as a possible source of new and more effective metal-based anticancer agents.[1] This interest was also fueled by the observation that gold compounds usually manifest a very different pharmacological profile compared to established anticancer platinum drugs, implying the occurrence of original and innovative modes of action. Hence, over the last two decades, several promising families of Au-based drug candidates, with the gold center in the oxidation states of +3 or +1, featuring diverse structural motifs, were prepared and characterized, and their biological and pharmacological profiles were initially assessed.[1] Relevant examples are offered by a few classical mononuclear gold(III) complexes,[2] such as gold(III) dithiocarbamates[3] and gold(III) porphyrins,[4] by some organogold(III) compounds,[5] a few binuclear gold(III) complexes,[6] various neutral, two-coordinate gold(I) complexes, inspired by auranofin,[7] a number of lipophilic cationic gold(I) complexes such as $[Au(dppe)_2]^+$, and others.[8]

N-Heterocyclic carbenes (NHCs) are very interesting gold(I) ligands as they manifest donor properties similar to phosphines, thus affording very stable gold(I) complexes; in addition, their imidazolium salt precursors are often more easily synthesized than similarly functionalized phosphines.[9] The azoles and azolium salts used in the synthesis of NHCs are generally air stable species, and their synthesis and purification is, in most cases, relatively straightforward. Hydrophilic/lipophilic properties can be readily fine-tuned by the incorporation of appropriate functional groups.[9c] Within this frame, several gold carbene complexes were prepared and characterized during the past few years that turned out particularly effective and promising from the biological and pharmacological point of view. Accordingly, a number of reports concerning cytotoxic gold(I/III) NHC complexes have

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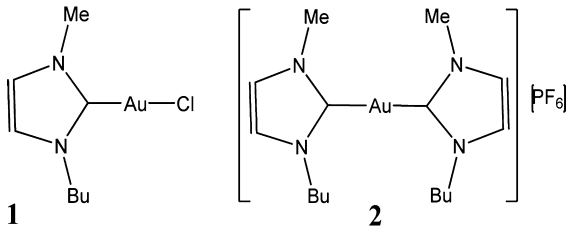
been published with many derivatives, showing highly promising antiproliferative activity with IC_{50} values in the low micromolar or even nanomolar range.[9,10]

In 2004 Barnard et al. documented the induction of "mitochondrial permeability transition" by various dinuclear gold(I) NHC complexes.[11] The interference of gold(I) NHC complexes with mitochondrial biochemistry is a feature of particular interest that was further stressed in subsequent studies. Baker et al. successfully synthesized a series of NHC gold(I) analogues of auranofin and reported on their biological activity.[12] More recently, Rubbiani et al. described a series of three structurally related Au(I) complexes with benzimidazole-derived NHC ligands that exerted selective thioredoxin reductase (TrxR) inhibition and significant antiproliferative effects.[13] On the basis of the various reported findings, gold carbene complexes are now mainly considered to be a class of antimitochondrial agents.[1b,9c,10a,14]

Even though several studies have been carried out so far on the cellular effects of gold carbene compounds and valuable mechanistic information has been gathered, the precise mode of the gold carbene complex action, at the molecular level, is still unclear. This led us to prepare and characterize two novel gold carbene complexes and investigate in depth their main chemical and biological features through a variety of physicochemical and biochemical tests. The gold carbene complexes that were designed and prepared for the present investigation are schematically represented in Chart 1. They feature mono-carbene gold(I) complex **1** and dicarbene gold(I) complex **2** having the same carbene ligand.

Chart 1. Structures of the NHC Au(I) Complexes Synthesized in This Study



In both cases 1-butyl-3-methyl-imidazole-2-ylidene was chosen as the NHC ligand to obtain gold complexes possessing a high lipophilicity, in line with previous studies.[9c,15a] Indeed, it is now well documented that selectivity for cancer cells over normal cells can be "tuned" by adjusting the hydrophilic/lipophilic balance in a series of related Au(I) compounds.[15] In complex **1** the second gold(I) ligand is a chloride ion that, in principle, is believed to act as a more labile ligand. The antiproliferative properties in vitro of these gold compounds were assessed toward the human ovarian carcinoma cell line A2780 either sensitive or resistant to cisplatin. Then, their reactivity with a few model proteins was explored, as it is commonly believed that gold(I) compounds produce their biological actions mainly through interactions with protein targets.

We also analyzed the interactions of these compounds with human Atox-1, a protein extremely prone to react with gold-containing species.[16] Specifically, Atox-1 is a cytoplasmic copper chaperone belonging to the copper trafficking system that interacts with the copper-binding domain of the membrane copper transporters ATP7A and ATP7B.[17] The copper trafficking system consists of proteins that help the uptake of copper into cells and then promote its transfer and delivery to copper-dependent cellular proteins. One of these "chaperones" is known as Atox-1. Most Atox-1 homologues are ∼70 amino acid proteins containing a conserved CXXC motif for copper(I) binding, located in a solvent-exposed loop, in the vicinity of the N terminus.[18] The latter CXXC motif confers to Atox-1 a high reactivity toward "soft" metal ions. Recently we showed that a few representative cytotoxic gold(III) compounds produce stable adducts upon reaction with the copper chaperone Atox-1; notably, such adducts contain gold in the oxidation state +1 as a consequence of gold(III) reduction.[16] Gold(I) ions are "soft" Lewis acids and, as such, should react readily with the copper(I) binding site of Atox-1.

Overall, the various results obtained in the present Work provide an initial picture of the potentialities of the examined gold(I) carbenes as prospective anticancer agents and offer some insights on the possible mode of action of these carbenes at the molecular level.

EXPERIMENTAL SECTION

Materials. Horse heart cytochrome c (C7752) and chicken hen egg white lysozyme (L7651) were purchased from Sigma and used as received. Atox-1—full length HAH1 (G09HA101)—was purchased from Giotto Biotech. RPMI 1640 cell culture medium, fetal calf serum (FCS), and phosphate-buffered saline (PBS) were obtained from Celbio (Milan, Italy); dimethyl sulfoxide (DMSO), sulforhodamine B (SRB), and cisplatin (purity >99.9%) were obtained from Sigma (Milan, Italy).

Instruments. Elemental analyses were performed by a Carlo Erba elemental analyzer model 1106. Infrared (IR) spectra were recorded with a Perkin-Elmer FT-IR spectrometer equipped with a universal attenuated total reflectance (UATR) sampling accessory. NMR spectra were recorded on a Varian Gemini 200 BB instrument (^{1}H, 200 MHz; ^{13}C, 50.3 MHz) at room temperature; frequencies are referenced to the residual resonances of the deuterated solvent. UV−visible (UV-vis) spectra were recorded on a Varian Cary 50 spectrophotometer. Electrospray ionization mass spectrometry (ESI-MS) measurements were performed on a linear trap quadrupole (LTQ) linear ion trap (Thermo, San Jose, CA) equipped with a conventional ESI source. Data collection for the sample was carried out on an Oxford Diffraction XCalibur Diffractometer with charge-coupled device (CCD) area detector, equipped with Mo Kα radiation (α = 0.7107 A) and a low-temperature device (data collection was performed at 120 K). The program suite used for the data collection was CrysAlis CCD,[19] while the data were reduced with the program CrysAlis RED, and the absorption correction was applied by the program ABSPACK.[20]

Synthesis of [(NHC)AuCl] (1). Ag_2O (60 mg, 0.26 mmol) was added to a solution of 1-butyl-3-methylimidazolium chloride (80 mg, 0.46 mmol) in a mixture of CH_2Cl_2 (4 mL) and MeOH (5 mL) under N_2 atmosphere and stirred overnight under protection from light. Then [(Me_2S)AuCl] (135 mg, 0.46 mmol) was added, and the suspension was stirred for additional 3 h under N_2. The black precipitate was separated by filtration over Celite, and the filtrate was evaporated to dryness under reduced pressure to yield a colorless oil. This was further purified by column chromatography on silica gel (CH_2Cl_2/hexane, 1:4) to give **1** as a colorless powder (107 mg; 63% yield). ^{1}H NMR (acetone-d_6, 293K): δ = 7.38 (d, J = 1.6 Hz, 1 H), 7.33 (d, J = 1.6 Hz, 1 H), 4.20 (t, J = 6.8 Hz, 2 H), 3.83 (s, 3 H), 1.85 (apparent quintet, J = 6.8 Hz, 2 H), 1.34 (apparent sextet, J = 7.4 Hz, 2 H), 0.94 ppm (t, J = 7.4 Hz, 3 H); ^{13}C{^{1}H} NMR (C_6D_6, 293K): δ = 171.9 (imidazolyl C2); 121.3, 120.9 (imidazolyl C4/C5), 50.7 (NCH_2), 37.4 (NCH_3), 33.0 (NCH_2CH_2), 19.7 ($NCH_2CH_2CH_2$), 13.8 ppm (CH_2CH_3); IR (solid state): ν = 3152, 3122, 2869, 1599, 1564, 1465, 1406, 1368, 1230, 1200, 752 cm^{-1}; Anal. Calcd (%) for $C_8H_{14}AuClN_2$: C 25.9, H 3.81, N 7.56. Found: C 25.7, H 3.65, N 7.39.

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Synthesis of $[Au(NHC)_2]PF_6$ (2). LiHMDS (111 mg, 0.78 mmol) was added to a solution of 1-butyl-3-methylimidazolium chloride (130 mg, 0.74 mmol) in dimethylformamide (DMF) (2 mL). After 30 min of stirring, a solution of $[(Me_2S)AuCl]$ (103 mg, 0.35 mmol) in DMF (1 mL) was added dropwise. The resulting mixture was stirred for 4 h, and the solvent was filtered and washed with ether. The hygroscopic solid was collected and then dissolved in water (2 mL). After the addition of saturated aqueous KPF_6, the resultant precipitate was collected, washed with water, and dried in vacuo, yielding a colorless solid (121 mg; 56% yield). ^{1}H NMR (acetone-d_6, 293K): δ = 7.49 (d, J = 1.6 Hz, 1 H), 7.43 (d, J = 1.6 Hz, 1 H), 4.33 (t, J = 7.0 Hz, 2 H), 3.99 (s, 3 H), 1.93 (apparent quintet, J = 7.0 Hz, 2 H), 1.37 (apparent sextet, J = 7.4 Hz, 2 H), 0.95 ppm (t, J = 7.4 Hz, 3 H); ^{13}C{^{1}H} NMR (acetone-d_6, 293K): δ = 184.8 (imidazolyl C2); 123.7, 122.6 (imidazolyl C4/C5), 51.4 (NCH_2), 38.2 (NCH_3), 34.3 (NCH_2CH_2), 20.3 ($NCH_2CH_2CH_2$), 13.9 ppm (CH_2CH_3); IR (solid state): ν = 3176, 3148, 2956, 2873, 1568, 1474, 1411, 1381, 1236, 1205, 1118, 1084, 877, 831, 739, 692 cm^{-1}; Anal. Calcd (%) for $C_{16}H_{28}AuF_6N_4P$: C 31.1, H 4.56, N 9.06. Found: C 30.9, H 4.71, N 8.81.

X-ray Crystallography. The structure of **2** was confirmed by X-ray crystallography. For compound **1** the data collected allow the determination of the structure but not reliably enough to be published (see Supporting Information). Crystal of the compound was grown by the diffusion of $CHCl_3$ vapors into an n-hexane solution of gold(I) complex.

The structures of compound **2** was solved by direct methods executed by the program SIR97[21] and then refined by full-matrix least-squares against F2 using all data (SHELXL 2012).[22] In **2** the Au(1), P(1), F(1), F(2), F(3), F(4), and F(5) atoms lie on a special position, and the occupancy factors are all 0.5. For both complexes, all non-hydrogen atoms were refined anisotropically, while the hydrogen atoms were set in calculated positions as riding atoms and refined accordingly.

Strong absorption has been detected for the crystal of **2**. This factor leads to strong peaks of residual electron density in the Fourier maps mainly, but not only, around the gold metal atom.

Geometrical calculations were completed with PARST,[23] and molecular plots were produced with ORTEP3 program[24a] and Mercury.[24b] Crystal data are reported in Table 1, while bond lengths and angles are reported in Table 2.

Cell Lines and Cell Culture. The human ovarian carcinoma cell line sensitive to cisplatin (A2780/S) and its cisplatin-resistant cell subline (A2780/R) were used for cytotoxicity studies. Cell lines were maintained in RPMI1640 medium supplemented with FCS (10%) and antibiotics at 37 °C in a 5% CO_2 atmosphere and subcultured twice weekly.

Table 1. Crystallographic Data for Complex 2

complex	**2**
empirical formula	$C_8H_{14}Au_{0.50}F_3N_2O_{0.75}P_{0.50}$
formula weight	321.18
temperature (K)	120(2)
wavelength (Å)	0.71073
crystal system, space group	monoclinic, $C2/m$
unit cell dimensions (Å, deg)	a = 18.3243(11) b = 13.0863(8) β = 105.088(5) c = 10.6892(5)
volume (Å^3)	2474.9(2)
Z, D_c (mg/cm^3)	8, 1.724
μ (mm^{-1})	6.069
F(000)	1248
crystal size (mm)	0.2 × 0.1 × 0.05
θ range (deg)	1.936 to 25.0
reflections collected	6738
data/restraints/parameters	3035/0/150
goodness-of-fit on F^2	1.047
final R indices [$I > 2\sigma(I)$]	$R1$ = 0.0562, w$R2$ = 0.1198
R indices (all data)	$R1$ = 0.0800, w$R2$ = 0.1480

Table 2. Bond Lengths (Å) and Angles (deg) for Compound 2

	2
Au(1)−C(1)	2.02(1)
C(1)−Au(1)−C(1′)[a]	178.0(4)
N(1)−C(1)−N(2)	105.0(8)
N(2)−C(5)−C(6)	112.2(7)
C(7)−C(6)−C(5)	119(1)
C(6)−C(7)−C(8)	111 (1)
N2−C5−C6−C7	57(4)
C5−C6−C7−C8	−179(3)

[a] ′ is reported by the symmetry operation x, $-y$, z.

Cell Growth Inhibition Studies. The cytotoxic effects of the studied gold carbenes were evaluated on the growth of A2780/S and A2780/R cell lines, according to the procedure described by Skehan et al.[25] Both compounds were initially diluted in DMSO as stock solutions (20 mM), and further dilutions were performed in PBS (0.5% DMSO present at the higher tested concentration).

Exponentially growing cells were seeded in 96-well microplates at a density of 5 × 10^3 cell/well. After cell inoculation, the microtiter plates were incubated under standard culture conditions (37 °C, 5% CO_2, 95% air, and 100% relative humidity) for 24 h prior to the addition of the studied compounds. After 24 h, the medium was removed and replaced with fresh medium containing drug concentrations ranging from 0.003 to 100 μM for a continuous exposure of 24 and 72 h for both compounds.

For comparison purposes the cytotoxicity effects of cisplatin (CDDP) measured in the same experimental conditions were also determined.

According to the procedure, the assay was terminated by the addition of cold trichloroacetic acid (TCA). Cells were fixed in situ by 10% TCA and stained by sulforhodamine B (SRB) solution at 0.4% (w/v) in 1% acetic acid. After staining, unbound dye was removed by washing five times with 1% acetic acid, and the plates were air-dried. Bound stain was subsequently solubilized with 10 mM tris-(hydroxymethyl)aminomethane (tris) base, and the absorbance was read on an automated plate reader at a wavelength of 540 nm.

The IC_{50} drug concentration resulting in a 50% reduction in the net protein content (as measured by SRB staining) in drug treated cells as compared to untreated control cells was determined after 72 h of drug exposure. The IC_{50} data for the two carbenes and cisplatin represent the mean of at least three independent experiments.

To evaluate presence or lack of cross-resistance to the studied compounds of cisplatin-resistant cells, A2780/R as compared to the parental A2780/S cells, the resistance ratio (R) was calculated as the IC_{50} values in the resistant cell line and the IC_{50} values in the sensitive one.

Interactions with Lysozyme and Cytochrome c. The gold complexes were added to the solution containing cytochrome c (cyt c) or lysozyme (HEWL) (3:1 metal/protein ratio) in 20 mM ammonium acetate buffer, pH 7.4. Mono and dicarbene/protein adducts were prepared by mixing equivalent amounts of the two proteins (10^{-4} M). The solution was incubated at 37 °C for 72 h. After a 20-fold dilution with water, ESI-MS spectra were recorded by direct introduction at 5 μL/min flow rate in an Orbitrap high-resolution mass spectrometer.

The working conditions were the following: spray voltage 3.1 kV, capillary voltage 45 V, and capillary temperature 220 °C. The sheath and the auxiliary gases were set, respectively, at 17 and 1 (arbitrary units). For acquisition, the Xcalibur 2.0. software (Thermo) was used, and monoisotopic and average deconvoluted masses were obtained by using the integrated Xtract tool. For spectrum acquisition a nominal resolution (at m/z 400) of 100 000 was used.

Interactions with Atox-1. Mono and dicarbene/protein adducts were prepared by mixing equivalent amounts of Atox-1 (10^{-4} M) in ammonuim acetate buffer at pH 7.4 and dithiothreitol (DTT) (10^{-4}

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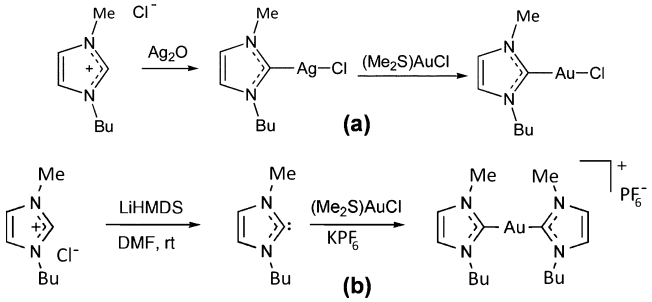
M) in a 1:1:1 metal/Atox-1/DTT molar ratio. The solution was incubated at room temperature for 24 h. After a 10-fold dilution with water and 1% HCOOH, ESI-MS spectra were recorded by direct introduction at 5 μL/min flow rate in an Orbitrap high-resolution mass spectrometer. The working conditions were the same as those indicated for the interactions with cyt c and HEWL.

RESULTS AND DISCUSSION

Synthesis and Structural Characterization. The gold(I) carbene complexes **1** and **2** were prepared by adapting the procedure developed by Baker and Berners-Price.[9c,26] The synthesis of complex **1** was achieved via the known route of transmetalation of the corresponding Ag(I) NHC complex with [(Me$_2$S)AuCl] (Scheme 1a). Complex **2** was synthesized by the

Scheme 1. (a) Synthesis of Compound 1; (b) Synthesis of Compound 2



reaction of [(Me$_2$S)AuCl] with 2 equiv of 1-butyl-3-methylimidazole-2-ylidene, prepared in situ through deprotonation of the imidazolium salt with lithium bis(trimethylsilyl)amide (lithium hexamethyldisilazide, LiHMDS) in DMF at room temperature (Scheme 1b). Complexes **1** and **2** were obtained in good yields as colorless solids, which are soluble in halogenated solvents, acetone, and DMSO. Complex **1** is soluble even in nonpolar solvents such as benzene, ether, hexane, etc.

The new compounds were characterized in the solid state by elemental analysis and IR spectroscopy and then in solution by ^{1}H and ^{13}C NMR spectroscopy. The solid-state structure for compound **2** was determined by single-crystal X-ray diffraction. For **1** the data are not complete but reported as Supporting Information (Figure S1 and Tables S1 and S2). The ORTEP view of compound **2** is shown in Figure 1, and crystallographic data are reported in Table 1. Compound **2** gave pale yellow crystals, which hold half of the complex and half of the PF_6^- counterion in the asymmetric unit.

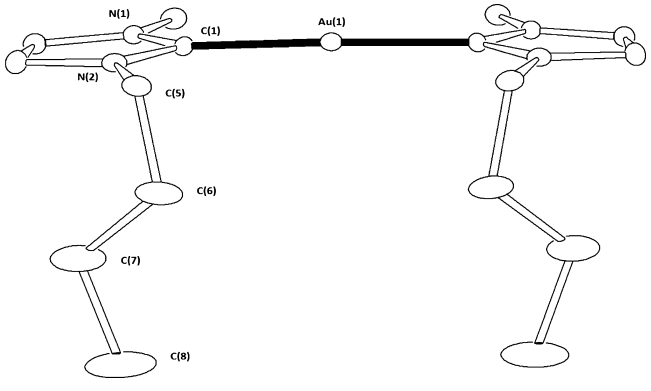


Figure 1. Molecular structure of **2**.

In both compounds, the NHC moiety is bound to the gold(I) center through the C atom. This gold(I) center displays the usual linear coordination with very modest distortions: in **2** the C(1)−Au(1)−C(1′) angle is 178.0(4)° (′ is reported by the symmetry operation x, $-y$, z). The bond length C(1)−Au(1) is 2.02(1) Å, and it is in the range found for similar compounds in a search performed in the Cambridge Structural Database (CSD) (v. 5.33 November 2011).[27] Differences in Au−C bond lengths might imply some differences in the bond strength in the two cases (for **1** the bond length is 1.94(2) Å) as previously observed by Ott et al. when working on a similar series of gold(I) complexes.[14a]

Also, the Au(1)−Cl(1) bond length is typical for these compounds (2.281(4) Å) as previously reported.[28] The aliphatic chain in the complex has the same dihedral conformation (see Table 2): the sequence of the dihedral angles being synclinal (sc) and antiperiplanar (ap).

Remarkably, in the crystal packing of **2** (Figure 2), it is possible to detect a relatively strong aurophilic interaction

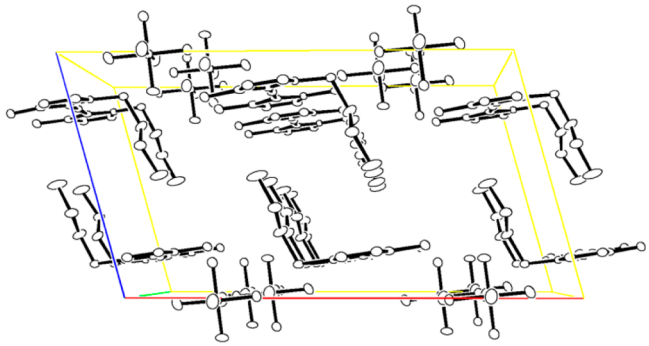


Figure 2. Views of the packing in crystals of **2**.

(Au···Au 3.5560(5) A) giving rise to dimers of the complex in the lattice (for detail, see Supporting Information, Figure S2). Moreover the crystal packing reveals a channel between the discrete molecule in which water molecules are located. The occupancy factors of these molecules are all set to 0.5, and this ribbon of water runs along the a axis (see Supporting Information, Figure S3).

Solution Chemistry. The solution chemistry of **1** and **2** was investigated through UV−vis spectrophotometry and ^{1}H NMR spectroscopy, under physiologically relevant conditions. The UV−vis spectra of the above complexes and, for comparison, of 1-butyl-3-methylimidazolium chloride, were recorded in 50 mM phosphate buffer at pH 7.4. All compounds are soluble in DMSO but poorly soluble in water. Thus, in line with previous studies, the compounds were first dissolved in DMSO, and the resulting solutions were diluted with a reference aqueous buffer (50 mM phosphate buffer, pH 7.4). The percentage of DMSO in the final aqueous solution is 1%. No precipitation was apparent at dilution, using the described experimental procedure. Solutions were examined spectrophotometrically over 24 h; the resulting spectral profiles are shown in Figure 3. Notably, the two compounds are soluble and stable within this medium, with full retention of their original structure. The spectra of the complexes showed absorbance maxima at ca. 250−260 nm, characteristic of the gold(I) chromophore, that may straightforwardly be assigned as metal-to-ligand charge-transfer (MLCT) bands. As gold(I) has a d^{10} configuration, ligand-to-metal charge-transfer (LMCT) tran-

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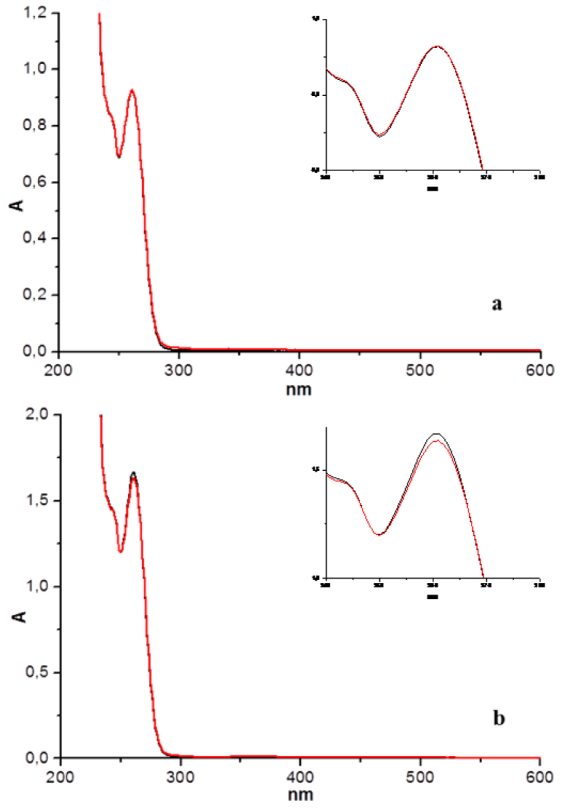

Figure 3. Hydrolysis profiles of complexes (a) **1** and (b) **2** dissolved in phosphate buffer (50 mM, pH 7.4). Spectra were recorded over 24 h at room temperature. (black) t = 0; (red) t = 24 h. Inset: zoom of the main peak.

sitions and ligand-field (LF) transitions are absent.[29] Since the imidazolium salt (λ_{max} = 210 nm) does not show any transition at ca. 250 and 260 nm, the bands were confidently assigned to MLCT transitions.

Furthermore, the stability of the two compounds toward biologically occurring reductants and/or thiols was evaluated. Ascorbic acid (AsA) and Glutathione (GSH) were chosen as reference agents. Interestingly, we observed that addition of even large molar excesses of the two species (up to 100:1 molar ratios) did not elicit any major spectral change, thus ruling out occurrence of gold(I) reduction (Supporting Information, Figure S4); also, no evidence was obtained for GSH coordination to the gold(I) center.

The ^{1}H and ^{13}C NMR spectra for **1** and **2** showed the signals commonly expected for these kinds of complexes. For complex **2** all signals are sharp (until −50 °C), as is usual for a structure where free rotation about the Au−C(carbene) bond occurs. As the formation of the gold(I) complexes takes place through deprotonation of the (NCHN) proton of the imidazolium chloride salt, the ^{1}H NMR spectra lack the characteristic NCHN resonance in the downfield region of the spectrum. The ^{13}C NMR spectrum, however, showed the appearance of the metal-bound carbene signal significantly downfield shifted in comparison to that of the corresponding imidazolium NCN carbon.

Both complexes turned out to be very stable in solution over 24 h observation; no appreciable release of the chloride group is noticed in the case of complex **1**. In addition, both compounds are highly resistant toward GSH and ascorbic acid treatment.

Cellular Studies/Antiproliferative Properties. Afterward, the antiproliferative properties of **1** and **2** were measured in vitro against the A2780 ovarian carcinoma human cell line sensitive to cisplatin (A2780/S) and its resistant subline (A2780/R), as reported in the Experimental Section. The IC_{50} values observed after 72 h exposure are reported in Table 3.

Table 3. In Vitro Growth Inhibition of the A2780 Cisplatin Sensitive or Resistant Cell Lines by 1 and 2 after a 72 h Drug Exposure

compounds	A2780/S IC_{50} (μM)	A2780/R IC_{50} (μM)	R^a
1	1.98 ± 0.17	0.68 ± 0.07	0.3
n^b	4	4	
2	1.68 ± 0.24	0.75 ± 0.05	0.4
n	4	4	
CDDP	1.84 ± 0.12	23.24 ± 0.75	12.6
n	6	6	

^{a}R, resistance ratio. ^{b}n, number of determinations.

Both these compounds show relevant antiproliferative effects, with IC_{50} values falling in the low micromolar range for either A2780/S (from 1.68 to 1.98 μM) or A2780/R cells (from 0.68 to 0.75 μM). This finding differs from the cytotoxicity results of Schuh et al.,[28] who suggest that the chloro-substituted NHC Au(I) complexes are expected to be less active since the chloride group may make the chloride derivatives more reactive and thus more prone to be deactivated by different cellular components.

Compound **2** was more effective than cisplatin even in the A2780/S cell line. Interestingly, these two gold carbene complexes were more active toward the cisplatin resistant cell line compared to the sensitive one (with resistance index (RI) values ranging from 0.4 to 0.5), implying that the molecular mechanism of resistance to cisplatin is totally ineffective toward them. Additional experiments were carried out to assess, at least qualitatively, the time dependence of gold-induced cytotoxic effects. This led us to measure the IC_{50} values of the two gold compounds at 24 h on both A2780/S and A2780/R cell lines. Far higher IC_{50} values were determined. Indeed, IC_{50} values at 24 h for **1** were 31.10 μM and 53.68 μM in A2780/S and A2780/R, respectively; those of **2** were 18.83 μM and 42.55 μM in A2780/S and A2780/R, respectively, to be compared to ∼1 μM values measured at 72 h. These results imply that the antiproliferative effects induced by these gold carbenes require relatively long times to show up, being mediated—most likely—through a specific and relatively slow cellular response. A similar situation holds for cisplatin: indeed IC_{50} values of cisplatin at 24 h were 32.24 μM and >100 μM in in A2780/S and A2780/R, respectively.

The available literature shows that NHC gold carbenes usually display a relevant cytotoxic activity. However, only the results of the study performed by Schuh et al.[28] are directly comparable with ours (same cell lines and same drug exposures). Results of Schuh show that their compounds have a slightly lower activity after a 72 h exposure (from 2.6 to 3.9 μM in A2780/S; from 1.6 to 6.8 μM in A2780/R) compared to our compounds, but higher activity after 24 h of drug exposure (from 3.2 to 11.5 μM in A2780/S; from 4.9 to 12.7 μM in A2780/R). Several other studies investigated the cytotoxic activity of NHC gold(I) carbenes but in tumor cell lines other than A2780.[13,14,30] Although most compounds

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included in these studies reached activities in the low micromolar range, only some of those reported by Rubbiani et al.[14] showed $IC_{50} < 1$ μM. Overall, our findings render both compounds well suitable for in vivo studies aimed at assessing their safety and anticancer activity in cisplatin-resistant animal models.

Reactions with Proteins. *Reactions with Cytochrome c and Lysozyme.* To gain a deeper insight into the specific reactivity of these NHC Au(I) complexes with their potential biomolecular targets, compounds **1** and **2** were reacted with HEWL and cyt c, which are often used as model proteins for metalation studies.[31] Reactions were monitored through ESI-MS spectrometry and UV−vis spectrophotometry, in accordance with protocols previously established in our laboratory.[32] Deconvoluted ESI-MS spectra of HEWL and cyt c samples reacted with compounds **1** and **2** were recorded, after 72 h incubation, at 37 °C. No evidence of protein metalation was gained in both cases. Even addition of an excess of the thiol agent DTT to the solution did not lead to formation of protein adducts (Supporting Information, Figure S5). The same reaction mixture (protein plus metallodrug), observed by UV−vis spectrophotometry, gave consistent results: the spectrum remains unchanged through the whole incubation time (Supporting Information, Figure S6).

Reactions with Atox-1. Similar reactivity studies were subsequently carried out in the presence of the copper chaperone Atox-1 as this protein seems to manifest a great affinity for gold compounds. The Atox-1 protein was reacted with the studied compounds by mixing equivalent amounts of Atox-1 (10^{-4} M) with **1** or **2**, in the presence of ammonium acetate buffer (pH 7.4) and DTT (10^{-4} M). Notably, the resulting ESI-MS spectra show that incubation of Atox-1 with NHC gold(I) complexes leads, after 24 h, to the formation of metal-protein adducts in appreciable amounts (Figure 4). This is clearly documented by the appearance of peaks of higher molecular mass than those assigned to the native protein falling at 7269 Da (protein without the first methionine, Met) and at 7400 Da (protein with the first methionine).

The new peaks may be straightforwardly assigned to formal binding of Au^+ to the protein. This implies that the starting gold(I) complexes, upon adduct formation, lose their original ligands. In both cases the monometalated derivative was the predominant adduct; however, a second peak, of lower intensity, is observed at 7661 Da, corresponding to protein binding of two Au^+ ions. All samples show additional peaks attributed to adducts formed between gold(I) and the Atox-1 protein still bearing the N-terminal methionine (Met-1).

From the above results it clearly emerges that **1** and **2** are NOT able to form adducts with the two model proteins, cytochrome c and lysozyme; in contrast, both react eagerly with the Atox-1 protein bearing a characteristic CXXC motif. Covalent adducts are formed where the NHC moiety is released and gold(I) ions are found associated with the protein. These observations imply that the CXXC motif possesses such a high affinity for the gold(I) center as to bind it coordinatively, in the place of chloride or of the first NHC ligand, and then to displace the other NHC ligand, oppositely located.

These results suggest that gold(I) NHC complexes are very selective in metalating proteins, having the chance to form stable adducts only with those few proteins possessing specific metal binding motifs.[10a] In this respect it can be stressed that the gold(I) center of the two gold carbenes is highly thiophilic and can recognise and bind the CXXC motif in Atox-1, but it is not able to bind the thiol group of GSH. The fact that both gold carbene compounds tightly bind to Atox-1, a key protein of copper metabolism, may be per se of particular interest as it may imply that these gold compounds are able to inhibit drastically the copper trafficking system with all the inherent cellular consequences.

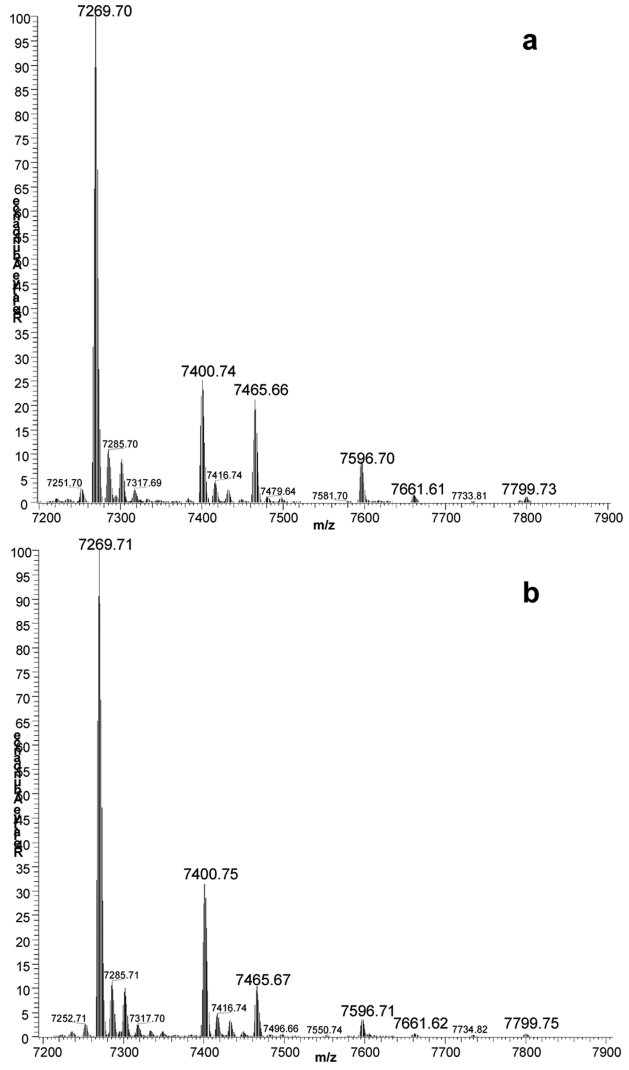

Figure 4. LTQ-Orbitrap ESI-MS spectra of Atox-1 (7269 Da) treated with gold compounds (a) **1** and (b) **2**. Atox-1 adducts are formed with Au^+ (7465 Da) or 2 Au^+ (7661 Da) bound to the protein. Incubation time was 24 h. The peak of Atox-1 with a methionine (7400 Da) is also visible, and the respective adducts with 1 Au^+ (7596 Da) and 2 Au^+ (7799 Da).

■ CONCLUSIONS

In conclusion, with the present study, we have designed, prepared, and characterized two novel gold carbene complexes as anticancer drug candidates. The two novel gold complexes were exhaustively described from the structural point of view thanks to the obtainment of their respective crystal structures. Further, their behavior in solution was elucidated. Both compounds show a high stability in physiological-like media with no evidence of NHC or chloride detachment even over long observation times or upon challenging them with ascorbic acid or GSH. Notably, both complexes manifested similar and very pronounced antiproliferative effects toward the reference

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cancer cell line A2780; cisplatin resistance was completely overcome. As gold carbene compounds are believed to act primarily on a few protein targets rather than on DNA, the reactivity of the present gold carbenes with three selected proteins was explored, mainly through ESI-MS analysis. Relevant metalation effects were highlighted only in the case of Atox-1 protein bearing a characteristic CXXC sequence, whereas adduct formation did not occur at all with cyt c and lysozyme. These findings support the view that the investigated gold carbene complexes work—most likely—through selective metalation of a few proteins bearing specific structural motifs for metal recognition. Selectivity in protein binding is expected to reduce the large toxic effects commonly associated with indiscriminate protein binding, which were previously observed for other cytotoxic gold compounds. On the basis of these observations the investigated gold carbenes look like very attractive and innovative antiproliferative agents, worthy of deeper pharmacological and mechanistic studies; in particular, identification and validation of their effective protein targets and elucidation of their molecular mechanism is an issue of paramount importance and urgency. In this regard, proteins such as TrxR and the poly(adenosine diphosphate)−ribose polymerases (PARPs) that are thought to constitute primary cellular targets for medicinal gold(I) complexes[28,33] will be taken into due consideration through future studies.

■ ASSOCIATED CONTENT

Supporting Information

Crystallographic data in CIF format. Crystallographic data has been deposited with the Cambridge Crystallographic Data Centre CCDC 936580 for 1, CCDC 936581 for 2. Figures illustrating the aurophilic interaction in the crystal packing of compound 2 and the water channel in the crystal structure of 2. UV−vis spectral profiles of compounds 1 and 2 with GSH. UV−vis spectral profiles of compounds 1 and 2 with cytochrome c. LTQ-Orbitrap ESI-MS spectrum of HEWL treated with compound 1 and DTT. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interests.

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■ REFERENCES

(1) (a) Mohr, F. *Gold Chemistry. Applications and Future Directions in the Life Sciences;* Wiley: Weinheim, Germany, 2009. (b) Nobili, S.; Mini, E.; Landini, I.; Gabbiani, C.; Casini, A.; Messori, L. *Med. Res. Rev.* **2010**, *30*, 550−580. (c) Berners-Price, S. J.; Filipovska, A. *Metallomics* **2011**, *3*, 863−873.

(2) Messori, L.; Abbate, F.; Marcon, G.; Orioli, P.; Fontani, M.; Mini, E.; Mazzei, T.; Carotti, S.; O'Connell, T.; Zanello, P. *J. Med. Chem.* **2000**, *43*, 3541−3548.

(3) (a) Ronconi, L.; Giovagnini, L.; Marzano, C.; Bettio, F.; Graziani, R.; Pilloni, G.; Fregona, D. *Inorg. Chem.* **2005**, *44*, 1867−1881. (b) Mlacic, V.; Chen, D.; Ronconi, L.; Landis-Piwowar, K. R.; Fregona, D.; Dou, Q. P. *Cancer Res.* **2006**, *66*, 10478−10486. (c) Saggioro, D.; Rigobello, M. P.; Paloschi, L.; Folda, A.; Moggach, S. A.; Parsons, S.; Ronconi, L.; Fregona, D.; Bindoli, A. *Chem. Biol.* **2007**, *14*, 1128−1139.

(4) (a) Sun, R. W.-Y.; Li, C. K.-L.; Ma, D.-L.; Yan, J. J.; Lok, C.-N.; Leung, C.-H.; Zhu, N.; Che, C.-M. *Chem.—Eur. J.* **2010**, *16*, 3097−3113. (b) Sun, R. W.-Y.; Che, C.-M. *Coord. Chem. Rev.* **2009**, *253*, 1682−1691. (c) Lum, C. T.; Yang, Z. F.; Li, H. Y.; Sun, R. W.-Y.; Fan, S. T.; Poon, R. T. P.; Lin, M. C. M.; Che, C.-M.; Kung, H. F. *Int. J. Cancer* **2006**, *118*, 1527−1538. (d) Wang, Y.; He, Q.-H.; Sun, R. W.-Y.; Che, C.-M.; Chiu, J.-F. *Cancer Res.* **2005**, *65*, 11553−11564.

(5) (a) Messori, L.; Marcon, G.; Cinellu, M. A.; Coronnello, M.; Mini, E.; Gabbiani, C.; Orioli, P. *Bioorg. Med. Chem.* **2004**, *12*, 6039−6043. (b) Coronnello, M.; Mini, E.; Caciagli, B.; Cinellu, M. A.; Bindoli, A.; Gabbiani, C.; Messori, L. *J. Med. Chem.* **2005**, *48*, 6761−6765.

(6) (a) Li, C. K.-L.; Sun, R. W.-Y.; Kui, S. C.-F.; Zhu, N.; Che, C.-M. *Chem.—Eur. J.* **2006**, *12*, 5253−5266. (b) Cinellu, M. A.; Maiore, L.; Manassero, M.; Casini, A.; Arca, M.; Fiebig, H.-H.; Kelter, G.; Michelucci, E.; Pieraccini, G.; Gabbiani, C.; Messori, L. *ACS Med. Chem. Lett.* **2010**, *1*, 336−339. (c) Gabbiani, C.; Casini, A.; Messori, L.; Guerri, A.; Cinellu, M. A.; Minghetti, G.; Corsini, M.; Rosani, C.; Zanello, P.; Arca, M. *Inorg. Chem.* **2008**, *47*, 2368−2379. (d) Casini, A.; Cinellu, M. A.; Minghetti, G.; Gabbiani, C.; Coronnello, M.; Mini, E.; Messori, L. *J. Med. Chem.* **2006**, *49*, 5524−5531.

(7) (a) Messori, L.; Marcon, G. In *Metal Ions in Biological Systems;* Siegel, A.; Siegel, H. Eds.; Marcel Dekker, Inc.: New York, 2004; Vol. *41*, pp 279−304. (b) Shaw, C. F., III *Inorg. Chem.* **1989**, *8*, 233−267.

(8) (a) Hoke, G. D.; Rush, G. F.; Bossard, G. E.; McArdle, J. V.; Jensen, B. D.; Mirabelli, C. K. *J. Biol. Chem.* **1988**, *263*, 11203−11210. (b) Modica-Napolitano, J. S.; Aprille, J. R. *Adv. Drug Delivery Rev.* **2001**, *49*, 63−70.

(9) (a) Herrmann, W. A.; Goossen, L. J.; Spiegler, M. *Organometallics* **1998**, *17*, 2162. (b) McGuinness, D. S.; Cavell, K. J.; Skelton, B. W.; White, A. H. *Organometallics* **1999**, *18*, 1596. (c) Baker, M. V.; Barnard, P. J.; Berners-Price, S. J.; Brayshaw, S. K.; Hickey, J. L.; Skelton, B. W.; White, A. H. *Dalton Trans.* **2006**, 3708−3715. (d) Oehninger, L.; Rubbiani, R.; Ott, I. *Dalton Trans.* **2013**, *42*, 3269−3284. (e) Liua, W.; Gust, R. *Chem. Soc. Rev.* **2013**, *42*, 755−773.

(10) (a) Hickey, J. L.; Ruhayel, R. A.; Barnard, P. J.; Baker, M. V.; Berners-Price, S. J.; Filipovska, A. *J. Am. Chem. Soc.* **2008**, *130*, 12570−12571. (b) Zou, T.; Lum, C. T.; Chui, S. S.-Y.; Che, C.-M. *Angew. Chem., Int. Ed.* **2013**, *52*, 2930−2933.

(11) Barnard, P. J.; Baker, M. V.; Berners-Price, S. J.; Day, D. A. *J. Inorg. Biochem.* **2004**, *98*, 1642−1647.

(12) Baker, M. V.; Barnard, P. J.; Berners-Price, S. J.; Brayshaw, S. K.; Hickey, J. L.; Skelton, B. W.; White, A. H. *J. Organomet. Chem.* **2005**, *690*, 5625−5635.

(13) Rubbiani, R.; Kitanovic, I.; Alborzinia, H.; Can, S.; Kitanovic, A.; Onambele, L. A.; Stefanopoulou, M.; Geldmacher, Y.; Sheldrick, W. S.; Wolber, G.; Prokop, A.; Wolfl, S.; Ott, I. *J. Med. Chem.* **2010**, *53*, 8608−8618.

(14) (a) Rubbiani, R.; Can, S.; Kitanovic, I.; Alborzinia, H.; Stefanopoulou, M.; Kokoschka, M.; Mönchgesang, S.; Sheldrick, W. S.; Wölfl, S.; Ott, I. *J. Med. Chem.* **2011**, *54*, 8646−8657. (b) Barnard, P. J.; Baker, M. V.; Berners-Price, S. J.; Day, D. A. *J. Inorg. Biochem.* **2004**, *98*, 1642−1647.

(15) (a) Weaver1, J.; Gaillard, S.; Toye, C.; Macpherson, S.; Nolan, S. P.; Riches, A. *Chem.—Eur. J.* **2011**, *17*, 6620−6624. (B) McKeage, M. J.; Berners-Price, S. J.; Galettis, P.; Bowen, R. J.; Brouwer, W.; Ding, L.; Zhuang, L.; Baguley, B. C. *Cancer Chemother. Pharmacol.* **2000**, *46*,

343−350. (c) Berners-Price, S. J.; Bowen, R. J.; Hambley, T. W.; Healy, P. C. *J. Chem. Soc., Dalton Trans.* **1999**, 1337−1346. (d) Berners-Price, S. J.; Bowen, R. J.; Galettis, P.; Healy, P. C.; McKeage, M. J. *Coord. Chem. Rev.* **1999**, *185−186*, 823−836.
(16) Gabbiani, C.; Scaletti, F.; Massai, L.; Michelucci, E.; Cinellu, M. A.; Messori, L. *Chem. Commun.* **2012**, *48*, 11623−11625.
(17) (a) Hamza, I.; Schafer, M.; Klomp, L. W.; Gitlin, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 13363−13368. (b) Boal, A. K.; Rosenzweig, A. C. *J. Am. Chem. Soc.* **2009**, *131*, 14196. (c) Hamza, I.; Prohaska, J.; Gitlin, J. D. *Proc. Natl. Acad. Sci. U.S.A* **2003**, *100*, 1215−1220.
(18) (a) Huffman, D. L.; O'Halloran, T. V. *Annu. Rev. Biochem.* **2001**, *70*, 677−701. (b) Arnesano, F.; Banci, L.; Bertini, I.; Ciofi-Baffoni, S.; Molteni, E.; Huffman, D. L.; O'Halloran, T. V. *Genome Res.* **2002**, *12*, 255−271.
(19) *CrysAlisPro*, Version 1.171.35.19; Agilent Technologies: San Jose, CA, (release 27−10−2011 CrysAlis171 .NET) (compiled Oct 27 2011,15:02:11).
(20) *CrysAlisPro*, Version 1.171.35.19; Agilent Technologies: San Jose, CA, (release 27−10−2011 CrysAlis171 .NET) (compiled Oct 27 2011,15:02:11) Empirical absorption correction using spherical harmonics, implemented in SCALE3 ABSPACK scaling algorithm.
(21) Altomare, A.; Burla, M. C.; Camalli, M.; Cascarano, G. L.; Giacovazzo, C.; Guagliardi, A.; Moliterni, A. G. G.; Polidori, G.; Spagna, R. *J. Appl. Crystallogr.* **1999**, *32*, 115.
(22) Sheldrick, G. M. *Acta Crystallogr.* **2008**, *A64*, 112−122.
(23) Nardelli, M. *J. Appl. Crystallogr.* **1995**, *28*, 659.
(24) (a) Farrugia, L. J. *J. Appl. Crystallogr.* **1997**, *30*, 565. (b) Macrae, I. J.; Bruno, J. A.; Chisholm, P. R.; Edgington, P.; McCabe, E.; Pidcock, L.; Rodriguez-Monge, R.; Taylor, J.; van de Streek; Wood, P. A. *J. Appl. Crystallogr.* **2008**, *41*, 466−470.
(25) Skekan, P.; Stroreng, R.; Scudiero, D.; Monks, A.; Mcmahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107−1112.
(26) Baker, M. V.; Barnard, P. J.; Brayshaw, S. K.; Hickey, J. L.; Skelton, B. W.; White, A. H. *Dalton Trans.* **2005**, 37−43.
(27) Allen, F. H. *Acta Cryst B* **2002**, *B58*, 380−388.
(28) Schuh, E.; Pflüger, C.; Citta, A.; Folda, A.; Rigobello, M. P.; Bindoli, A.; Casini, A.; Mohr, F. *J. Med. Chem.* **2012**, *55*, 5518−5528.
(29) (a) Kunkely, H.; Vogler, A. *J. Organomet. Chem.* **2003**, *684*, 113. (b) Kunkely, H.; Vogler, A. *Inorg. Chem. Commun.* **2000**, *3*, 143.
(30) (a) Lemke, J.; Pinto, A.; Niehoff, P.; Vasylyeva, V.; Metzler-Nolte, N. *Dalton Trans.* **2009**, *21*, 7063−7070. (b) Kaps, L.; Biersack, B.; Müller-Bunz, H.; Mahal, K.; Münzner, J.; Tacke, M.; Mueller, T.; Schobert, R. *J. Inorg. Biochem.* **2012**, *106*, 52−58. (c) Pellei, M.; Gandin, V.; Marinelli, M.; Marzano, C.; Yousufuddin, M.; Dias, H. V.; Santini, C. *Inorg. Chem.* **2012**, *51*, 9873−9882.
(31) Casini, A.; Guerri, A.; Gabbiani, C.; Messori, L. *J. Inorg. Biochem.* **2008**, *102*, 995−1006.
(32) Casini, A.; Gabbiani, C.; Mastrobuoni, G.; Messori, L.; Moneti, G.; Pieraccini, G. *ChemMedChem* **2006**, *1*, 413−417.
(33) (a) Bindoli, A.; Rigobello, M. P.; Scutari, G.; Gabbiani, C.; Casini, A.; Messori, L. *Coord. Chem. Rev.* **2009**, *253*, 1692−1707. (b) Mendes, F.; Groessl, M.; Nazarov, A. A.; Tsybin, Y. O.; Sava, G.; Santos, I.; Dyson, P. J.; Casini, A. *J. Med. Chem.* **2011**, *54*, 2196−2206. (c) Serratrice, M.; Edafe, F.; Mendes, F.; Scopelliti, R.; Zakeeruddin, S. M.; Grätzel, M.; Santos, I.; Cinellu, M. A.; Casini, A. *Dalton Trans.* **2012**, *41*, 3287−3293.