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ESI MS studies highlight the selective interaction of Auranofin with protein free thiols†

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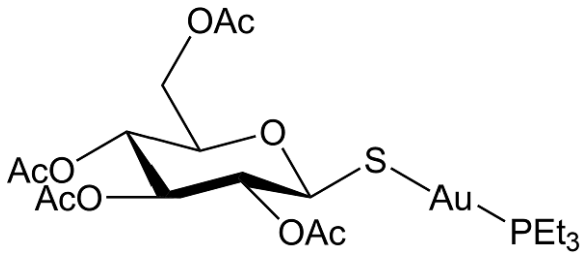
The clinically established gold drug Auranofin was reacted individually with a group of representative proteins, namely ubiquitin, RNase A, carbonic anhydrase, haemoglobin and superoxide dismutase, and adduct formation was monitored in the various cases by ESI-MS analysis. We found that the reaction is highly selective for solvent exposed free cysteines that are modified through coordination of the $AuPEt_3^+$ fragment. Indeed, ESI-Q-TOF MS spectra carried out on protein samples incubated with a three fold molar excess of Auranofin allowed direct detection of the native proteins bearing bound $AuPEt_3^+$ fragments in the cases of carbonic anhydrase and haemoglobin. At variance, the two proteins that do not possess any free cysteine residue, i.e. ubiquitin and RNase A, were unable to bind the gold fragment. In the case of SOD, adduct formation is hindered by the scarce solvent accessibility of the free Cys residue. These findings were further confirmed by a series of competition binding experiments with ebselen, a potent and selective cysteine-modifying reagent; we observed that pre-treatment with ebselen prevents the binding of the $AuPEt_3^+$ fragment to both carbonic anhydrase and haemoglobin.

Introduction

Starting from the discovery of cisplatin in the 1960's, Pt-based metallodrugs have been extensively investigated and successfully implemented in the clinical treatment of cancer where they still play a pivotal role.[1-3] Indeed, Pt drugs are present in about 50% of standard chemotherapeutic regimens for cancer. Pairwise, many other inorganic compounds bearing metal centres of different nature like gold,[4-6] ruthenium[7-9] and copper[10,11] have been designed, prepared and evaluated as experimental anticancer drugs with some encouraging results. Among them, gold-based compounds seem very promising.[12-14] Gold has been used in medicine since the ancient times; its application in modern pharmacology started with Robert Koch's work in 1890s and the discovery of the antimicrobial properties of the gold cyanide complex against *tubercule bacilli*. Later on, gold therapy was found to reduce joint pain; accordingly, during the 1920s a variety of injectable gold(I) thiolates were introduced in the clinics to treat rheumatoid arthritis initiating the so-called chrysotherapy *e.g.* sodium aurothiomalate (Myocrisin) and auro-thioglucose (Solganol).[15]



Scheme 1. Auranofin (AF).

Auranofin, a mixed ligand gold(I) complex, is a more recent FDA approved gold-based drug suitable for oral administration, in clinical use for the treatment of rheumatoid arthritis (RA) since 1988. Auranofin (1-Thio-β-D-gluco pyranosato triethylphosphine gold-2,3,4,6-tetraacetate – AF hereafter, Scheme 1), consists of a mononuclear gold(I) centre linearly coordinated to a triethylphosphine molecule and to 1-thio-β-D-glucose-2,3,4,6-tetraacetate.

During the last two decades, repurposing studies revealed that AF is a promising candidate drug for a variety of other therapeutic indications beyond RA including several microbial and parasitic diseases, and also some forms of cancer. Currently, a few clinical trials for the repurposing of AF in oncological applications are ongoing with quite encouraging results.[16,17] From these trials the remarkable anticancer

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versatility of this gold-based drug is clearly emerging, ranging from chronic lymphocytic leukaemia (NCT01419691)[18] to non-small and/or small cell lung cancer (NCT01737502)[19] and ovarian cancer (NCT03456700).[20]

Owing to its pharmaceutical importance, AF has been the subject of numerous mechanistic studies that explored its solution behaviour, its reactions with biomolecules [21-23] and its cellular effects; in particular, the alterations of the cellular redox state are believed to represent the actual basis of the mechanism of action of AF, specifically through the inhibition of important redox enzymes, such as thioredoxin reductase (TrxR).[24-28]

In a majority of cases, anticancer metal-based drugs are metal complexes behaving as prodrugs: this implies that they become able to form coordinative bonds with target biomolecules, and thus exert their pharmacological actions, only after the release of a weak ligand acting as the leaving group.[4-6,29] Strong coordinative bonding of the metal centre to specific functional groups of biomolecules is apparently at the origin of their biological actions.[30,31] Though nucleic acids are the putative primary targets for clinically established platinum-based drugs,[32] there is now a growing evidence that proteins rather than nucleic acids are the main targets for other classes of anticancer metal based drugs.[33]

This seems to be the case of AF, and to this respect a few important early studies were contributed by Frank Shaw III.[34] Indeed, Frank Shaw highlighted a binding preference of AF for proteins while the interactions with nucleic acids turned out to be very modest and nearly negligible.[35,36] Also, he could establish that under biologically relevant conditions AF preferentially releases the thiosugar ligand in such a way that the gold(I) centre retaining the phosphane ligand, i.e. the $AuPEt_3^+$ fragment, becomes available for coordination to protein side chains.[21-23,29] The reactions of AF with a number of proteins were thus described but the precise structures of the resulting adducts could be elucidated only in very rare instances.[37,38]

In any case, the clear binding preference of AF for proteins containing free cysteines (and, even more, free selenocysteines) [21-23] has been supported during the past years, in agreement with hard-soft acid-base considerations. More recently, important experimental evidences further strengthened the hypothesis for such reactivity,[29,39,40] without providing conclusive and unequivocal evidence.

This prompted us to characterize in a deeper detail, through high-resolution MS analysis, the process of protein metalation by AF and to elucidate the nature of the formed adducts at the molecular level.

Indeed, mass spectrometry has emerged in the last few years as an election tool to characterize the adducts formed by metal-based drugs with biomolecules, in particular with proteins of small size[41,42] but even of medium/large size.[43] When associated to parallel crystallographic data, MS studies turned out to provide an exhaustive description of the process of protein metalation induced by a variety of metal based drugs.[44,45] Accordingly, we have exploited the MS approach to study the interaction of AF with a few representative proteins to single out general trends in reactivity. The feasibility and potential of this approach was clearly documented in a recent study of ours concerning the adducts that BSA forms with auranofin and its analogues.[29] This kind of studies is made possible by the acquisition of a new high-resolution TripleTOF® 5600+ ESI-Q-TOF mass spectrometer (from AB Sciex) with improved technical performances and by the use of a well-established experimental protocol developed in our laboratory.[46,47]

Results and Discussion

The General Strategy. To prove the importance of the presence and accessibility of cysteine residues for the binding of AF to proteins, we have investigated this feature comparatively working with two groups of representative proteins. The first group included two small proteins i.e. ubiquitin (Ub) and ribonuclease A (RNase A), devoid of the free cysteine (or selenocysteine as well) residue; the second group included three medium-size proteins, i.e. carbonic anhydrase I (hCA I), human haemoglobin (Hb) and bovine superoxide dismutase (SOD), all possessing at least one free cysteine residue. All the above proteins are commercially available, and the stock solutions were prepared in the typical ESI-MS compatible solvent, in this case ammonium acetate (2mM) at pH 6.8.

The proteins solutions were individually incubated with AF (at a fixed 3:1 metal to protein molar ratio) for increasing time periods up to 72 hours. Subsequently, ESI mass spectra were recorded for the various samples under standard conditions and direct infusion (see the ESI for details) after 24 and 72 h of incubation in order to evaluate the eventual time course of adduct formation.

Ubiquitin and ribonuclease A. Interestingly, we observed that during the 72 h of incubation no adducts are formed when reacting AF with the two small model proteins ubiquitin and RNase A. The deconvoluted mass spectrum of ubiquitin, Figure 1, displays mainly the signal at 8563 Da attributed to the protein;

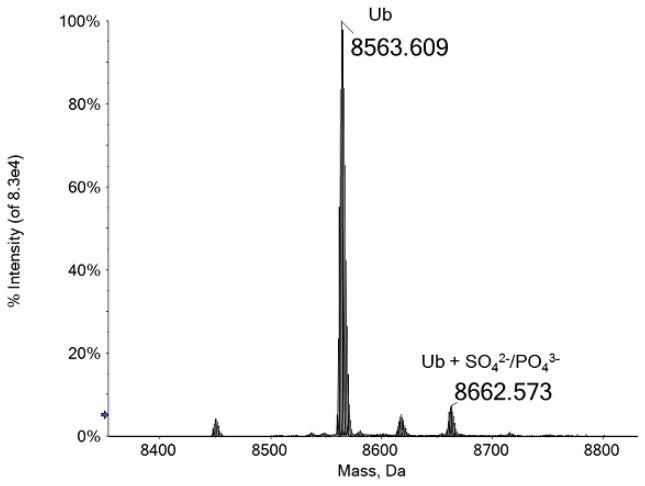

Figure 1. Deconvoluted ESI-Q-TOF mass spectrum of Ub solution 10^{-7} M incubated for 24 h at 37 °C with AF (3:1 metal to protein ratio) in 2mM ammonium acetate solution, pH 6.8.

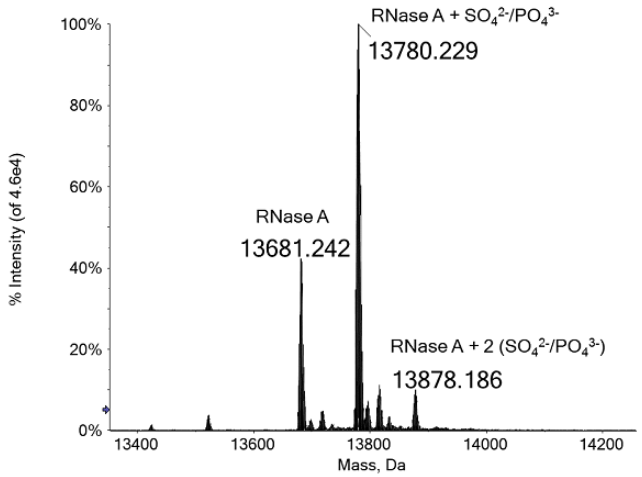

Figure 2. Deconvoluted ESI-Q-TOF mass spectrum of RNase A solution 10^{-7} M incubated for 24 h at 37 °C with AF (3:1 metal to protein ratio) in 2mM ammonium acetate solution, pH 6.8.

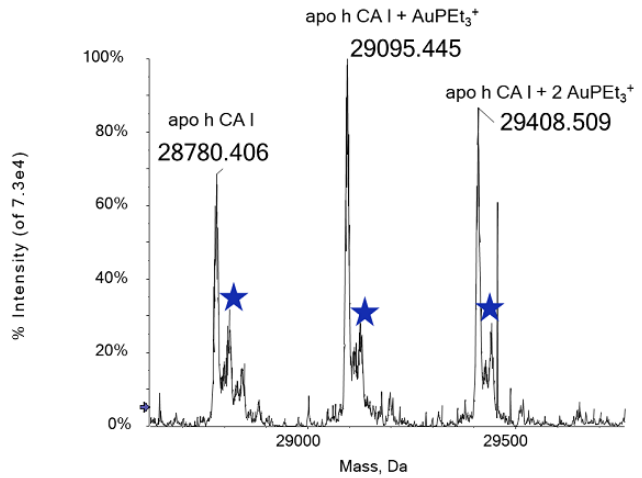

Figure 3. Deconvoluted ESI-Q-TOF mass spectrum of CA I solution 7×10^{-7} M incubated for 24 h at 37 °C with AF (3:1 metal to protein ratio) in 2 mM ammonium acetate solution, pH 6.8. The respective Zn-CA I adducts are marked with a blue star.

another signal shifted of +99 Da (8662 Da) is observed an it is referred to the protein plus the isobaric SO_4^{2-} or PO_4^{3-} ion.
This signal is characteristic for certain proteins and is due to the extraction/purification method used by the manufacturer.[48]
Similarly, three characteristic signals are detected in the case of RNase A incubated with AF (Figure 2): the first at 13681 Da is the protein signal (with four disulphide bridges); the second, shifted of +99 Da is the signal of the RNase A plus one SO_4^{2-} (or PO_4^{3-}) ion, while the third signal at 13878 Da represents the bis adduct with the same anion.
The results obtained for Ub and RNase clearly pointed out that these two proteins showed no reactivity toward AF. Since these two proteins are representative models devoid of free and solvent-accessible thiol group, this may be the true reason for lack of adduct formation.

Carbonic anhydrase I. On the other hand, the spectrum shown in Figure 3 recorded on a hCA I sample reacted with AF, is suggestive of the opposite situation where well-defined metallodrug/protein adducts are formed and are straightforwardly observed. Indeed, in this mass spectrum, beyond the peak at 28780 Da attributed to the apo-protein (with the loss of Met1 and the presence of the acetylation on Ala2), two more intense peaks are seen. The first centered at 29095 Da (+315 Da) is assigned to the adduct between apo-hCA I and one $AuPEt_3^+$ residue, and the second, at 29408 Da, is attributed to the corresponding bis adduct [apo-hCA I + 2$AuPEt_3^+$]. Moreover, smaller peaks at +65,5 Da (marked with blue stars) are also well detectable and correspond to the respective adducts of holo-hCA I.
This difference in relative abundance between the apo- and holo-protein is probably due to the experimental ionization and pH conditions that could cause the loss of Zn ions from hCA I.[49,50] Indeed, the zinc ion is held by four histidine residues (His3, His94, His96, His119), which undergo protonation at low pH values (0.1% formic acid was added before infusion in the mass spectrometer) leading to zinc release.[49-52]

Other information about the adducts stability can be inferred evaluating the deconvoluted ESI mass spectrum recorded after 72 h of incubation; indeed, no differences from the spectrum after 24 h of incubation are observed, in terms of the number and the relative intensities of the signals (see the ESI). The adducts formed in the first 24 h of incubation appear to be stable over the considered time lapse.

Haemoglobin. Similarly, the AF/haemoglobin system was prepared and analysed in the same way. Again, the resulting deconvoluted ESI mass spectrum is highly informative and suggests a significant degree of adduct formation, as reported in Figure 4. Indeed, while the spectrum of the native protein is only characterised by two intense peaks at 15125 Da, and 15898 Da that correspond respectively to the Hb α-chain and to the oxidized β-chain (each without the Met1 residue), the sample reacted with AF shows two additional peaks at 15439 and 16213 Da.

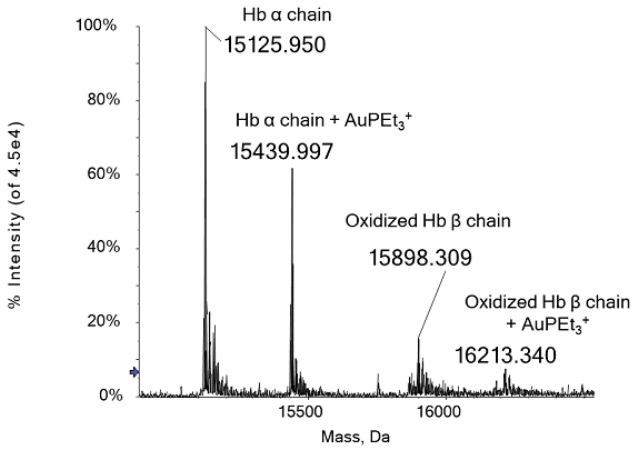

Figure 4. Deconvoluted ESI-Q-TOF mass spectrum of Hb solution 10^{-7} M incubated for 24 h at 37 °C with AF (3:1 metal to protein ratio) in 2 mM ammonium acetate solution, pH 6.8.

The signal at 15439 Da differs from the α-chain peak by +315 Da, so it was attributed to the mono adduct with a bound $AuPEt_3^+$ moiety; the signal at 16213 Da (shift = +314 Da from the oxidized β-chain peak) was attributed to the mono adduct of the β-chain bearing the same $AuPEt_3^+$ fragment.
The degree of metalation of the two chains, empirically deduced from the peak's intensities ratio, is almost the same for both α and β chain. Even in this case, the adducts formed appear to be stable; indeed, the deconvoluted ESI mass spectrum recorded after 72 h of incubation doesn't significantly differ from the one after 24 h.

Superoxide dismutase. With the aim to completely characterize the reactivity of AF towards free Cys-containing proteins, the bovine superoxide dismutase was also evaluated and the relative ESI mass spectrum is depicted in Figure 5. In this case no adducts formation is observed and the main peaks registered are only attributable to different forms of the commercial protein; in particular, the signal at 15715 Da is attributed to the [apoSOD+Zn^{2+}+Cu^{2+}-$4H^+$] specie (with the loss of Met1, the presence of a disulphide bridge and of one acetylation on Ala2) where the protons are involved in the active site of the enzyme and partly belong to the amino acid residues implicated in the metal ions coordination. In detail, these protons belong to His61 residue in common with both the metal ions, and to Asp81 that binds Zn.[53] The small peak at 15751 Da (marked with a green circle) differs from the main protein signal by +35 Da and it was tentatively attributed to the adduct between the SOD and two NH_4^+ ions from the buffer. The signal at 15814 Da is shifted by +98 Da from the main peak and it was attributed to a SOD phosphorylation.
It is noteworthy that in the case of SOD no adducts were formed although this protein possesses one free cysteine for subunit (e.g. Cys6).[54] A credible explanation for this unexpected behaviour probably relies in the spatial arrangement of these two residues inside the protein folding of each subunit.

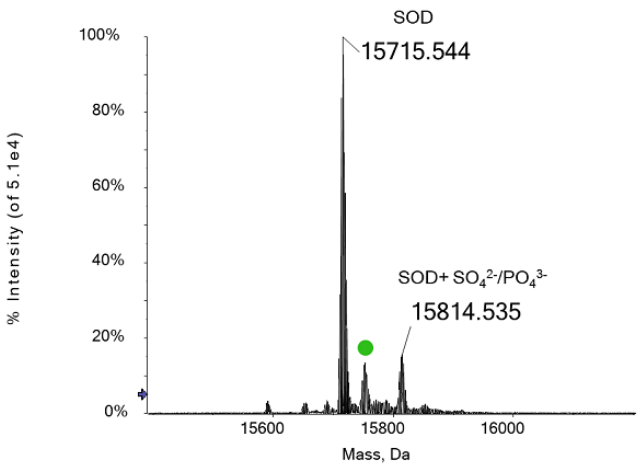

Figure 5. Deconvoluted ESI-Q-TOF mass spectrum of SOD solution 10^{-7} M incubated for 24 h at 37 °C with AF (3:1 metal to protein ratio) in 2 mM ammonium acetate solution, pH 6.8. The green circle indicates the [SOD+$2NH_4^+$] adduct (15751 Da).

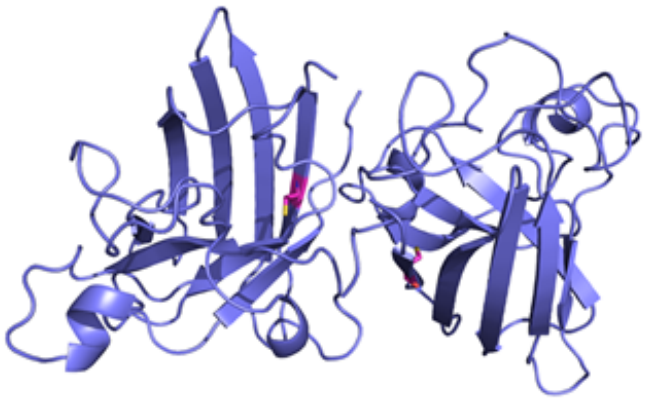

Figure 6. Ribbon representation of the overall structure of the bovine SOD. The side chain of the free cysteine is shown in ball and stick for each subunit. From Protein Data Bank, entry 1cb4.

In fact, as displayed in Figure 6, the two cysteines are poorly solvent-exposed, and not fully accessible to other molecules,[55,56] (as previously noted) penalising almost completely the reaction with AF.

Competition binding experiments using Ebselen. It is remarkable that AF is able to form stable adducts with hCA I and Hb but not with the small model proteins tested, i.e. ubiquitin and RNase A. This different behaviour may be tentatively explained by considering that both Hb and hCA I -similarly to bovine serum albumin (BSA) but at variance with RNase A and ubiquitin- possess one solvent accessible free cysteine residue. This consideration implies that other side chains like those of methionine and histidine are unable to form stable adducts with the $AuPEt_3^+$ fragment and that free cysteine residues are the specific anchoring sites for this gold(I) species.
In order to definitively prove that the free cysteine residue is the main gold binding site, we investigated whether BSA is still able to bind AF after selective chemical modification of its free thiol group by ebselen. This latter is an organoselenium compound, whose molecular weight is 274 Da, known to bind covalently to thiols to form stable seleno disulfides.[57,58] This type of molecules, capable of reacting strongly and selectively with specific side chains are named "covalent inhibitors". Reactive cysteine residues on proteins are a common target for certain covalent inhibitors, whereby the high nucleophilicity of the cysteine thiol under physiological conditions provides an ideal anchoring site for electrophilic small molecules. These inhibitors are particularly important to identify functional cysteines that lead to modulation of protein activity through covalent modification and are extremely useful for the study of the cysteine-proteome.[59,60]
To this respect, owing to its high plasma concentration and to the presence of a reactive thiol group, BSA is an excellent target model for ebselen, that binds the protein covalently at Cys34.[57,58] Notably, we found that pre-treatment of BSA with ebselen prevents the subsequent binding of the gold triethylphosphane fragment provided by AF. This was demonstrated by first reacting the protein with ebselen for 24h:

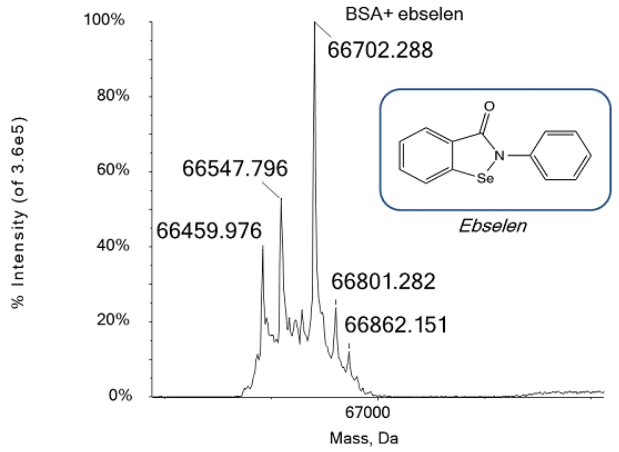

Figure 7. Deconvoluted ESI-Q-TOF mass spectrum of BSA solution $5x10^{-7}$ M incubated for 24 h at 37 °C with ebselen (1.5:1 metal to protein ratio) in 2 mM ammonium acetate solution, pH 6.8. The inset shows the molecular structure of ebselen.

the resulting ESI mass spectrum reported in Figure 7 shows a main peak at 66702 Da corresponding to the covalent adduct between ebselen and BSA, plus other signals belonging to characteristic PTMs of the native protein.[29]

Subsequently, AF was added to the same sample, in slight excess: after further 24 h of incubation the newly recorded spectrum did not show any changes (See the ESI), confirming that the ebselen-blocked Cys34 was not able to react any more with AF.

Thus, with the aim to prove this behaviour also with the studied proteins and further confirm the results obtained with BSA, the entire panel of selected proteins was reacted with ebselen in 1:1.5 protein/ebselen molar ratio and incubated up to 72 h at 37 °C.

At this point the ESI mass spectra were recorded and two distinct reactivity profiles highlighted:

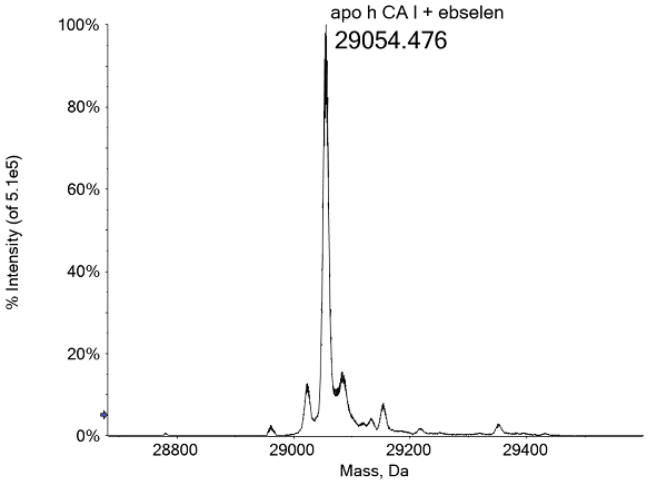

Figure 8. Deconvoluted ESI-Q-TOF mass spectrum of CAI solution 7×10^{-7} M incubated for 24 h with ebselen (1.5:1 metal to protein ratio) in 2 mM ammonium acetate solution, pH 6.8.

1) those proteins that did not react with AF were completely inert also with ebselen (i.e. ubiquitin, RNase A and SOD); 2) the proteins reactive towards AF were also reactive with ebselen, giving rise to the respective covalent monoadduct (i.e. hCA I, Hb and BSA).

All the mass spectra were recorded after 24, 48 and 72 h of incubation at 37 °C, confirming the lack of reactivity for the first group and the stability over time of the protein/ebselen adducts formed in the second group (see the ESI for the spectra).

The hCA I gives rise to the intense adduct signal at 29054 Da (Figure 8), shifted of +274 Da from the free protein signal.

Likewise, the deconvoluted ESI mass spectrum of Hb shows the alpha and the oxidized beta chain signals shifted of +274 Da, indicating the formation of the adduct with ebselen (Figure 9).

The adducts are stable up to 72 h of incubation, since the spectra recorded didn't differ from the ones acquired after 24 h of incubation (see ESI). After 72 h, 1.5 eq. of AF were added to all samples and the ESI-MS newly recorded after 2 and 24 h of additional incubation. Once again, the results were in perfect agreement with the previous observations. The first group of proteins were still unreactive confirming that the presence of a free and accessible cysteine residue is essential for the interaction with the gold compound. Notably, even the second group of proteins turned out to be unreactive toward AF, proving the unicity of the binding site for AF and ebselen (see ESI); indeed, once the competitor blocked this site, AF was no more able to replace ebselen on the thiol group.

Another interesting aspect that can be deduced from these results is related to the reactivity of SOD. In fact, as already observed in case of AF, its cysteine residue resulted not to be highly accessible, also preventing its direct reaction with the selective cysteine modifier ebselen.

Experimental section

Materials. Auranofin was purchased from Enzo Life Sciences (Famingdale, New York). Lyophilized human carbonic anhydrase (hCA I), superoxide dismutase bovine (SOD1), bovine serum albumin (BSA), human haemoglobin (Hb), ribonuclease pancreatic bovine (RNase A), bovine ubiquitin (Ub) and ebselen were purchased from Sigma-Aldrich and used without further purification or manipulation. Water, methanol and ammonium acetate were of LC-MS grade and were purchased from Sigma-Aldrich.

Sample preparation. Stock solution of AF was freshly prepared in LC-MS grade water and methanol (50:50 v/v) to a final concentration of 3×10^{-3} M. Stock solutions of the proteins were prepared in 2 mM ammonium acetate solution, pH 6.8, at 10^{-3} M.

For each AF/protein pair, appropriate aliquots of these stock solutions were mixed and diluted with 2 mM ammonium acetate solution (pH 6.8) to a final protein concentration of 10^{-4} M and a protein-to-metal molar ratio of 1:3. The solutions were incubated up to 72 h at 37 °C.

For the competition experiments with ebselen, a stock solution of this cysteine modifier was prepared in DMSO to a final

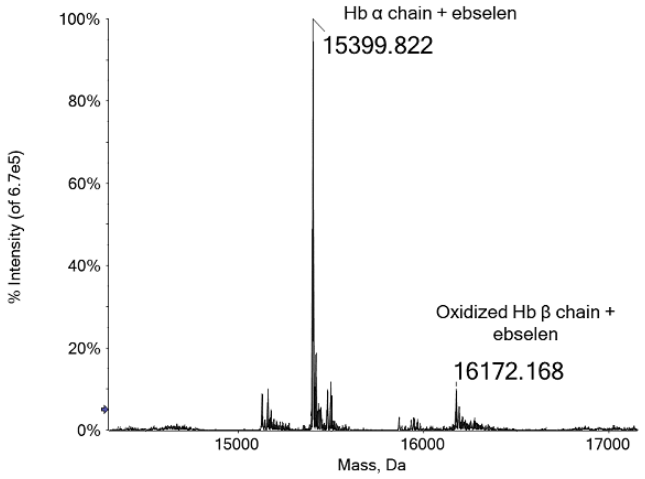

Figure 9. Deconvoluted ESI-Q-TOF mass spectrum of Hb solution 10^{-7} M incubated for 24 h with ebselen (1.5:1 metal to protein ratio) in 2 mM ammonium acetate solution, pH 6.8.

concentration of 10^{-2} M. The protein's stock solutions were the same used with AF. For each ebselen/protein pair, appropriate aliquots of these stock solutions were mixed and diluted with 2 mM ammonium acetate solution (pH 6.8) to a final protein concentration of 10^{-4} M and a protein-to-metal molar ratio of 1:1.5. The solutions were incubated up to 72 h at 37 °C.

ESI-MS analysis: final dilutions. Aliquots of the solutions of AF with each protein were sampled after 24 and 72 h of incubation and were diluted as follows: BSA solution with 2 mM ammonium acetate solution (pH 6.8) at 5×10^{-7} M protein final concentration; SOD solutions with LC-MS grade water at 10^{-7} M protein final concentration; Hb solution with 2 mM ammonium acetate solution (pH 6.8) at 10^{-7} M protein final concentration; hCA I solution with LC-MS grade water at 7×10^{-7} M protein final concentration; RNase A solution with LC-MS grade water at 10^{-7} M protein final concentration; Ub solution with LC-MS grade water at 10^{-7} M protein final concentration. In all the final solutions 0.1% v/v of LC-MS grade formic acid was added just before infusion in the mass spectrometer.
For the competition experiments with ebselen, ESI-MS spectra were recorded on the sampled solution at 24, 48, 72 h of incubation and properly diluted as already described. After 72 h, 1.5 eq. of AF were added to the same mixtures and further incubated up to 24 h. Additional ESI-MS spectra were recorded on these solutions after 2 and 24 h at 37 °C.

ESI-MS analysis: instrumental parameters. The ESI mass study was performed in accordance with a well-established protocol[29] using a TripleTOF® 5600+ high-resolution mass spectrometer (Sciex, Framingham, MA, U.S.A.), equipped with a DuoSpray® interface operating with an ESI probe. Respective ESI mass spectra were acquired through direct infusion at 7 µL/min flow rate.
The ESI source parameters were optimized for each protein and were as follows: for BSA positive polarity, Ionspray Voltage Floating 5500 V, Temperature 0, Ion source Gas 1 (GS1) 45 L/min; Ion source Gas 2 (GS2) 0; Curtain Gas (CUR) 12 L/min, Declustering Potential (DP) 150 V, Collision Energy (CE) 10 V, range 1000-2600 *m/z*; for Hb positive polarity, Ionspray Voltage Floating 5500 V, Temperature 0, Ion source Gas 1 (GS1) 45 L/min; Ion source Gas 2 (GS2) 0; Curtain Gas (CUR) 15 L/min, Declustering Potential (DP) 60 V, Collision Energy (CE) 10 V, range 570-1300 *m/z*; for SOD positive polarity, Ionspray Voltage Floating 5500 V, Temperature 0, Ion source Gas 1 (GS1) 40 L/min; Ion source Gas 2 (GS2) 0; Curtain Gas (CUR) 15 L/min, Declustering Potential (DP) 200 V, Collision Energy (CE) 10 V, range 1500-3500 *m/z*; for CA I positive polarity, Ionspray Voltage Floating 5500 V, Temperature 0, Ion source Gas 1 (GS1) 50 L/min; Ion source Gas 2 (GS2) 0; Curtain Gas (CUR) 20 L/min, Declustering Potential (DP) 50 V, Collision Energy (CE) 10 V, range 600-1400 *m/z*, for RNaseA positive polarity, Ionspray Voltage Floating 5500 V, Temperature 0, Ion source Gas 1 (GS1) 40 L/min; Ion source Gas 2 (GS2) 0; Curtain Gas (CUR) 15 L/min, Declustering Potential (DP) 100 V, Collision Energy (CE) 10 V, range 1000-3000 *m/z*; for Ub positive polarity, Ionspray Voltage Floating 5500 V, Temperature 0, Ion source Gas 1 (GS1) 35 L/min; Ion source Gas 2 (GS2) 0; Curtain Gas (CUR) 20 L/min, Declustering Potential (DP) 180 V, Collision Energy (CE) 10 V, range 900-1900 *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).

Conclusions

ESI-Q-TOF MS measurements allowed us to obtain a very detailed description of the reaction of AF with proteins and to elucidate the nature of the resulting adducts at the molecular level. Five representative proteins were utilized for these studies, namely ubiquitin, RNase A, hCA I, haemoglobin and superoxide dismutase. Adduct formation is nicely monitored in real time by ESI-Q-TOF mass spectra. We have gained strong evidence that AF binds only proteins bearing free and solvent accessible thiol groups such as hCA I and haemoglobin. This explains why no adducts are obtained with ubiquitin and RNase A. The impossibility of forming adduct with SOD is ascribed to the fact that its only free cysteine residue is poorly accessible. Binding occurs through release of the thiosugar ligand and coordination of the $AuPEt_3^+$ fragment to the deprotonated thiol group. A further confirmation of this picture comes from competition binding experiments with ebselen, a selenium compound known to be a strong and selective cysteine modifier. Protein pre-treatment with ebselen prevents in all cases AF protein binding. In the light of these findings it is very realistic that the mode of action of AF relies on the direct blockade of active site cysteines (or selenocysteines) in crucial proteins.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Notes and references

‡ Only with a very recent study by Pickering *et al*. (2020) it has been clearly demonstrated, by EXAFS measurements, that AF binds through the gold atom directly to the Se of TrxR1, suggesting that the inhibition of the enzyme by AF is due to the direct binding of the metal centre to the functional selenocysteine inside the enzyme's active site.[27]

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