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New gold carbene complexes as candidate anticancer agents

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Abstract Three structurally related gold(I) carbene complexes with bulky hydrophobic ligands i.e. **1–3** were investigated in solution for further consideration as candidate anticancer agents. Cytotoxic assays were subsequently conducted on bone marrow-derived preosteoclast cell line of human origin (FLG 29.1) and human colon cancer cells (HCT-116). A far greater cytotoxic activity was measured for compound **1** against HCT-116 cells compared to **2** and **3**; conversely, all compounds were highly and similarly active against FLG 29.1 cells. Results obtained for the reaction of complexes **1** and **2** with RNase A documented the occurrence of a weak interaction with this model protein and the formation of a tiny amount of the corresponding adduct. Moreover, a certain reactivity of the complex **2** was also detected toward GSH. The general implications of the obtained results are discussed.

Keywords Anticancer drugs · Gold carbene · ESI–MS · Protein interaction

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Introduction

Coordination compounds of gold(I/III) manifest interesting anti-arthritic, anti-tuberculosis and anti-tumor activities, but the mechanisms of these biological activities remain largely unexplained. Another difficulty in the field of gold chemistry is the limited stability of gold compounds under biological related conditions. Accordingly, there is an ongoing search for new gold complexes with a wider window of stability, pH independent solubility and fewer side effects, this being today a real challenge in bioinorganic and medicinal chemistry (Nobili et al. 2010).

The first metal-carbene complexes, known since 1968, were synthesized independently by Wanzlick and Schönherr (1968), and Öfele (1968). This investigation field has grown dramatically afterward. The current status of gold(I/III)-carbene complexes includes various NHC systems ranging from five-membered to seven-membered rings, including imidazolium, benzimidazolium, pyrimidinium, pyrazolium, naphthimidazolium and other moieties (Lin and Vasam 2007). In connection to some positive

biological results of Au-carbene complexes (Garner et al. 2015; Bertrand et al. 2015; Pratesi et al. 2014), in particular the remarkable antimicrobial activity of Au(I)-NHCs (Ray et al. 2007) and anticancer activity (MTT assay) of Au(I)-NACs, the main goal of the present study is to analyse the reactivity of this kind of complexes toward a variety of biologically relevant nucleophiles. The final aim is to evaluate the widely adopted application of Au(I) complexes in medicine and to capacitate a new generation of gold complexes.

Gold carbene complexes deserve particular attention as the carbene ligand appears to confer a higher stability to the resulting compounds (Bertrand and Casini 2014). This prompted us to prepare a new series of gold(I) carbene complexes with a relatively large hydrophobic ligand. The study complexes are drawn in Fig. 1. They consist of one Au(I)-bis-N-acyclic carbene (NAC), **1**, one Au(I) N-acyclic carbene (NAC), **2** and one symmetrical unsaturated five-membered Au(I)-bis-N-heterocyclic carbene (NHC), **3**. The two symmetrical Au(I)-bis-carbene compounds, **1** and **3**, bear two identical carbene ligands linearly coordinated to a gold(I) centre, while the Au(I)-*mono*-carbene compound **3** is asymmetric with a chloride atom as second ligand.

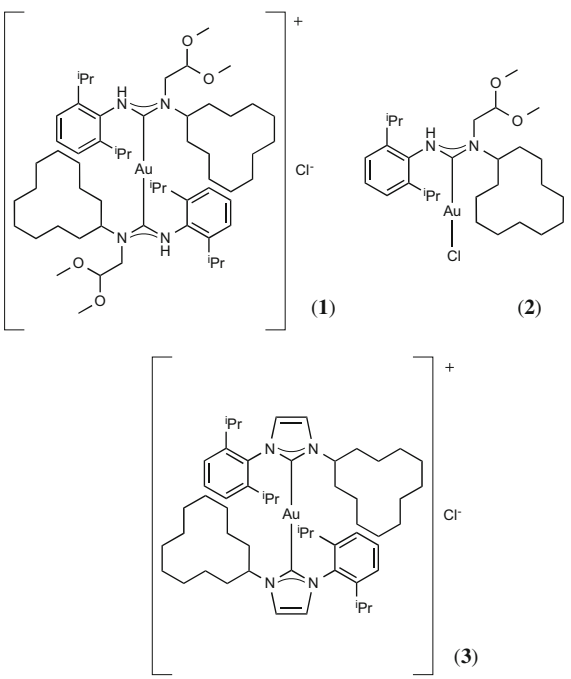

Fig. 1 The three gold(I) carbene studied complexes; **1–3**

Materials and methods

Materials

The gold carbene **1–3** were synthesized as previously described (Hashmi et al. 2011; Jones and Lautner 1988; Đurović et al. 2016). RNase and GSH were purchased from Sigma and used without further purification.

^{1}H-NMR studies

For the stability studies, solutions of the three gold carbenes were prepared in DMSO-d_6 at a concentration of 10^{-3} M. ^{1}H-MNR spectra were recorded up to 24 h on a Bruker Ultrashield 400 Plus (Bruker, Billerica, MA, USA). Measurements took place at 400.13 MHz (^{1}H).

For the interaction studies with small molecules, to the previous solutions were added, separately, small amounts of GSH and Imidazole to a final 3:1 (small molecule-to-gold compound) molar ratio. ^{1}H-MNR spectra were recorded on the obtained solution up to 24 h at room temperature.

UV–Visible experiments

UV–Vis spectra were recorded on a Varian Cary 50 UV–Vis spectrophotometer (Varian, Palo Alto, CA, USA) in the range of 200–700 nm. Stock solution of the three studied gold carbenes (3×10^{-3} M) was prepared by dissolving the complexes under investigation in DMSO.

The electronic spectra were recorded diluting small amounts of freshly prepared stock solutions of the individual complexes in DMSO and the relative amount of RNase A at a stoichiometric ratio of 3:1 (metal-to-protein). The final concentration of each gold compound in the final sample after dilution in 10 mM phosphate, pH 7.4 was 10^{-5} M. The resulting solutions were monitored collecting the electronic spectra over 48 h at room temperature.

The UV–Vis interaction experiments with small molecules were performed using the same gold carbenes stock solutions (3×10^{-3} M) in DMSO. DMSO stock solutions (3×10^{-3} M) of GSH and Imidazole were prepared. A small amount of these solutions were added, separately, to 100 μL of the stock solution of the gold carbenes in order to obtain a

10:1 (small molecule-to-metal) ratio. The mixtures were diluted with DMSO at a final concentration of 10^{-4} M of the gold compounds. The resulting solutions were monitored collecting the electronic spectra over 48 h at room temperature.

RNase-A interaction studies: ESI–MS

Metal complexes/protein were prepared by adding the selected gold carbene dissolved in DMSO to the solution of protein (10^{-3} M) in 20 mM ammonium acetate buffer, pH 6.8. The final metal complex/protein ratio was 3:1 and the concentration was 10^{-5} M. The solutions were incubated for 24 and 48 h at 37 °C. After a 20-fold dilution with water, ESI–MS spectra were recorded by direct introduction of the sample at a flow rate of 5 μL/min in an LTQ Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA, USA), equipped with a conventional ESI source. 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 (arbitrary units) and 1 (arbitrary units). Xcalibur 2.0 software (Thermo) was used for acquisition, 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.

Cellular studies

Cell cultures

HCT-116 and FLG 29.1 cells were cultured in RPMI 1640 (Euroclone; Milan, Italy) with 10 % Fetal Bovine Serum (FBS) (Euroclone; Milan, Italy). All the cell lines were cultured at 37 °C in humidified atmosphere containing 5 % CO_2 in air.

Pharmacology experiments

Cells were seeded in a 96-well flat-bottomed plate (Corning-Costar, Corning, NY, USA) at a cell density of 1×10^4 cells per well in either RPMI or DMEM complete medium. The compounds **1–3** were used, after solubilisation in water with DMSO, in range of concentration of 0–200 nM. After 24 and 48 h, viable cells (determined by Trypan blue exclusion) were counted in triplicate using a haemocytometer. Each experimental point represents the mean of a single experiment carried out in triplicate.

Trypan blue assay

Cells viability was assessed by the Trypan blue exclusion assay. In brief, 10 μL of 0.4 % Trypan blue solution was added to 10 μL cell suspensions in culture medium. The suspension was gently mixed and transferred to a haemocytometer. Viable and dead cells were identified and counted under a light microscope. Blue cells failing to exclude the dyes were considered nonviable, and transparent cells were considered viable. The percentage of viable cells was calculated on the basis of the total number of cells (viable plus nonviable). The IC_{50} value (i.e., the dose that caused apoptosis of 50 % of cells) was calculated by fitting the data points (after 24 h and 48 h of incubation) with a sigmoidal curve using Calcusyn software (Biosoft, Cambridge, UK).

Results and discussion

^{1}H-NMR stability studies and interaction with GSH and imidazole of gold(I) carbenes

Prior to performing the biological assays and the protein interaction studies, the solution behavior of three gold carbenes was analyzed by ^{1}H-NMR spectroscopy. Unfortunately, due to the pronounced non-polar chemical nature of the bulky substituents on the carbenic moiety, neither water nor phosphate buffered solutions seemed to be suitable solvents for these metallocarbenes. Therefore, all experiments were performed in pure DMSO or in DMSO-containing buffer solutions.

In this case, all the measurements were conducted on the freshly prepared solution at 0, 6, 12 and 24 h. In Fig. 2 the spectra at 0 and 24 h related to the compound **2** are shown.

Despite the chemical nature of non-symmetrical carbene, characterized by the presence of a potentially hydrolysable chloride ligand, the gold carbene **2** manifested a high stability in solution, resulting into a fully stable complex for at least 24 h in DMSO solution, as can be noticed from Fig. 2. In fact, after 24 h, no significant spectral changes in intensity and in chemical shift attributable to chemical modifications

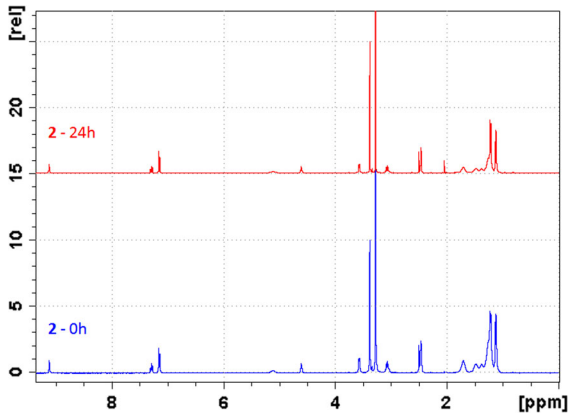

Fig. 2 ^{1}H-NMR spectra recorded for compound **2** in DMSO-d_6 at 0 (*blue line*) and 24 h (*red line*). (Color figure online)

of the Au-carbene were detected. The other two symmetrical gold compounds, **1** and **3**, manifested fundamentally similar stability properties (data not shown).

Afterwards, we decided to test the reactivity of these Au-carbenes against two small molecules that can be representative of some protein binding-sites, i.e. GSH and Imidazole. Also in this case the ^{1}H-NMR spectrum of the starting Au carbene was compared to the spectra obtained after incubation with the selected small molecule.

In Fig. 3 the NMR spectra obtained for compound **2** incubated with GSH are reported. As can be noticed, the appearance of the four new signals in the range between 7.8 and 8.5 ppm offers evidence for some reactivity of carbene **2** toward GSH. Also in this case, compounds **1** and **3** were perfectly unreactive with GSH and Imidazole. This behaviour may be explained by substitution of chloride present in the *mono*-carbene complex **2**, in contrast to the bis-carbene complexes **1** and **3**.

UV–Vis interaction experiments

Starting from the evidence of interaction of Au-carbene **2** with GSH revealed by the NMR spectroscopy, the UV–Vis spectrophotometric analysis was then employed in order to further clarify this type of interaction.

In particular, the three gold compounds were incubated with GSH and Imidazole, respectively. The obtained results were in agreement with those observed in the NMR spectra, as none of the studied compounds showed appreciable amount of adducts formed with Imidazole. In the case of GSH, only compound **2** manifested an interesting and significant change in the electronic absorption spectrum, as depicted in Fig. 4.

In this spectrum the progressive formation and growing up of the absorption band with the max at 294 nm is evident, accompanied by the concomitant strong increment in absorbance of the band at 265 nm.

To definitely assign these absorption bands to an interaction of the metal compound with GSH, there was the necessity to exclude that these signals might arise from a progressive oxidation in solution of GSH. For this reason, some UV–Vis spectra of GSH in DMSO solution were recorded over 24 h and also in

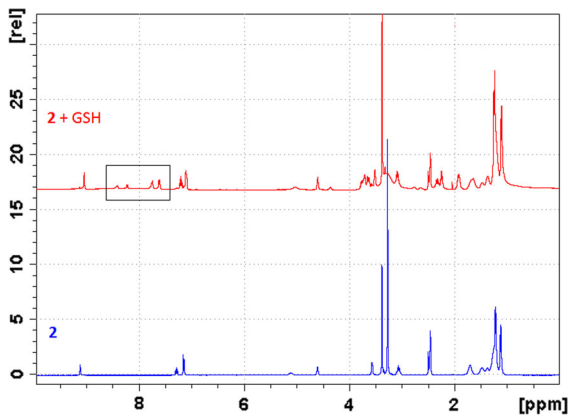

Fig. 3 ^{1}H-NMR spectra recorded for compound **2** in DMSO-*d6* (*lower panel*) at 0 h and in presence of GSH (*upper panel*) incubated for 24 h at r.t.; 3:1 (GSH-to-metal) molar ratio

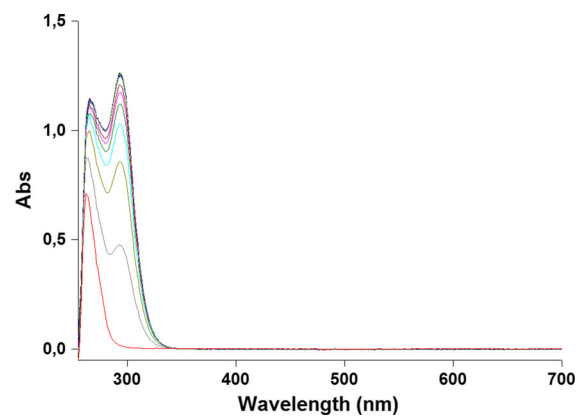

Fig. 4 Time-dependent UV–Vis spectra (up to 48 h) of compound **2** 10^{-4} M dissolved in DMSO in the presence of GSH 3 × 10^{-4} M, complex/GSH = 1:3 molar ratio

the presence of an added oxidizing agent, H_2O_2. While GSH dissolved in DMSO did not reveal any absorption band, oxidized GSH showed only one intense band at 268 nm. At this point it was straightforward that the absorption bands shown in Fig. 4 can be assigned to an interaction of **2** with GSH; in particular we can hypothesize the loss of the chloride ligand the subsequent formation of an adduct between the gold centre and the thiol group of glutathione.

Subsequently, the interactions of these gold(I) compounds with the RNase-A model protein were explored. UV–Vis spectrophotometric analysis of metallodrug-protein samples allows the continuous monitoring of the various gold(I) carbenes in the presence of this protein. Remarkably, also in this case, the compounds **1** and **3** did not show appreciable interactions with the model protein, confirming one more time their extreme stability in solution. On the contrary, the UV–Vis spectra recorded for compound **2** in the presence of RNase suggested the occurrence of a modest interaction with the protein, as highlighted in Fig. 5. In fact, during 48 h, a slight modification of the electronic spectrum was observed with the appearance of a small, but meaningful, absorption band at 280 nm.

RNase-A interaction studies: ESI–MS

Metal binding properties of the model protein versus these gold(I) carbenes were studied in detail through high resolution ESI–MS technique. ESI–MS measurements permitted disclosing the nature of protein bound

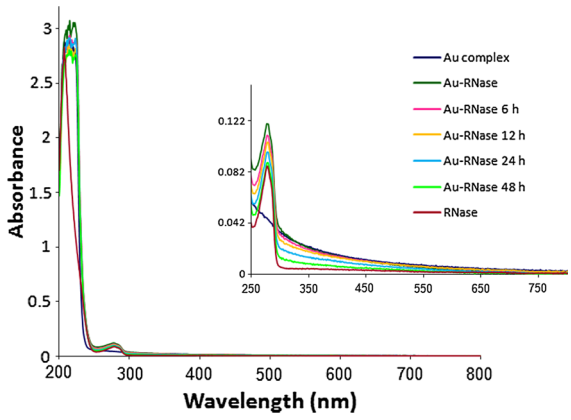

Fig. 5 Time-dependent UV–Vis spectra (up to 48 h) of compound **2** 10^{-5} M dissolved in 10 mM phosphate buffer, pH 7.4 in the presence of RNase-A 3×10^{-5} M, complex/protein = 3:1 molar ratio

metallic fragments and their binding stoichiometry thus providing mechanistic insight into the respective metalation processes.

In order to demonstrate the nature, and thus confirming the interaction of these gold-carbenes with RNase, compounds **1**, **2** and **3** were incubated for 24 and 48 h in presence of RNase. The resulting mixtures were injected, after dilution in MilliQ water, into the mass spectrometer. The obtained ESI–MS spectra showed several peaks and most of them were not attributable to a metalated protein. As a matter of fact, from the literature (Chowdhury et al. 1990) it was well established that the solution media, i.e. the saline buffer, can contain small amount of phosphoric acid or sulphuric acid that are responsible of the formation of undesired and isobaric adducts with Ribonuclease A (+98 *m/z*). Hence, from the inspection of ESI–MS spectra in Fig. 6 recorded for **1** (A), **2** (B) and **3** (C) in presence of Ribonuclease A (*m/z* 13681.2) it was clear that compound **1** led to the formation of an adduct specie at *m/z* 13878.20 (panel A), corresponding to RNase bound to a bare gold atom. The other peaks at *m/z* 13779.20 and 13976.13 were easily assigned to the protein's mono- and bis- phosphate/sulphate adducts, respectively. Probably, the bis-phosphate/sulphate adduct was formed on the RNase-Au complex.

In case of compound **2**, the pattern of signals was the same (panel B): the unique RNase-Au adduct was present at *m/z* 13878.12, and the two phosphate/sulphate adducts with the protein were detected at *m/z* 13779.16 and 13976.08.

As a further indication of the chemical inertness of carbene **3**, the Fig. 6 (panel C) showed only three adducts of RNase with phosphate/sulphate ions without any evidence of protein metalation.

In vitro cytotoxicity assays on representative cancer cell lines

Aiming to completely characterize the compounds **1**–**3** as potential anticancer agents, we decided to evaluate their cellular effects against a small panel of cancer cell lines. The cancer cell panel included the following cell lines: FLG 29.1 (human acute myeloid leukemia) taken as model of a liquid tumor, HCT-116 (human colorectal adenocarcinoma) representative for a classical solid tumor and the non-cancerous cell line L929 (mouse fibroblast) as negative control. For the determination of the cytotoxic effects of compounds

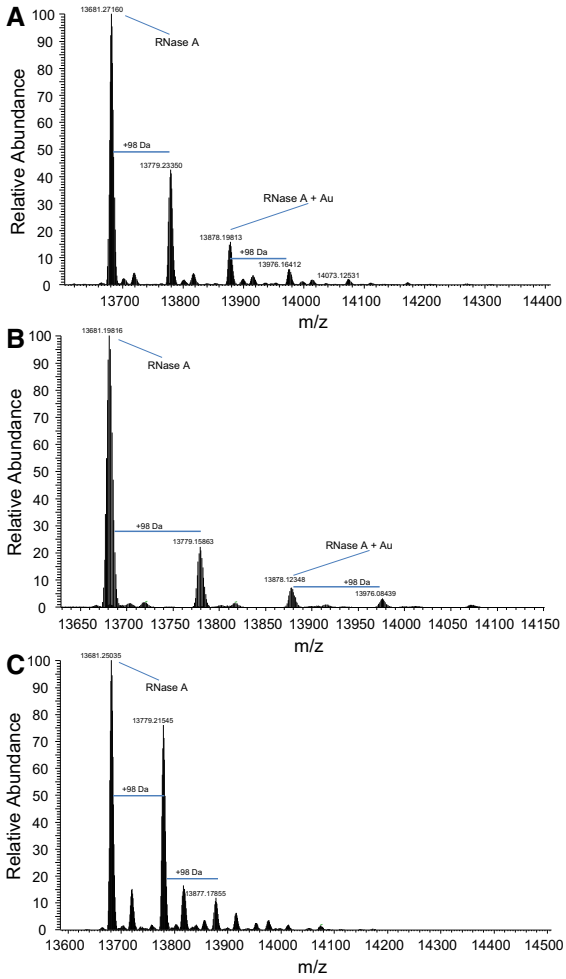

Fig. 6 Deconvoluted ESI–MS spectra of RNase A in 20 mM ammonium acetate buffer, pH 6.8 incubated with compound **1** (*panel a*), **2** (*panel b*) and **3** (*panel c*) after 48 h at 37 °C. The protein concentration was 10^{-4} M (with complex to protein molar ratio of 3:1)

1–3 on the above cancer lines we used the Trypan blue method as detailed in the experimental section, and the obtained IC_{50} values were reported in Table 1.

As can be noticed in Table 1, compounds **1–3** manifested a comparable and very high cytotoxic potential against the FLG 29.1 cell line with low IC_{50} values. Differently, the HCT-116 cell line turned out to be less sensitive to compound **2** and **3**, but the cytotoxicity potential was increased in case of compound **1**.

Moreover, the cytotoxic activity against the non-cancerous cell line L929 (mouse fibroblast), was also assessed under the same conditions. Normal cells

Table 1 IC_{50} values (μM) of **1–3** complexes in representative cancer cell lines

Compound	FLG 29.1	HCT-116	L929
1	2.59 ± 0.13	1.81 ± 0.23	12.74 ± 0.85
2	2.95 ± 0.21	36.73 ± 0.45	16.92 ± 2.31
3	2.94 ± 0.37	13.39 ± 0.42	25.65 ± 3.06
Cisplatin	24.41 ± 0.81	25.52 ± 2.13	–

FLG 29.1 bone marrow-derived preosteoclast cell line of human origin, *HCT-116* human colon cancer cells *L929* mouse fibroblast

Table 2 IC_{50} values (μM) of compound **1** for cancer cell lines at different sensitivity to cisplatin

Compound	HT-29	HCT-8
1	4.48 ± 0.14	1.39 ± 0.14
Cisplatin	16.61 ± 0.91	8.73 ± 1.45

HT-29, HCT-8 human colorectal adenocarcinoma cells

turned out to be less sensitive to compound **1** and **3** than cancer cells, in agreement with the general minor reactivity of these two compounds in comparison with **2**. Also, the IC_{50} values of cisplatin for the same cancer cell lines were compared to those of the three gold-carbenes, showing in all cases a lower antiproliferative potential of the cisplatin.

In the same experimental conditions other two cancer cell lines, HT-29 and HCT-8, were also tested. These cell lines were representative for different sensitivity to cisplatin and were tested only for the most bio-active compound **1** (Table 2).

The obtained data clearly showed that compound **1** was extremely cytotoxic also for these cell line, and in particular for the HCT-8.

In conclusion, these results offer some initial evidence that the mode of action of these three Au(I)-carbenes markedly differs from cisplatin.

Conclusions

In conclusion, we have studied here three novel gold carbene complexes, two of which are symmetrical bis-carbene gold complexes while the third one is a *mono*-carbene complex. These compounds were tested as antiproliferative agents in two representative cancer cell lines. The three compounds turned out to be highly cytotoxic toward a leukemia cell line with IC_{50} values

in the low micromolar range; in contrast only compound **1** was highly active toward the colon cancer cell line while the other two gold compounds manifested moderate to low activity. The reactivity toward small molecules, i.e. GSH and Imidazole was tested. Compounds **1**–**3** seemed to be unreactive with Imidazole and only compound **2** reacted with GSH. When challenged against the model protein RNase A only compounds **1** and **2** were able to form small quantities of a protein adduct. In the adduct a gold(I) ions is directly bound to the protein with loss of the carbene ligand.

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