Protein metalation by two structurally related gold(I) carbene complexes: an ESI MS study

Carlotta Zoppi[a], Lara Massai[a*], Damiano Cirri[b], Chiara Gabbiani[b], Alessandro Pratesi[b*], Luigi Messori[a]

[a]Laboratory of Metals in Medicine (MetMed), Department of Chemistry "U. Schiff", University of Florence, via della Lastruccia 3, 50019 Sesto Fiorentino, Italy. E-mail: lara.massai@unifi.it

[b]Department of Chemistry and Industrial Chemistry, University of Pisa, Via G. Moruzzi 13, 56124 Pisa, Italy; E-mail: alessandro.pratesi@unipi.it

Abstract

The reactions with a few model proteins of two structurally related gold carbene compounds, namely the gold(I) monocarbene complex Au(NHC)Cl and the corresponding bis-carbene complex $[Au(NHC)_2]PF_6$ (where NHC is an N-heterocyclic carbene ligand), were comparatively studied by ESI MS measurements. The investigated proteins were: human serum albumin, human carbonic anhydrase and bovine superoxide dismutase; in addition, the reactions of the two gold carbenes with the C-terminal synthetic dodecapeptide of thioredoxin reductase were also analyzed. Formation of metallodrug-protein adducts was observed in all cases made exception for the reactions of $[Au(NHC)_2]PF_6$ with carbonic anhydrase and superoxide dismutase. Notably, in line with expectations, the monocarbene gold complex turned out to be more effective than its dicarbene counterpart in forming protein adducts. The reactivity of these gold carbene complexes with model proteins is compared to that of a few other gold(III) and gold(I) complexes whose reactions with model proteins had been previously investigated with the same methodology; it emerges that the two gold carbenes react more selectively with proteins at well-defined anchoring sites. The implications of these results are discussed in the frame of the current knowledge on medicinal gold compounds.

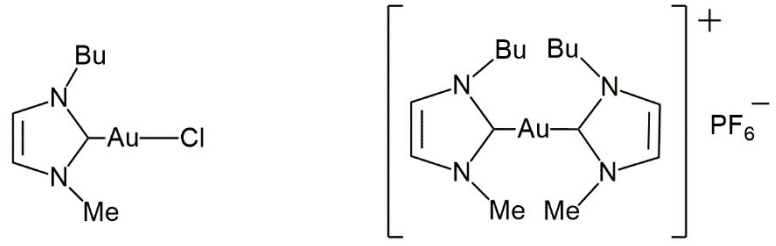
Keywords: protein metalation; gold-NHC compounds; mass spectrometry, metallodrugs

1. Introduction

Gold compounds form an interesting class of metal-based drug candidates with a potentially wide field of applications [1–4]. The real medicinal interest for gold-based compounds started in 1890 with the discovery made by Robert Koch on the inhibition potential of gold cyanide against the growth of Tubercle bacillus. During the last century, some Au(I) complexes have been successfully employed in the clinics as antiarthritic drugs [5], leading in the 1980s, to the development by Sutton and co-workers of the orally active Au(I) phosphine compound auranofin. Auranofin was the first oral gold compound approved for the clinical treatment of rheumatoid [6,7].

Beyond its antiarthritic properties, between the end of 1970s and the mid-1980s, the *in vitro* antiproliferative properties of auranofin against tumour cell lines were also highlighted [8,9], sparking new interest for gold compounds as potential anticancer agents [10–13].

As a result, during the last years, a lot of attention was paid to anticancer gold compounds with the final aim to replace platinum-based drugs in view of the severe side effects of the latter [10,14–16]. In this frame, extensive studies were conducted by many research groups with the purpose of finding gold complexes endowed with favourable chemical and biological properties. Within a multi-year research project financed by AIRC, our laboratory has deeply investigated a panel of seven representative gold compounds with rather encouraging results [17–21]. The panel comprises the following compounds: Auoxo6, Au_2Phen, Aubipic, AuL12, Au(NHC)Cl, $[Au(NHC)_2]PF_6$ and auranofin, as previously reported. Within this panel, two gold(I) carbenes were included, namely the monocarbene gold complex Au(NHC)Cl and the corresponding bis-carbene complex $[Au(NHC)_2]PF_6$ (Figure 1); this inclusion is motivated by the growing interest of the scientific community toward metal carbene complexes for medicinal applications [22–24]. Moreover, gold(I) compounds usually exhibit greater stability in solution in comparison with gold(III) metal complexes, being these latter quickly degraded by ultraviolet light and weak reducing agents [25].



(a) *(b)*

Figure 1 *Chemical structure of gold(I)-N-heterocyclic carbene complexes. (A) Au(NHC)Cl and (B) [Au(NHC)$_2$]PF$_6$.*

Since gold compounds are commonly believed to exert their biological effects through interactions with proteins rather than with nucleic acids [26], we decided to investigate systematically the reactions of the above-mentioned compounds with a number of representative proteins to model the occurring reactions and identify common trends of reactivity. The reactions of auranofin, Auoxo6, Au$_2$Phen, Aubipyc and AuL12 with a set of model proteins were already discussed in two papers previously published by this research group [26,27]. Here we analyze comparatively the reactivity of the two gold carbene compounds (*i.e.* Au(NHC)Cl and [Au(NHC)$_2$]PF$_6$) with the same proteins panel (Figure2).

It is worth reminding that substantial biological information has been gained on these two gold carbene compounds [24,28–30]. These compounds performed very well within the chosen set of gold compounds in terms of *in vitro* antiproliferative properties, with the dicarbene gold complex being more effective than its monocarbene counterpart. The cellular effects of these two gold carbenes were investigated in detail in a recent paper of our [31], where we demonstrated, through a comparative proteomic analysis, that the number of modulated proteins is far larger in [Au(NHC)$_2$]PF$_6$-treated than in Au(NHC)Cl-treated A2780 cells. In any case, both gold compounds mainly affected proteins belonging to the following functional classes: protein synthesis, metabolism, cytoskeleton and stress response and chaperones. Particularly, [Au(NHC)$_2$]PF$_6$ gave rise to an evident upregulation of several glycolytic enzymes. Moreover, only [Au(NHC)$_2$]PF$_6$ triggered a net impairment of respiration and a metabolic shift towards glycolysis, suggesting that mitochondria are relevant cellular targets. We also found that both carbenes, similarly to the gold(I) compound auranofin, caused a strong inhibition of the seleno-enzyme thioredoxin reductase (TrxR). From these studies, it emerged that TrxR inhibition and metabolic impairment probably play a major role in the [Au(NHC)$_2$]PF$_6$ cytotoxicity. These

antiproliferative effects were also confirmed in two additional human ovarian cancer cell lines (*i.e.*, SKOV3 and IGROV1) [31].

The following protein systems have been considered here to characterize gold carbenes-proteins interactions: human serum albumin (HSA), human carbonic anhydrase I (hCA I), bovine superoxide dismutase (SOD) and the C-terminal dodecapeptide of thioredoxin reductase (dTrxR). This choice was dictated by the fact that all the above three proteins possess a free cysteine residue amenable for gold(I) coordination [27]. In turn, the C-terminal dodecapeptide of TrxR1 possesses the peculiar -Cys-Sec- reactive motif, mimicking the enzyme's active site [30].

The analysis was carried out according to the general protocol developed in our laboratory as detailed below [27,32–34].

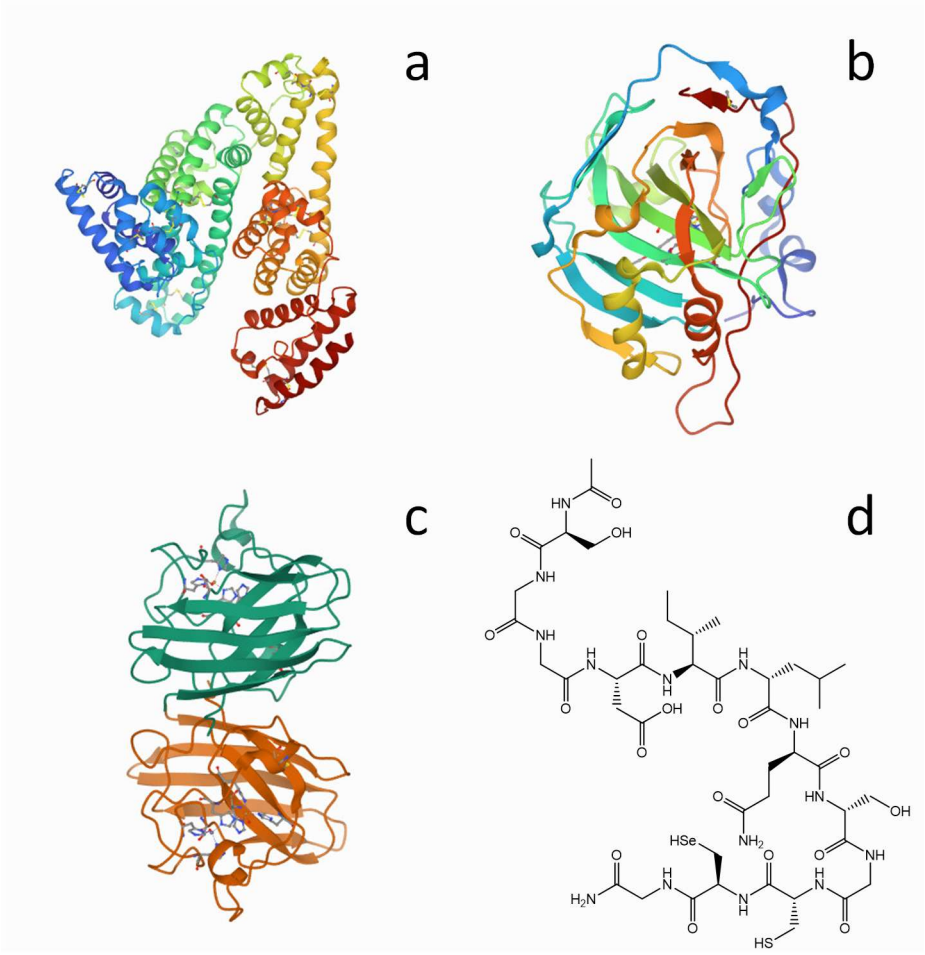


Figure 2. *Ribbon representation of the overall structure of a) human serum albumin (PDB entry 1AO6); b) human carbonic anhydrase I (PDB entry 2NN7); c) bovine superoxide dismutase (PDB entry 1CB4); d) C-terminal dodecapeptide of TrxR1.*

Materials and methods

2.1 Materials

Lyophilized hCA I, HSA and SOD were purchased from Sigma-Aldrich and used without further purification or manipulation. Au(NHC)Cl and $[Au(NHC)_2]PF_6$ as well as the C-terminal dodecapeptide of thioredoxin reductase (dTrxR), were synthesized in the MetMed Laboratories at the Department of Chemistry, University of Florence, following already established procedures [24,30,35]. DTT and dimethyl sulfoxide (DMSO) were purchased from Fluka. LC-MS grade solvents and salts (water, methanol and ammonium acetate) were purchased from Sigma-Aldrich.

2.2 Electrospray Ionization Mass Spectrometry Experimental Conditions

2.2.1 Sample Preparation

Stock solutions of hCA I 10^{-4} M, HSA, SOD 10^{-3} M, and dTrxR 10^{-3} M were prepared by dissolving the proteins and the peptide in LC-MS grade water. Stock solutions 10^{-2} M of the gold(I) carbene compounds were prepared by dissolving the samples in DMSO. Stock solution 10^{-1} M of DTT was prepared in H_2O. For the experiments with hCA I, HSA and SOD, aliquots of the respective stock solutions were mixed with aliquots of each gold carbene at protein-to-metal ratio 1:3 and diluted with ammonium acetate solution 2×10^{-3} M (pH 6.8) to 10^{-4} M final protein concentration. In case of hCA I the final protein concentration was 10^{-5} M. The mixtures were incubated at 37 °C up to 24 h. For the experiments with dTrxR aliquots of its stock solution were diluted with ammonium acetate solution 2×10^{-3} M (pH 6.8) to 10^{-4} M final peptide concentration. Then, aliquots of DTT stock solution were added in peptide to reducing agent ratios 1:10. The mixtures were incubated at 37 °C for 30 min and then aliquots of the gold carbenes solutions were added in peptide-to-metal compound ratio 1:3. The obtained mixtures were incubated at 37 °C up to 72 h.

2.2.2 Electrospray Ionization Mass Spectrometry Analysis: Final Dilutions

After the incubation time, all solutions were sampled and diluted to a final protein concentration of 7×10^{-7} M for hCA I, 5×10^{-7} M for HSA, 10^{-7} M for SOD and 5×10^{-7} M for dodecapeptide of TrxR using ammonium acetate solution 2×10^{-3} M, pH 6.8. The HSA and the peptide final solution were also added with 0.1% v/v of formic acid just before the infusion in the mass spectrometer. The hCA I and SOD final solutions were analysed both with and without the addition of 0.1% v/v of formic acid.

2.2.3 Instrumental Parameters

The ESI mass study was performed using a TripleTOF® 5600+ high-resolution mass spectrometer (AB Sciex, Framingham, MA, United States), equipped with a DuoSpray® interface operating with an ESI probe. Respective ESI mass spectra were acquired through direct infusion at 5 µL/min of flow rate. The general ESI source parameters optimized for each protein and peptide analysis were as follows: HSA: positive polarity, ion spray voltage floating 5500V, temperature 25 °C, ion source Gas 1 (GS1) 45 L/min; ion source Gas 2 (GS2) 0; curtain gas (CUR) 12 L/min, collision energy (CE) 10 V; declustering potential (DP) 150 V, acquisition range 1000–2600 *m/z*. hCA I: positive polarity, ion spray voltage floating 5500 V, temperature 25 °C, ion source Gas 1 (GS1) 40 L/min; ion source Gas 2 (GS2) 0; CUR 10 L/min, CE 10 V; DP 50 V, acquisition range 760–990 *m/z*. dTrxR: positive polarity, ion spray voltage floating 5500 V, temperature 100 °C, ion source Gas 1 (GS1) 25 L/min; ion source Gas 2 (GS2) 25 L/min; CUR 30 L/min, CE 10 V; DP 50 V, acquisition range 1000–2000 *m/z*. SOD: positive polarity, ion spray voltage floating 5500 V, temperature 25 °C, ion source Gas 1 (GS1) 40 L/min; ion source Gas 2 (GS2) 0; Curtain Gas (CUR) 15 L/min, collision energy (CE) 10 V; declustering potential (DP) 200 V, acquisition range 1500–3500 *m/z*. For acquisition, Analyst TF software 1.7.1 (Sciex) was used, and deconvoluted spectra were obtained by using the Bio Tool Kit micro-application v.2.2 embedded in PeakView™ software v.2.2 (Sciex).

2. Results and Discussion

3.1 The general design of the ESI MS experiments

All studies were conducted according to a well-defined experimental protocol that was developed in our laboratory in recent years and is now documented by several papers [26,27]. According to this protocol, lyophilized proteins were dissolved in H_2O. To each protein solution, a 3:1 amount of the gold complex was added and the incubation was carried out for 72 hours. Then the samples were analyzed with an ESI-Q-TOF mass spectrometer by direct infusion of the sample in the positive ESI mode. In some cases, the experimental protocol provides for the addition of 0.1% v/v of formic acid to the solution just before the injection in order to promote the ionization process into the ESI source.

3.2 Human serum albumin

The results obtained reacting human serum albumin (HSA) with the two gold carbene compounds in a 1:3 protein to metal ratio are reported below in comparison to the ESI mass spectrum of HSA alone, figure 3. The deconvoluted mass spectrum of the protein, figure 3A, is characterized by the main signal at 66437 Da assigned to the native protein, while the signals at 66556 Da and 66601 Da are attributed to different kinds of post-translational modifications of the albumin. The signal at 66556 Da is perfectly in agreement with an additional cysteine bound to the Cys34. The peak at 66601 Da belongs to a glycated form of HSA, also in this case present in native serum proteins. Indeed, both human and bovine serum albumin show characteristic post-translational modifications (PTMs), such as cysteinylation, involving the unique cysteine residue normally present as a free thiol [32,36].
Upon reaction with the gold carbene compounds, the native protein signal is no more observed, while a few signals of greater mass are detected, indicating extensive protein metalation. Specifically, the metal fragment that binds the protein corresponds, for both carbenes, to 1-butyl-3-methylimidazole-2-ylidene-gold(I). In the case of Au(NHC)Cl a large degree of HSA metalation is observed already after 24 h of incubation (Figure 1S); in figure 3B a clear signal with 90% of relative intensity at 67105 Da is attributed to the adduct of the native protein with two gold(I) fragments, after 72 h of incubation. Upon considering the high affinity of gold(I) species for sulphur atoms [1,27], we might assume that the Cys residue could be a preferential binding site for the gold(I) carbene. Indeed, human serum albumin possesses one free cysteine residue (Cys34) that is accessible to ligands [37,38]. The formation of a bis adduct on a single cysteine residue is not entirely surprising as it has been already observed for the gold(I) drug auranofin by Frank Shaw III who proposed for bovine serum albumin the formation of a thiolate-bridged bis-gold(I) adduct on the same Cys34 residue [39]. Therefore, we might assume that two gold carbene moieties bind on the same sulphur atom of the Cys34, in a similar

way to what happens for auranofin. Furthermore, lower intensity signals are also present, at 66889 Da and 67439 Da respectively assigned to the cysteinylated protein with a gold(I) moiety and to the native protein with three metal fragments. These adducts could be explained by considering that a few additional amino acid residues other than cysteines are possible binding sites for gold compounds, *i.e.* His, Gln and Lys residues, as widely reported in the literature [40]. As a matter of fact, several of our works have shown that His and Gln residues of model proteins such as lysozyme (HEWL) and ribonuclease A (RNase A) can bind gold species [19,41–43]. For the bis carbene compound, generally, lesser reactivity respect to the mono carbene is observed; as a result, in figure 3C after 72 h of incubation at 37 °C, only the signal at 67105 Da is detected with a 60% relative intensity that is attributable to the adduct formed with two Au(I) carbene moieties.

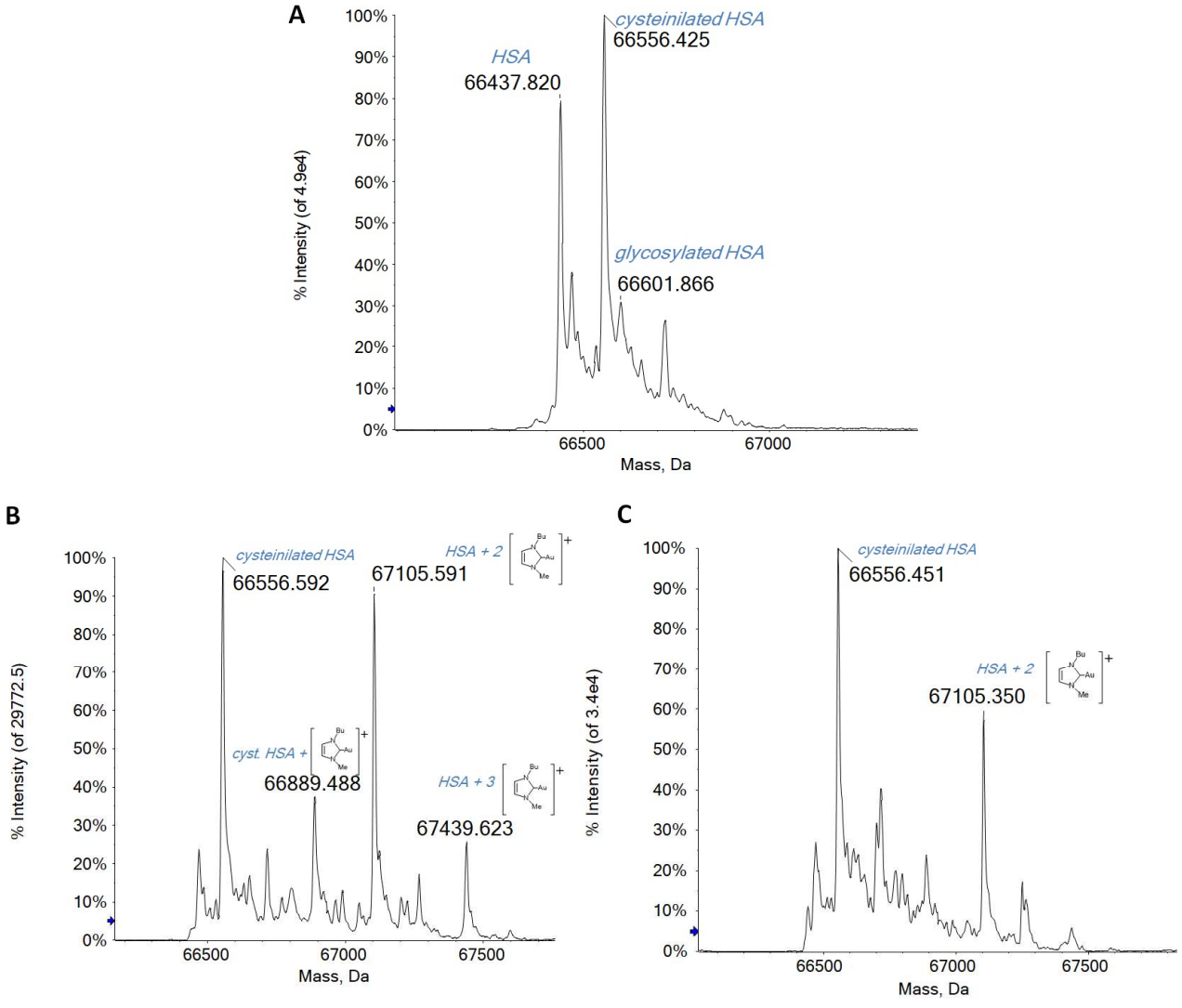


Figure 3 *(A) Deconvoluted mass spectra of HSA 5 × 10^{-7} M in 2 mM of ammonium acetate solution at pH 6.8 and incubated at 37 °C for 72 h with (B) Au(NHC)Cl and (C) $[Au(NHC)_2]PF_6$ in 1:3 protein-to-gold ratio; 0.1% v/v of formic acid was added just before infusion.*

3.3 Human carbonic anhydrase

Our standard and well consolidated experimental procedure for the analysis of proteins usually requires the addition of a small volume (0.1 % v/v) of acid to the sample just before the introduction into the spectrometer, to improve the ionization process in the source. For the specific case of the human carbonic anhydrase I (hCAI), this slightly acidic condition was found to cause partial denaturation of the enzyme, leading to the loss of the zinc ion from the active site and to some unfolding of the protein. The experimental conditions to observe this protein in the native state were defined in depth in a recent paper of ours [34]. So, we decided to investigate the interactions of gold carbenes with hCA I by following the experimental procedure which preserves the native state of the protein. Then, we repeated the experiment also adding the 0.1% of acid to the solution to gather useful information about the binding mode of the gold compounds both with the protein in its native state and in the denatured one. The obtained results are highly informative; the deconvoluted mass spectra under native and denatured conditions are reported in Figure 4 and Figure 5, respectively.

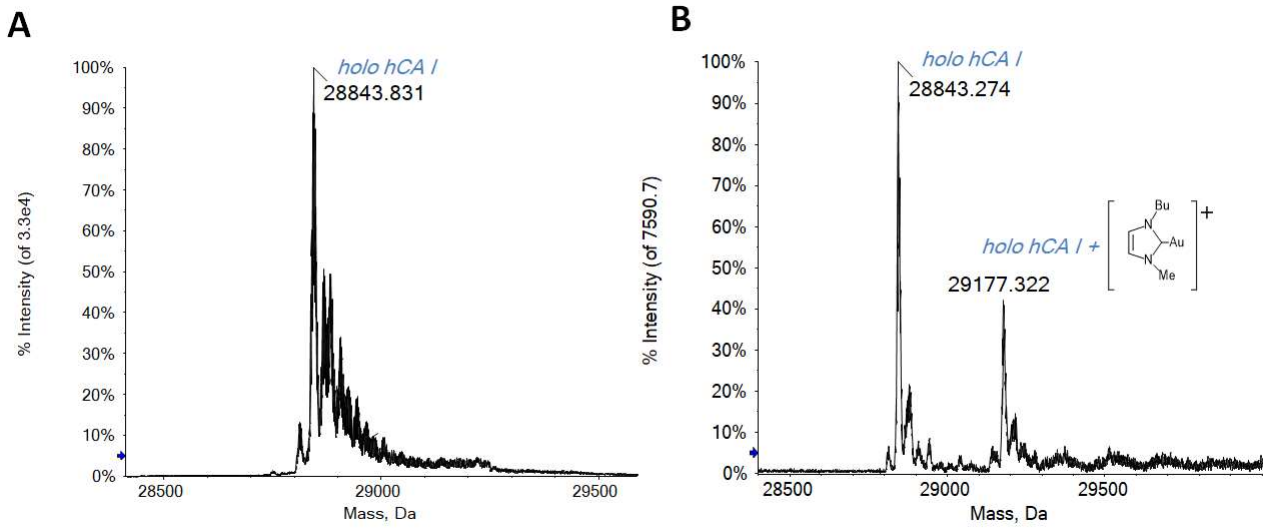


Figure 4 *(A) Deconvoluted mass spectra of hCA I 7 × 10^{-7} M in 2 mM ammonium acetate solution at pH 6.8 and incubated at 37 °C for 24 h with (B) Au(NHC)Cl in a 1:3 protein-to-gold ratio.*

The deconvoluted mass spectrum of native hCA I alone is reported in figure 4A: the signal at 28843 Da is attributed to the holo-enzyme. In figure 5A the deconvoluted mass spectrum of carbonic anhydrase, after the acid addition, shows the signal at 28780 Da attributed to the apo-protein. The instrumental parameters were optimized differently for the native and the denaturing conditions as extensively commented in our previous paper [34].

Under native conditions the reaction of hCA I with these gold complexes is selective for the monocarbene; indeed, Au(NHC)Cl converts hCA I partially into the gold derivative through the usual 1-butyl-3-methylimidazole-2-ylidene-gold(I) fragment, whereas the bis-carbene complex does not react at all over 72 h of incubation (Figure S2). In figure 4B the deconvoluted mass spectrum displays the signal of the monoadduct at 29177 Da with a relative intensity of 40% compared to the protein signal; the intensity of the signal decreases by about 10% over 72 hours of incubation, revealing the appreciable stability of this adduct (Figure S3) without showing transformations in thermodynamically more stable complexes of different stoichiometry [32]. After acid addition, some interesting changes in reactivity were noted. Again, the Au(NHC)Cl complex appears to be more reactive compared to the bis carbene one. However, in the presence of the acid, the reaction with the gold mono carbene is far more pronounced, respect to the native state conditions, and the protein seems to react completely with the gold species. Indeed, upon reacting hCA I with Au(NHC)Cl, figure 5B, the protein signal is no more detected and a 100% intensity signal at 29449 Da emerges, assigned to the adduct of the apo-protein with two 1-butyl-3-methylimidazole-2-ylidene-gold(I) fragments. This adduct is stable over 72 h of incubation at 37 °C as the mass spectrum is perfectly superimposable with the one at 24 h (Figure S4A). The same adduct signal is barely detectable in the case of $[Au(NHC)_2]PF_6$ while the apo-protein signal is the main one, indicating a very scarce tendency of the bis gold carbene to react with this protein over 72 h of incubation, figure 5C (Figure S4B). The 0.1% v/v addition of formic acid to the solution lowers the pH from pH 6.8 to pH 2.9. This causes the protonation of the imidazole rings of the three histidine residues (*i.e.* His94, His96, and His119) into the active site of the enzyme, leading to the loss of the Zn^{2+} ion. Moreover, the acidic condition seems to be responsible of a partial protein unfolding, exposing amino acid residues that in the native state are not accessible and making them prone to react with metal species, as we already noted for the case of the gold(I)-based drug auranofin. [34]. The mono carbene gold(I) case could be similar: in the denaturing environment it is highly reactive while in a more native-like condition is less. Human carbonic anhydrase I possesses a unique free cysteine residue (Cys212) that is not solvent accessible when the enzyme is folded [34]. Again, the well-known affinity of gold(I)-containing species for sulphur atoms, specifically for free cysteine residues, let us to infer some explanation in the observed differences in reactivity of hCA I with gold carbene when it is in denaturing or native conditions. When the hCA I is partially denatured, it exposes the free Cys212 residue to the solvent offering a possible binding site for the gold(I) carbene and, indeed, a strong interaction is noted (Figure 5B): the gold(I) mono carbene Au(NHC)Cl reacts completely with the protein, giving a bis adduct thought the bonding of the gold(I) containing fragments. The formation of a bis adduct on a single cysteine residue may be interpreted as already seen for HSA: two carbene gold(I) fragments can bind on the

same sulphur atom of the Cys212. At variance, when hCA I is in its native state, the Cys212 residue cannot bind to the gold(I) species anymore; nevertheless, the formation of a mono adduct is anyway observed which could indicate the involvement of other amino acid residues, in the binding of gold(I) fragment.

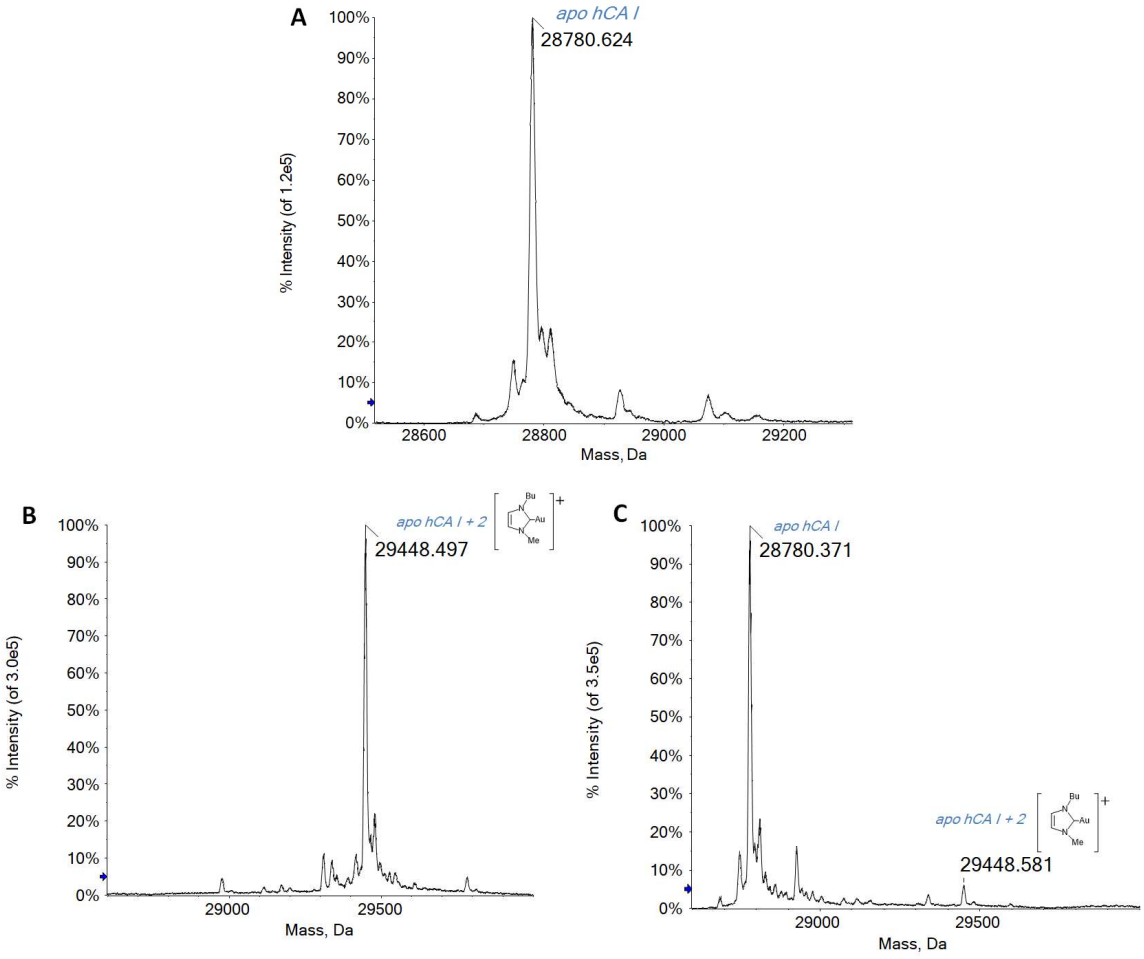


Figure 5 *(A) Deconvoluted mass spectra of hCA I 7 × 10−7 M in 2mM ammonium acetate solution at pH 6.8 and incubated at 37 °C for 24 h with (B) Au(NHC)Cl and (C) [Au(NHC)$_2$]PF$_6$ in 1:3 protein-to-gold ratio. 0.1% v/v of formic acid was added just before infusion.*

3.4 SOD

As already shown for hCA I, also in the case of superoxide dismutase (SOD) a native approach has been attempted and no acid has been added to the final solution of the protein. Significantly, no difference in the mass spectrum of the protein is observed compared to the case with acid addition: the holo-enzyme is clearly detectable in both cases. Indeed, the acid addition does not appear to alter the binding of Cu^{2+} and Zn^{2+} ions in the enzyme active site nor the conformation of the protein itself.

In fact, from the comparison of the charge state distribution spectra, the enzyme seems to charge nearly to the same extent with acid addition or not, suggesting that no denaturation and unfolding occur (Figure S5 and S6) [34]. Also, no differences in the reactivity trend with gold(I)-carbene species are detected, as we repeated the interactions experiments both in native-like (Figure 6) and acidic conditions (Figures S7 and S8).

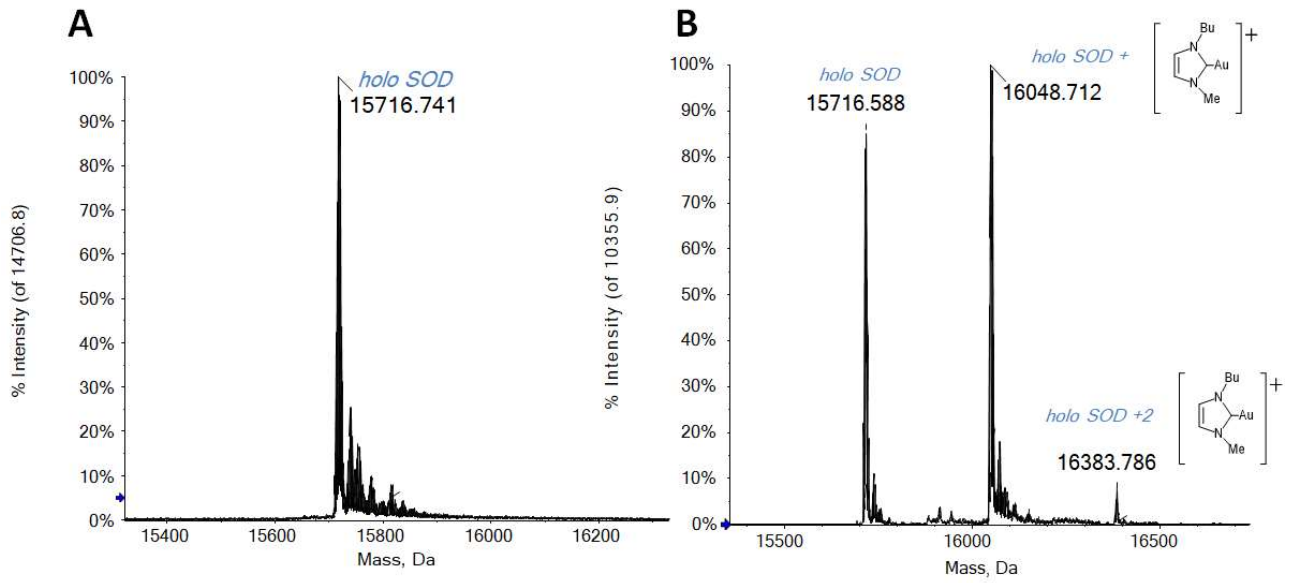


Figure 6 *(A) Deconvoluted mass spectra of SOD 10^{-7} M in 2mM of ammonium acetate solution at pH 6.8 and incubated at 37 °C for 24 h with (B) Au(NHC)Cl in a 1:3 protein-to-gold ratio.*

In figure 6A, the deconvoluted mass spectrum of the protein alone shows a very well resolved signal at 15715 Da that is assigned to holo SOD, as described in detail in a recent paper [27]. Upon reaction with SOD, the mono gold(I) carbene shows a large reactivity already after 24 h of incubation, giving rise to a very intense mono adduct signal with the 1-butyl-3-methylimidazole-2-ylidene-gold(I) moiety and a less intense, yet remarkable, bis adduct signal, Figure 6B. The unreacted SOD signal is still present, indicating that the metalation of the protein is not complete. Notably, the adducts seem to be stable over 72 h of incubation at 37 °C (Figure S10) without loss of the bound metal-fragment or formation of adducts with different stoichiometry. Instead, as confirmation of the less reactive character of the bis-gold(I) carbene, no metalation of the protein has been observed over 72 h of incubation, and just a clear holo SOD signal was seen (Figure S11).

We previously demonstrated that the two free cysteine residues of dimeric SOD are poorly solvent accessible, so that the cysteine-modifier reagent ebselen does not bind them [27]. Therefore, these

results confirm what already observed in the case of native carbonic anhydrase and human serum albumin and suggest that the Cys residue is not the unique binding site for the gold(I) carbene: other amino acid residues are probably involved.

3.5 Trx dodecapeptide

The enzyme thioredoxin reductase is unanimously considered one of the main targets for cytotoxic gold compounds. So, we selected the C-terminal dodecapeptide that mimics the TrxR1 active site, to study its reactivity with gold(I) carbenes. The peptide possesses the -Cys-Sec- reactive motif as a possible binding site for the gold species. The intramolecular -S-Se- bridge between the aforesaid residues needs to be reduced in order to favour the reaction with the gold compounds. So, the reducing agent dithiothreitol (DTT) was added in 10:1 ratio DTT/peptide 30 minutes before the incubation of the dodecapeptide with each gold carbene. The mass/charge spectrum in Figure 7A shows the signals of the peptide and of its adducts with Na^+ and NH_4^+ ions from the solution. Upon reaction with the two gold carbenes, a common reactivity trend has been identified: the two gold(I) carbenes completely lose their ligands and bind to the peptide as naked Au(I) ions. In figure 7B and 7C, the mass/charge spectra of the gold carbenes incubated with the peptide up to 24 h are reported: in both cases, besides the unreacted peptide signal, an *ensemble* of signals of greater mass is also present, indicating the formation of peptide adducts with Au(I) ion (1381 Da). Moreover, ancillary signals of the same Na^+ and NH_4^+ adducts are detected. Again, the higher reactivity of Au(NHC)Cl compared to the bis-carbene compound is confirmed for this dodecapeptide. In fact, the signal adduct in Figure 7B shows greater intensity respect to the unreacted peptide, pointing out an almost complete peptide metalation. On the other hand, the opposite situation is observed for the bis carbene, where the unreacted peptide represents the most abundant species with respect to the metal adduct. Interestingly, in this latter case, the adducts are not stable over time and after 72 h of incubation their signals are far less intense or almost absent (Figure S12).

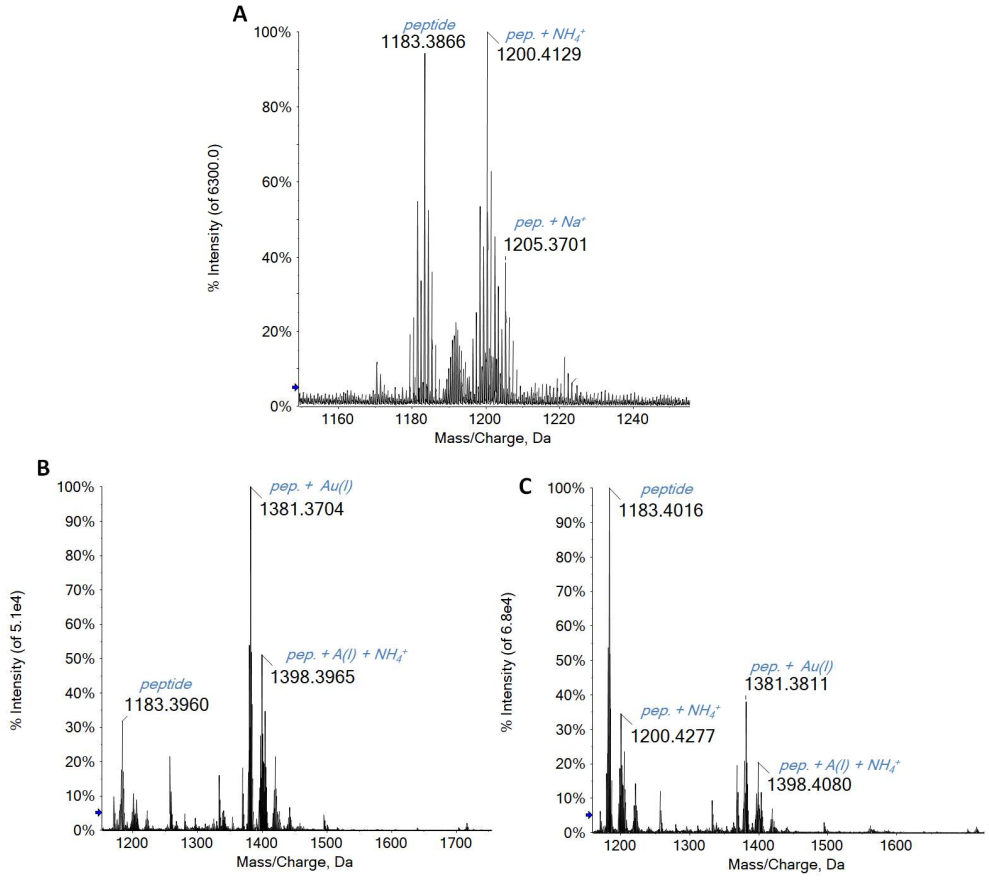


Figure 7 *(A) ESI mass spectra of dTrxR 5 × 10^{-7} M in 2mM ammonium acetate solution at pH 6.8 and incubated at 37 °C for 24 h with (B) Au(NHC)Cl and (C) [Au(NHC)$_2$]PF$_6$ at 1:3:10 peptide-to-gold-to-DTT ratio. 0.1% v/v of formic acid was added just before infusion.*

Conclusions

The recent literature points out that medicinal gold compounds produce their pharmacological effects mostly by reacting with a variety of protein targets. The interactions with nucleic acids seem to be less important. The metalation of proteins by gold complexes has been investigated in depth during the last few years through combined X-ray diffraction and ESI MS experiments. Substantial structural and functional insights have been gained. Very recently the ESI-MS technique has been extended to consider a number of proteins of a bigger size that may be physiologically more relevant. A study concerning auranofin revealed a selective interaction of this drug with solvent accessible free cysteines of target proteins [27]. Another study investigated the protein interactions of a small group of gold(III) compounds with the same panel of biomolecules here analysed, revealing a common oxidising character that leads to a rapid reduction of the metal compound to the resulting Au(I) species that, in turn, is able to bind the biomolecule [26].

In this work, we have applied the ESI MS approach to analyse the interactions of two representative gold carbene compounds, namely Au(NHC)Cl and $Au(NHC)_2PF_6$, with a pool of selected and biologically relevant targets. These gold complexes were comparatively challenged against three representative proteins and a small oligopeptide that reproduces the C-terminal sequence of the enzyme thioredoxin reductase. Notably, all these proteins contain a free cysteine residue; in addition, the dodecapeptide also contains the peculiar -S-Se- reactive motif. Analysis of the obtained results allowed us to identify a few trends in the reactivity of these gold carbene complexes with biosubstrates:

- In general, the reactivity of gold carbenes seems to be less pronounced than in the case of other gold compounds and more selective.
- Free cysteines appear to be the preferential binding sites for gold carbenes.
- The monocarbene complex is more reactive than its bis-carbene counterpart, possibly as the consequence of the strength of the direct gold-carbon and its high kinetic stability. Nevertheless, the higher antiproliferative activity of the bis-carbene might be attributed to the characteristics like those of the delocalized lipophilic cations (DLCs), in fact, a greater mitochondrial accumulation, due to the formation of cationic species $[Au(NHC)_2]^+$, might increase the cytotoxic effects [31].
- The protein-bound metallic fragment always retains one carbene ligand, except in the case of the dodecapeptide where the carbene ligands are completely lost.
- The dodecapeptide, probably owing to the presence of -S-Se- motif, is unique for its ability to stabilize the respective gold adduct thanks to the soft character if the two elements. Probably, this stabilization concurs to the loss of the original gold ligands both from the monocarbene and the biscarbene complex.

The here discussed features suggest that these gold carbene complexes are characterised by a more selective reactivity towards protein targets than other medicinal gold compounds. In other words, they appear to prefer free cysteine or selenocysteine residues for protein anchoring. Notably, when these residues are not present or accessible, a far reduced protein reactivity is detected. These observations open new interesting perspectives in the design and development of new medicinal gold compounds endowed with a remarkable selectivity for the so-called cysteinome, *i.e*. the cysteine proteome.

Acknowledgements

The authors thank AIRC and Fondazione Cassa Risparmio Firenze for funding the projects "Advanced mass spectrometry tools for cancer research: novel applications in proteomics,

metabolomics and nanomedicine" (Multi-user Equipment Program 2016, grant number 19650) for financial support. CIRCMSB is also acknowledged.

References

[1] J.C. Dabrowiak, Metals in Medicine, 2009. https://doi.org/10.1002/9780470684986.

[2] K.D. Mjos, C. Orvig, Metallodrugs in medicinal inorganic chemistry, Chem. Rev. 114 (2014) 4540–4563. https://doi.org/10.1021/cr400460s.

[3] E.J. Anthony, E.M. Bolitho, H.E. Bridgewater, O.W.L. Carter, J.M. Donnelly, C. Imberti, E.C. Lant, F. Lermyte, R.J. Needham, P.J. Sadler, Chemical Science Metallodrugs are unique : opportunities and challenges of discovery and development †, Chem. Sci. (2020).

[4] F. Magherini, A. Modesti, L. Bini, M. Puglia, I. Landini, S. Nobili, E. Mini, M.A. Cinellu, C. Gabbiani, L. Messori, Exploring the biochemical mechanisms of cytotoxic gold compounds: A proteomic study, J. Biol. Inorg. Chem. 15 (2010) 573–582. https://doi.org/10.1007/s00775-010-0624-3.

[5] W.F. Kean, F. Forestier, Y. Kassam, W.W. Buchanan, P.J. Rooney, The history of gold therapy in rheumatoid disease, Semin. Arthritis Rheum. (1985). https://doi.org/10.1016/0049-0172(85)90037-X.

[6] B.M. Sutton, E. Mcgusty, D.T. Walz, M.J. Dimartino, Oral Gold. Antiarthritic Properties of Alkylphosphinegold Coordination Complexes, J. Med. Chem. 15 (1972) 1095–1098. https://doi.org/10.1021/jm00281a001.

[7] B.M. Sutton, Gold compounds for rheumatoid arthritis, Gold Bull. (1986). https://doi.org/10.1007/BF03214639.

[8] T.N. Simon, D.H. Kunishima, G.J. Vibert, A. Lorber, Cellular antiproliferative action exerted by auranofin, J. Rheumatol. (1979).

[9] C.K. Mirabelli, R.K. Johnson, C.M. Sung, L. Faucette, K. Muirhead, S.T. Crooke, Evaluation of the in Vivo Antitumor Activity and in Vitro Cytotoxic Properties of Auranofin, a Coordinated Gold Compound, in Murine Tumor Models, Cancer Res. 45 (1985) 32–39. https://europepmc.org/article/med/3917372 (accessed October 9, 2020).

[10] C. Yeo, K. Ooi, E. Tiekink, Gold-Based Medicine: A Paradigm Shift in Anti-Cancer Therapy?, Molecules. 23 (2018) 1410. https://doi.org/10.3390/molecules23061410.

[11] L. Massai, A. Pratesi, J. Bogojeski, M. Banchini, S. Pillozzi, L. Messori, Ž.D. Bugarčić, Antiproliferative properties and biomolecular interactions of three Pd(II) and Pt(II) complexes, J. Inorg. Biochem. 165 (2016) 1–6. https://doi.org/10.1016/j.jinorgbio.2016.09.016.

[12] T. Marzo, L. Massai, A. Pratesi, M. Stefanini, D. Cirri, F. Magherini, M. Becatti, I. Landini, S. Nobili, E. Mini, O. Crociani, A. Arcangeli, S. Pillozzi, T. Gamberi, L. Messori, Replacement of the Thiosugar of Auranofin with Iodide Enhances the Anticancer Potency in

a Mouse Model of Ovarian Cancer, ACS Med. Chem. Lett. 10 (2019) 656–660. https://doi.org/10.1021/acsmedchemlett.9b00007.

[13] S. Yue, M. Luo, H. Liu, S. Wei, Recent Advances of Gold Compounds in Anticancer Immunity, Front. Chem. (2020). https://doi.org/10.3389/fchem.2020.00543.

[14] A. Casini, R.W.Y. Sun, I. Ott, Medicinal chemistry of gold anticancer metallodrugs, in: Met. Dev. Action Anticancer Agents, 2018. https://doi.org/10.1515/9783110470734-007.

[15] I. Ott, On the medicinal chemistry of gold complexes as anticancer drugs, Coord. Chem. Rev. 253 (2009) 1670–1681. https://doi.org/10.1016/j.ccr.2009.02.019.

[16] V. Fernández-Moreira, R.P. Herrera, M.C. Gimeno, Anticancer properties of gold complexes with biologically relevant ligands, in: Pure Appl. Chem., 2019. https://doi.org/10.1515/pac-2018-0901.

[17] P. Gratteri, L. Massai, E. Michelucci, R. Rigo, L. Messori, M.A. Cinellu, C. Musetti, C. Sissi, C. Bazzicalupi, Interactions of selected gold(iii) complexes with DNA G quadruplexes, Dalt. Trans. (2015). https://doi.org/10.1039/c4dt02698e.

[18] T. Gamberi, L. Massai, F. Magherini, I. Landini, T. Fiaschi, F. Scaletti, C. Gabbiani, L. Bianchi, L. Bini, S. Nobili, G. Perrone, E. Mini, L. Messori, A. Modesti, Proteomic analysis of A2780/S ovarian cancer cell response to the cytotoxic organogold(III) compound Aubipyc, J. Proteomics. 103 (2014) 103–120. https://doi.org/10.1016/j.jprot.2014.03.032.

[19] L. Messori, F. Scaletti, L. Massai, M.A. Cinellu, C. Gabbiani, A. Vergara, A. Merlino, The mode of action of anticancer gold-based drugs: A structural perspective, Chem. Commun. 49 (2013) 10100–10102. https://doi.org/10.1039/c3cc46400h.

[20] F. Guidi, I. Landini, M. Puglia, F. Magherini, C. Gabbiani, M.A. Cinellu, S. Nobili, T. Fiaschi, L. Bini, E. Mini, L. Messori, A. Modesti, Proteomic analysis of ovarian cancer cell responses to cytotoxic gold compounds, Metallomics. 4 (2012) 307–314. https://doi.org/10.1039/c2mt00083k.

[21] N. Micale, T. Schirmeister, R. Ettari, M.A. Cinellu, L. Maiore, M. Serratrice, C. Gabbiani, L. Massai, L. Messori, Selected cytotoxic gold compounds cause significant inhibition of 20S proteasome catalytic activities, J. Inorg. Biochem. 141 (2014) 79–82. https://doi.org/10.1016/j.jinorgbio.2014.08.001.

[22] F. Guarra, N. Busto, A. Guerri, L. Marchetti, T. Marzo, B. García, T. Biver, C. Gabbiani, Cytotoxic Ag(I) and Au(I) NHC-carbenes bind DNA and show TrxR inhibition, J. Inorg. Biochem. (2020). https://doi.org/10.1016/j.jinorgbio.2020.110998.

[23] N. Estrada-Ortiz, F. Guarra, I.A.M. de Graaf, L. Marchetti, M.H. de Jager, G.M.M. Groothuis, C. Gabbiani, A. Casini, Anticancer Gold N-Heterocyclic Carbene Complexes: A

Comparative in vitro and ex vivo Study, ChemMedChem. (2017). https://doi.org/10.1002/cmdc.201700316.
[24] L. Messori, L. Marchetti, L. Massai, F. Scaletti, A. Guerri, I. Landini, S. Nobili, G. Perrone, E. Mini, P. Leoni, M. Pasquali, C. Gabbiani, Chemistry and biology of two novel gold(I) carbene complexes as prospective anticancer agents, Inorg. Chem. 53 (2014) 2396–2403. https://doi.org/10.1021/ic401731a.
[25] M.D. Đurović, Ž.D. Bugarčić, R. van Eldik, Stability and reactivity of gold compounds – From fundamental aspects to applications, Coord. Chem. Rev. (2017). https://doi.org/10.1016/j.ccr.2017.02.015.
[26] L. Massai, C. Zoppi, D. Cirri, A. Pratesi, L. Messori, Reactions of Medicinal Gold(III) Compounds With Proteins and Peptides Explored by Electrospray Ionization Mass Spectrometry and Complementary Biophysical Methods, Front. Chem. 8 (2020) 966. https://doi.org/10.3389/fchem.2020.581648.
[27] C. Zoppi, L. Messori, A. Pratesi, ESI MS studies highlight the selective interaction of Auranofin with protein free thiols, Dalt. Trans. 49 (2020) 5906–5913. https://doi.org/10.1039/d0dt00283f.
[28] F. Guarra, T. Marzo, M. Ferraroni, F. Papi, C. Bazzicalupi, P. Gratteri, G. Pescitelli, L. Messori, T. Biver, C. Gabbiani, Interaction of a gold(i) dicarbene anticancer drug with human telomeric DNA G-quadruplex: solution and computationally aided X-ray diffraction analysis, Dalt. Trans. (2018). https://doi.org/10.1039/c8dt03607a.
[29] G. Ferraro, C. Gabbiani, A. Merlino, First Crystal Structure for a Gold Carbene-Protein Adduct, Bioconjug. Chem. (2016). https://doi.org/10.1021/acs.bioconjchem.6b00298.
[30] A. Pratesi, C. Gabbiani, E. Michelucci, M. Ginanneschi, A.M. Papini, R. Rubbiani, I. Ott, L. Messori, Insights on the mechanism of thioredoxin reductase inhibition by Gold N-heterocyclic carbene compounds using the synthetic linear Selenocysteine containing C-terminal peptide hTrxR(488-499): An ESI-MS investigation, J. Inorg. Biochem. 136 (2014) 161–169. https://doi.org/10.1016/j.jinorgbio.2014.01.009.
[31] F. Magherini, T. Fiaschi, E. Valocchia, M. Becatti, A. Pratesi, T. Marzo, L. Massai, C. Gabbiani, I. Landini, S. Nobili, E. Mini, L. Messori, A. Modesti, T. Gamberi, Antiproliferative effects of two gold(I)-N-heterocyclic carbene complexes in A2780 human ovarian cancer cells: A comparative proteomic study, Oncotarget. 9 (2018) 28042–28068. https://doi.org/10.18632/oncotarget.25556.
[32] A. Pratesi, D. Cirri, L. Ciofi, L. Messori, Reactions of Auranofin and Its Pseudohalide Derivatives with Serum Albumin Investigated through ESI-Q-TOF MS, Inorg. Chem. 57

(2018) 10507–10510. https://doi.org/10.1021/acs.inorgchem.8b02177.
[33] A. Pratesi, D. Cirri, D. Fregona, G. Ferraro, A. Giorgio, A. Merlino, L. Messori, Structural Characterization of a Gold/Serum Albumin Complex, Inorg. Chem. 58 (2019) 10616–10619. https://doi.org/10.1021/acs.inorgchem.9b01900.
[34] C. Zoppi, A. Nocentini, C.T. Supuran, A. Pratesi, L. Messori, Native mass spectrometry of human carbonic anhydrase I and its inhibitor complexes, JBIC J. Biol. Inorg. Chem. 1 (2020) 3. https://doi.org/10.1007/s00775-020-01818-8.
[35] A. Pratesi, C. Gabbiani, M. Ginanneschi, L. Messori, Reactions of medicinally relevant gold compounds with the C-terminal motif of thioredoxin reductase elucidated by MS analysis, Chem. Commun. 46 (2010) 7001–7003. https://doi.org/10.1039/c0cc01465f.
[36] J. Talib, J.L. Beck, S.F. Ralph, A mass spectrometric investigation of the binding of gold antiarthritic agents and the metabolite [Au(CN)2]- to human serum albumin, J. Biol. Inorg. Chem. 11 (2006) 559–570. https://doi.org/10.1007/s00775-006-0103-z.
[37] F. Nakashima, T. Shibata, K. Kamiya, J. Yoshitake, R. Kikuchi, T. Matsushita, I. Ishii, J.A. Giménez-Bastida, C. Schneider, K. Uchida, Structural and functional insights into S-thiolation of human serum albumins, Sci. Rep. (2018). https://doi.org/10.1038/s41598-018-19610-9.
[38] J.L. Beck, S. Ambahera, S.R. Yong, M.M. Sheil, J. De Jersey, S.F. Ralph, Direct observation of covalent adducts with Cys34 of human serum albumin using mass spectrometry, Anal. Biochem. (2004). https://doi.org/10.1016/j.ab.2003.10.041.
[39] A.A. Isab, C. Frank Shaw, J. Lockela, J.D. Hoeschele, Reactions of Trimethylphosphine Analogues of Auranofin with Bovine Serum Albumin, Inorg. Chem. 27 (1988) 3588–3592. https://doi.org/10.1021/ic00293a029.
[40] L. Messori, A. Merlino, Protein metalation by metal-based drugs: X-ray crystallography and mass spectrometry studies, Chem. Commun. (2017). https://doi.org/10.1039/c7cc06442j.
[41] L. Messori, F. Scaletti, L. Massai, M.A. Cinellu, I. Russo Krauss, G. Di Martino, A. Vergara, L. Paduano, A. Merlino, Interactions of gold-based drugs with proteins: Crystal structure of the adduct formed between ribonuclease A and a cytotoxic gold(iii) compound, in: Metallomics, 2014. https://doi.org/10.1039/c3mt00265a.
[42] L. Messori, M.A. Cinellu, A. Merlino, Protein recognition of gold-based drugs: 3d structure of the complex formed when lysozyme reacts with Aubipyc, ACS Med. Chem. Lett. (2014). https://doi.org/10.1021/ml500231b.
[43] A. Merlino, T. Marzo, L. Messori, Protein Metalation by Anticancer Metallodrugs: A Joint ESI MS and XRD Investigative Strategy, Chem. - A Eur. J. 23 (2017) 6942–6947.

https://doi.org/10.1002/chem.201605801.