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Gold-phosphine binding to *de novo* designed coiled coil peptides

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ABSTRACT

The coordination of the therapeutically interesting [AuCl(PEt_3)] to the *de novo* designed peptide, TRIL23C, under aqueous conditions, is reported here. TRIL23C represents an ideal model to investigate the binding of [AuCl(PEt_3)] to small proteins in an effort to develop novel gold(I) phosphine peptide adducts capable of mimicking biological recognition and targeting. This is due to the small size of TRIL23C (30 amino acids), yet stable secondary and tertiary fold, symmetric nature and the availability of only one thiol binding site. [AuCl(PEt_3)] was found to react readily with the Cys side chain in a 1:1 ratio as confirmed by UV-visible, ^{31}P NMR and mass spectrometry. Circular dichroism confirmed that the coiled coil structure was retained on coordination of the $\{Au(PEt_3)\}^+$ unit. Redesign of the exterior of TRIL23C based on a biologically relevant recognition sequence found in GCN4, did not alter the coordination chemistry of [AuCl(PEt_3)]. To the best of our knowledge, this represents the first report on the coordination of gold(I) phosphine compounds to *de novo* designed peptides, and could lead to the generation of novel gold(I) phosphine peptide therapeutics in the future.

1. Introduction

The search for new and improved metal based drugs is fueled primarily by the success of the first platinum-based chemotherapeutic drug, cisplatin, and the second generation analogues such as carboplatin and oxaliplatin. Both the success of platinum drugs and the severe limitations associated with their use in the clinic have driven extensive research into alternative metal complexes for drug development. Gold is a promising non-platinum metal which has been investigated for the development of new therapeutics, and for which promising results are increasingly being reported. Notably the phosphine gold(I) complex auranofin (marketed as Ridaura) is currently used in the clinic for the treatment of rheumatoid arthritis. Furthermore, promising anticancer and anti-HIV activity have been reported for auranofin and related gold(I) complexes [1,2].

Various studies suggest that the phosphine gold(I) unit in auranofin is important for antitumor activity, but that the thiol carbohydrate moiety is replaced in vivo [2]. It has been recognized that this offers an opportunity to incorporate a bioactive ligand in its place [3–7]. One attractive class of bioactive and biocompatible ligands are peptides, which offer targeting and therapeutic opportunities, e.g. nuclear localization sequences (NLS), cell penetrating peptides (CPPs) and tumour targeting sequences such as RGD.

Reports exist in the literature of short peptide gold(I) phosphine complexes, which have been synthesized through the incorporation of the gold(I) phosphine unit into a non-natural amino acid. These are generally derivatives of the thiol containing amino acid cysteine (Cys), and are subsequently used in solid phase peptide synthesis as a means of incorporating the gold(I) phosphine into the peptide sequence [5]. This approach has been successfully employed; however, these synthetic procedures can potentially be time consuming, restricted by the harsh chemical synthesis conditions required and ultimately would be limited to peptide sequences that can readily be synthesized.

Long peptides and proteins can achieve biomolecular recognition through the formation of many favorable non-covalent interactions between amino acid side chains and the target. In addition to the primary structure, it is the correct secondary structure, such as the α-helix or β-sheet, which is crucial for the correct positioning of these side chains, thereby achieving strong and selective binding to the target. Not surprisingly biological recognition is achieved on a larger length scale (nm) than traditional small molecule chemistry (Å). It is therefore our intention to investigate the coordination of gold(I) triethylphosphine based on auranofin, to protein-type sequences with well defined secondary structure elements (nm). Ultimately the goal would be to develop targeted derivatives of auranofin, with improved biodistribution and bioactivity.

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A potential challenge associated with this approach is the large size and complexity of the natural proteins we are intending to mimic in our design. Therefore our intention is to exploit the advantages offered by *de novo* peptide design (from "first principles"), which provides an opportunity to study the coordination of gold(I) phosphine to simplified protein folds in aqueous solution. Due to the high affinity of gold(I) for soft ligands such as the thiol of Cys, it should be possible to readily incorporate the gold(I) phosphine into a peptide sequence under mild aqueous conditions, as has previously been reported for natural proteins including albumin [8], zinc fingers [9–11], glutathione reductase [12] and cyclophilin [13]. In this manuscript we will focus on *de novo* designed α-helical coiled coil peptides based on TRI, Ac-G(LKALEEK)$_4$G-NH$_2$, developed by Pecoraro, DeGrado and co-workers [14,15]. Cys derivatives of these have been shown to readily coordinate soft heavy metal ions such as mercury, cadmium, bismuth, arsenic and lead [14–23]. However, to the best of our knowledge, the study reported here represents the first on the coordination of gold(I) phosphine compounds to *de novo* designed peptides with well defined secondary and tertiary structure. This approach could ultimately result in the development of a new generation of gold(I) phosphine peptide therapeutics.

2. Results and discussion

2.1. Peptide design

TRI is a 30 amino acid peptide based on the heptad repeat approach, Ac-G($\mathbf{L_a}K_\mathbf{b}A_\mathbf{c}L_\mathbf{d}E_\mathbf{e}E_\mathbf{f}K_\mathbf{g}$)G-NH$_2$, which spontaneously self-assembles in water to generate a two stranded coiled coil at low pH, and a three stranded coiled coil at higher pH (>5.5) [14]. This self-assembly is the result of a hydrophobic core of leucine residues (**a** and **d** sites) and interhelical salt bridges (between **e** and **g** sites). Gold(I) complexes have been reported to bind to both histidine (His) and Cys side chains, though with a greater affinity for the latter. As such, a thiol metal binding site was designed in the interior of the coiled coil by replacing a leucine (Leu) residue (at the 23 position) with a Cys, to generate the previously reported TRIL23C, Ac-G-LKALEEK-LKALEEK-LKALEEK-CKALEEK-G-NH$_2$ [23]. A control peptide which lacked the necessary thiol group required for gold(I) coordination, was prepared. Tryptophan (Trp) was incorporated into this peptide sequence, Ac-G-WKALEEK-LKALEEK-LKALEEK-LKALEEK-G-NH$_2$ (TRIL2W), to aid in concentration determination.[1] These sequences were synthesised, purified and characterized by routine methods [24].

2.2. UV-visible spectroscopy

Starting with the more reactive chloride derivative of auranofin, [AuCl(PEt$_3$)], its ability to bind to TRIL23C under mild conditions and in aqueous solution, was investigated by UV-visible spectroscopy. Aliquots of a stock solution of [AuCl(PEt$_3$)] were titrated into a 30 μM solution of TRIL23C monomer in 10 mM phosphate buffer pH 8. This resulted in the steady increase in the absorbance at 250 nm (Fig. 1A), which plateaus on addition of 30 μM [AuCl(PEt$_3$)] (Fig. 1B), indicating a 1:1 ratio between the gold(I) complex and the peptide monomer TRIL23C. The UV-visible spectra obtained (Fig. 1A) are not dissimilar to those previously reported for [AuCl(PEt$_3$)] binding to cysteine, penicilamine and small model peptides based on zinc finger proteins [10,25]. The total metal concentration was used to calculate an extinction coefficient of λ_{250} 2935 M^{-1} cm^{-1} for the complex [Au(PEt$_3$)(TRIL23C-*S*)]. This compares well to similar species in the literature [25].

In order to establish if binding was indeed through the intended Cys side chain, the concentration of available thiols was measured prior to, and at the end of, the titration using the Ellman's test [26,27]. This confirmed 30 μM of available thiols prior to the addition of any [AuCl(PEt$_3$)]. However, once the absorbance at 250 nm had reached its plateau, the Ellman's test indicated that no available thiols were present in solution. This observation is further supported by an analogous titration performed with a peptide sequence which does not contain a Cys residue, TRIL2W.[1] The titration of a stock solution of [AuCl(PEt$_3$)] into a 30 μM solution of TRIL2W monomer in 10 mM phosphate buffer pH 8, did not result in any increase in the absorbance at 250 nm (Fig. 1B).

These results and the ratio of 1 Au(I): 1 TRIL23C monomer are consistent with substitution of the chloride group with the deprotonated thiol of Cys, so as to generate [Au(PEt$_3$)(TRIL23C-*S*)]. In contrast, soft metal ions such as mercury, cadmium, bismuth, arsenic and lead are capable of binding in the interior of the three stranded coiled coil, (TRIL23C)$_3$, to all three equivalent Cys side chains, one from each peptide chain [14–23]. Despite reports of three and four coordinate gold(I) complexes [2], linear two coordinate gold(I) is more common and we observe the replacement of the highly labile chloride ligand by a single thiol from one peptide chain. This suggests that the triethylphosphine ligand remains bound (vide infra) and prevents the gold(I) from being accommodated in the interior of the coiled coil, as a result of steric clashes.

2.3. pH dependent binding

In order to assess the pH dependence of gold(I) complexation, the pH of an aqueous solution containing 30 μM [AuCl(PEt$_3$)] and 30 μM

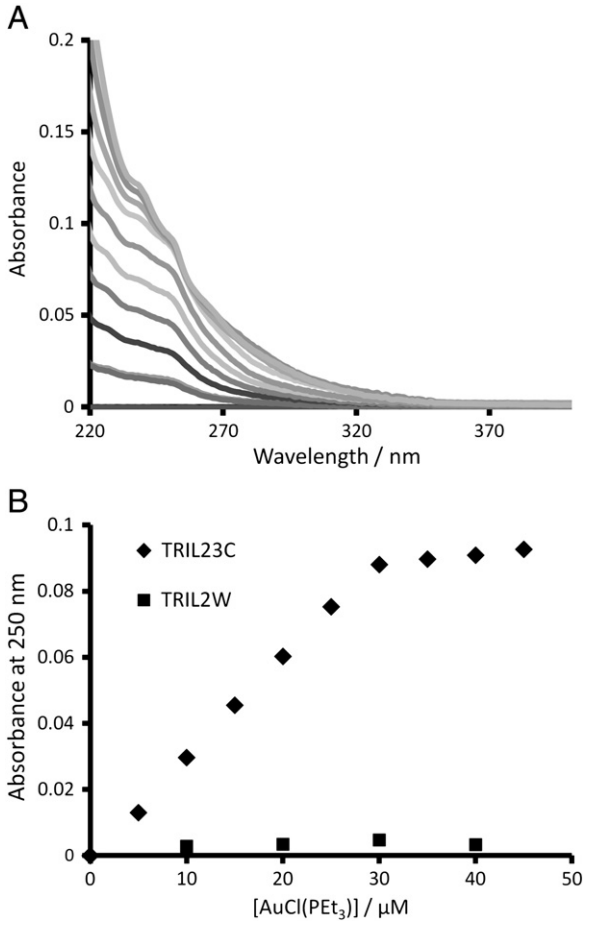

Fig. 1. (A) UV-vis spectra for a titration of [AuCl(PEt$_3$)] into a solution containing 30 μM TRIL23C in 10 mM phosphate pH 8. (B) A plot of the absorbance at 250 nm as a function of [AuCl(PEt$_3$)]/μM titrated into a 30 μM solution of TRIL23C and TRIL2W, respectively.

[1] In the absence of a Cys thiol side chain, a single Trp residue is required to determine the peptide concentration by monitoring the absorbance at 280 nm.

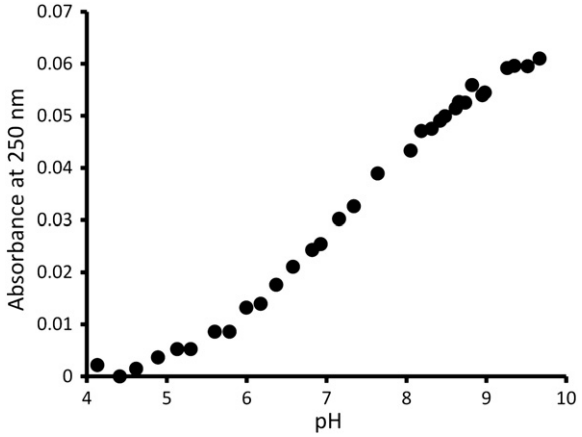

Fig. 2. UV-visible pH titration of a 30 μM solution of TRIL23C in the presence of 30 μM [AuCl(PEt_3)]. Absorbance monitored at 250 nm as a function of pH.

TRIL23C was gradually raised from ca. 4 to 9.5, and monitored by UV-visible spectroscopy. This increase in pH was accompanied by a steady increase in the absorbance at 250 nm, with a final spectrum which resembles that shown earlier in Fig. 1A. A plot of the absorbance at 250 nm, associated with coordination to the Cys, as a function of pH is shown in Fig. 2. The absorbance at 250 nm does not reach a plateau until ca. pH 9.5, suggesting complete formation of [Au(PEt_3)(TRIL23C-*S*)] at this pH. An apparent pK_a of 7.3 has been extracted from the pH profile shown in Fig. 2, for the deprotonation of the thiol side chain on gold(I) coordination. A reverse pH titration was performed by lowering the pH gradually from ca. 9.5 to 4.5. This resulted in a drop in absorbance at 250 nm, suggesting reversible binding of [AuCl(PEt_3)] to TRIL23C as a function of pH. The observation of reversible binding is further supported by the identical elution times of [Au(PEt_3)(TRIL23C-*S*)] and TRIL23C under the acidic conditions of our reversed phase HPLC experiments (data not shown).

These experiments suggest that binding of $\{Au(PEt_3)\}^+$ to the thiol Cys side chain of TRIL23C is a pH dependent process, and that [Au(PEt_3)(TRIL23C-*S*)] is only fully formed under basic conditions. This implies that at biological pH only a portion of the $\{Au(PEt_3)\}^+$ would remain bound to the peptide. However, as the $\{Au(PEt_3)\}^+$ unit is thought to be important for the antitumor activity of auranofin, its release from the targeting peptide, triggered by a pH drop, could be considered an attractive activation mechanism. Notably, cancerous cells are thought to be more acidic than healthy cells, and this feature could subsequently be responsible for triggering the release of more $\{Au(PEt_3)\}^+$ than in healthy cells.

2.4. Mass spectrometry characterisation

Electrospray ionisation (ESI)-mass spectrometry (MS) has been used to examine the reaction of the gold(I) phosphine complex [AuCl(PEt_3)] with our *de novo* designed peptide, TRIL23C. The inherent sensitivity, speed and specificity of MS-based techniques can provide significant insight into both the design and mechanism of action of metallodrugs. Here we investigate the application of a tandem MS approach, electron transfer dissociation (ETD), to allow rapid determination of the binding site for [AuCl(PEt_3)] reacted with TRIL23C.

ETD [28] is a powerful fragmentation technique, complementary to collision-induced dissociation (CID), and was proved particularly useful for determining sites of labile post-translational modifications (PTMs) of peptides and proteins which can be difficult to characterize using conventional CID methods. ETD is a radical-driven fragmentation technique and results in cleavage of the peptide N–Cα bond to give c and $z^\bullet$ type peptide product ions (cf. cleavage of the amide bonds producing b and y" type ions using CID). It has previously been demonstrated that the binding site could rapidly be obtained following the reaction of $[(\eta^6\text{-bip})Ru(en)Cl]^+$, a potential anticancer complex, to the neurotransmitter Substance-P peptide (RPKPQQFFGLM) using ETD [29].

Initially, TRIL23C (2 μM) was analyzed denatured in an aqueous solution of 50% methanol (Figure S1). Abundant multiply charged ions were detected from $[TRIL23C + 4\,H]^{4+}$ — $[TRIL23C + 8\,H]^{8+}$ over the *m/z* acquisition range shown (annotated as A4–A8, Figure S1). The inset in Figure S1 shows the Maximum Entropy deconvoluted ESI mass spectrum which provided the molecular weight of 3411.0 Da (sequence average mass of 3411.1 Da). A dimeric species, $(TRIL23C)_2$, is also detected (annotated as B6–B11, Figure S1), and can be attributed to oxidation of Cys side chains in the denatured peptide, due to the oxidizing conditions of ESI [30,31].

Following the reaction of 100 μM TRIL23C with 1000 μM [AuCl(PEt_3)] (1:10), abundant multiply charged ions were detected for the $\{Au(PEt_3)\}^+$-peptide complex from $[TRIL23C + \{Au(PEt_3)\} + 2\,H]^{3+}$ — $[TRIL23C + \{Au(PEt_3)\} + 6\,H]^{7+}$ as shown in Figure S2A. The Maximum Entropy deconvoluted ESI mass spectrum of the {TRIL23C + 10 equivalents [AuCl(PEt_3)]} spectrum is shown in Figure S2B. The spectra contain adducts, labelled A-F, which are assigned as TRIL23C, {Au}TRIL23C, $\{Au(PEt_3)\}$TRIL23C, $\{Au(Au(PEt_3))\}$TRIL23C, $\{Au(PEt_3)\}_2$-TRIL23C and $(TRIL23C)_2$ respectively. The high ratio of [AuCl(PEt_3)]:TRIL23C (10:1) used in these experiments, is responsible for the formation of species containing multiple gold(I) units. The formation of $\{Au(PEt_3)\}_2$(Cys-*S*) has previously been reported for reaction of excess [AuCl(PEt_3)] with albumin [32].

In theory the precise site of binding of $\{Au(PEt_3)\}^+$ to TRIL23C can be determined by both ETD and CID fragmentation methods. The choice of method will depend on the multiply charged precursor ion detected and thus selected to undergo fragmentation. Following CID of $[TRIL23C + \{Au(PEt_3)\} + 4\,H]^{5+}$ and $[TRIL23C + \{Au(PEt_3)\} + 6\,H]^{7+}$ no sequence-specific ions containing the gold(I) based complex were observed. The only peak containing the gold(I) complex was that of $\{Au(PEt_3)\}^+$, a direct result from the loss of bound TRIL23C (data not shown). The reverse was found following ETD-generated mass spectra of the $[TRIL23C + \{Au(PEt_3)\} + 4\,H]^{5+}$ and $[TRIL23C + \{Au(PEt_3)\} + 6\,H]^{7+}$ multiply charged precursor ions. Fig. 3 shows the ETD spectrum obtained from $[TRIL23C + \{Au(PEt_3)\} + 4\,H]^{5+}$. This spectrum shows efficient ETD from which the precise site of binding of the $\{Au(PEt_3)\}^+$ unit could be determined upon the Cys residue at position 23 of the peptide TRIL23C.

The structural information inferred from the amount of peptide sequence coverage obtained using ETD has previously been shown to be charge-state dependent [33]. It is known that in comparison to doubly charged peptide ions, triply charged ions provide greater sequence coverage upon fragmentation using ETD. The same observation has been obtained in these experiments. Limited sequence coverage was obtained for the triply and quadruply charged $\{Au(PEt_3)\}^+$-peptide adducts. These ions produced abundant charge-reduced species which did not allow the site of the gold(I) binding to be precisely determined. However, the sequence coverage obtained by fragmentation of the 5+ and 7+ $\{Au(PEt_3)\}^+$-peptide adduct allowed the site of gold(I) binding to be located upon the Cys residue at position 23, as expected. The percentage of sequence coverage was 97% and 100% for the 5+ and 7+ respectively. The 6+ ion was not selected as a precursor ion to undergo fragmentation since an interfering ion was observed on the shoulder of the isotopic distribution. These MS experiments confirm binding of $\{Au(PEt_3)\}^+$ to the thiol side chain of Cys in position 23, as originally intended.

2.5. ^{31}P NMR characterisation

The substitution of the trans chloride of [AuCl(PEt_3)] with the peptide, TRIL23C, can readily be monitored by ^{31}P NMR spectroscopy. The addition of 1/3 equivalents of [AuCl(PEt_3)] to a 4.96 mM solution of TRIL23C monomer in 10% D_2O at pH 2.3, resulted in one major resonance at 48.3 ppm and a very small resonance at 37.4 ppm (Fig. 4A).

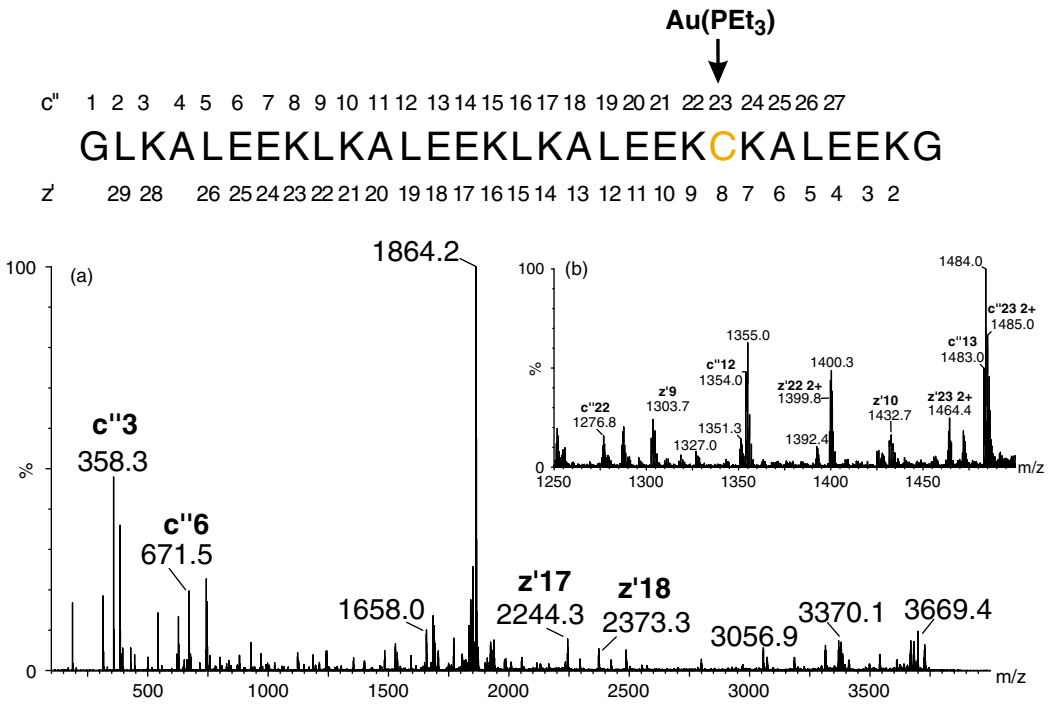

Fig. 3. ETD mass spectrum of the precursor ion [TRIL23C + {AuPEt$_3$} + 4H]$^{5+}$, detected at m/z 746, showing 97% sequence coverage and identifying the attachment of the gold(I) triethylphosphine to the cysteine residue in the 23 position.

The nature of the resonance at 48.3 ppm is not clear, but could be due to formation of $\{Au(PEt_3)_2\}^+$ [34,35]. The UV-visible pH titration (vide supra) has demonstrated that basic conditions are required for deprotonation of the thiol and binding of the $\{Au(PEt_3)\}^+$ unit to the peptide, confirmed by MS to be through the Cys side chain. The pH of the NMR solution was therefore raised to 8.1, which resulted in the disappearance of the original resonances, and the appearance of a new resonance at 39.2 ppm (accounting for 57% of the overall ^{31}P signal), with two smaller resonances at 39.9 (32%) and 40.5 ppm (11%), respectively (Fig. 4B). These three signals all fall close to the range expected for $\{Au(PEt_3)\}^+$ bound to thiols, with previous reports citing resonances ranging between 34.2 and 39.1 ppm [8,34–37,39]. Under these experimental conditions we have less $\{Au(PEt_3)\}^+$ than available thiol ligands (1:3) and as such a mixture of species will be formed. The three resonances observed are most likely due to coiled coils with differing numbers of thiol bound to $\{Au(PEt_3)\}^+$. For example, one resonance could be due to a coiled coil with all Cys side chains coordinated to $\{Au(PEt_3)\}^+$, e.g. $([Au(PEt_3)(TRIL23C\text{-}S)])_3$. The two remaining resonances are likely the result of coiled coils containing a mixture of metallated and un-metallated peptide strands, e.g. $(TRIL23C)_2([Au(PEt_3)(TRIL23C\text{-}S)])$ and $(TRIL23C)([Au(PEt_3)(TRIL23C\text{-}S)])_2$, respectively. The affinity of gold(I) for a thiol group has previously been demonstrated to be inversely proportional to its pK_{SH} [38]. Similarly a relationship was reported between the ^{31}P NMR chemical shift and the thermodynamic stability of the resulting $[Au(PEt_3)(SR)]^+$ complex [39]. Based on these correlations it would seem that the Cys side chain in TRIL23C has a high affinity for gold(I) forming stable complexes with the $\{Au(PEt_3)\}^+$ unit.

On further addition of $[AuCl(PEt_3)]$, to achieve a final ratio of 0.8 equivalents $[AuCl(PEt_3)]$ per TRIL23C peptide monomer, the resonance at 39.2 ppm grew in intensity, accounting for 83% of the overall ^{31}P signal. This is consistent with this signal being responsible for the fully metallated coiled coil, $([Au(PEt_3)(TRIL23C\text{-}S)])_3$. The addition of more $[AuCl(PEt_3)]$ reduces the number of un-metallated peptide strands and the two peaks at 39.9 and 40.5 ppm reduce in intensity and correspond to significantly less of the overall ^{31}P signal (<2 and <14%, respectively) (Fig. 4C). Except from an extremely small resonance at 66.3 ppm (<1%) most likely due to formation of PEt_3O [39], there were no additional resonances detected over the range 240 to −50 ppm.

An analogous experiment performed on addition of $[AuCl(PEt_3)]$ to a 10% D_2O solution (resulting concentration would have been 6.8 mM) in the absence of TRIL23C, resulted in precipitation of the $[AuCl(PEt_3)]$ complex and no resonances were detected in the ^{31}P NMR spectrum. The insolubility of $[AuCl(PEt_3)]$ in the absence of TRIL23C compared to

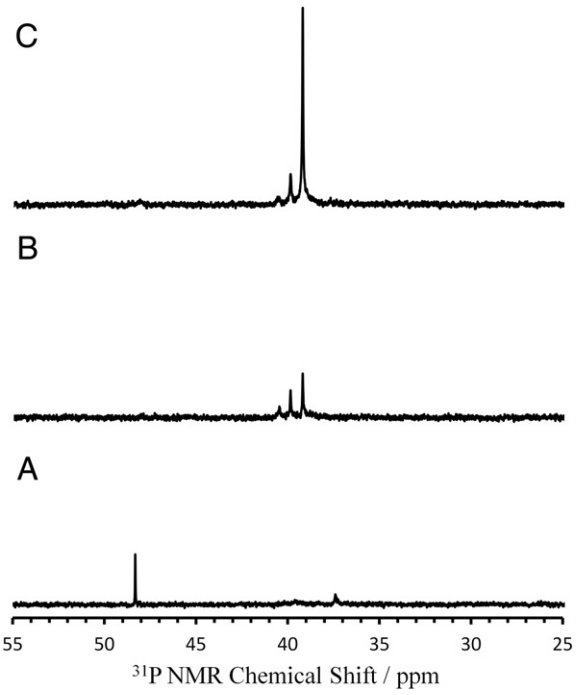

Fig. 4. $^{31}P\{^1H\}$ NMR spectra from the reaction of $[AuCl(PEt_3)]$ and TRIL23C in 10% D_2O. (A) $[AuCl(PEt_3)]$ and TRIL23C in a 1:3 ratio at pH 2.3, (B) in a 1:3 ratio at pH 8.1 and (C) in a 0.8:1 ratio at pH 8.1.

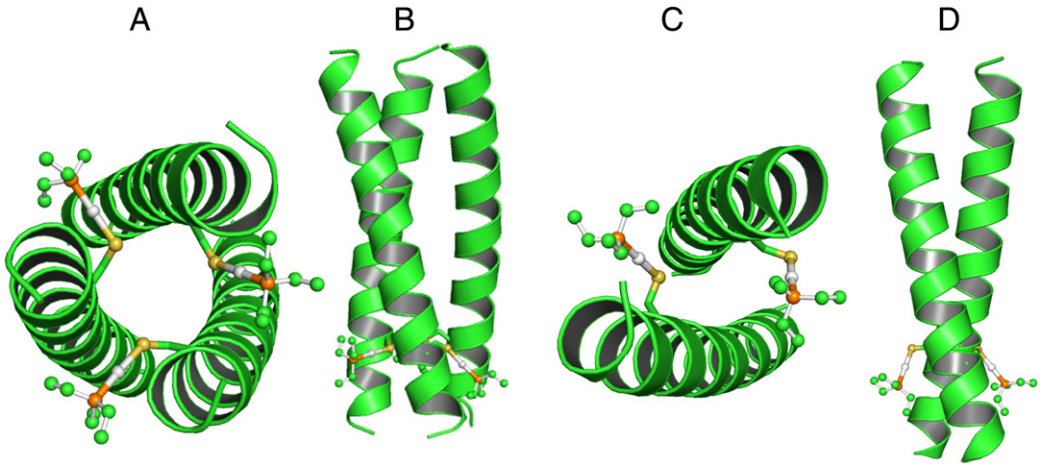

Fig. 5. Ribbon diagrams of models of $\{Au(PEt_3)\}^+$ bound to the Cys residue at the alpha-helical interface of either a three stranded coiled coil, $([Au(PEt_3)(TRIL23C\text{-}S)])_3$, or two stranded coiled coil, $([Au(PEt_3)(TRIL23C\text{-}S)])_2$. Shown are the main chain atoms represented as helical ribbons and the Cys side chains in stick form with the thiol group and the Au(I) shown as spheres (orange and grey, respectively), as a (A or C) top-down view from the C-terminus and a (B or D) side-on view of the coiled coil.

experiments performed in the presence of TRIL23C, for which no precipitation was observed, would be consistent with the coordination of $[AuCl(PEt_3)]$ to TRIL23C.

2.6. Circular dichroism

Although the UV-visible, MS and ^{31}P NMR results confirm binding of one $\{Au(PEt_3)\}^+$ unit to the Cys side chain of TRIL23C in a 1:1 ratio, they are unable to provide information concerning the folding state of the peptide. One option is that the designed coiled coil structure can tolerate the binding of $\{Au(PEt_3)\}^+$ to the Cys side chain. This would require the thiol side chain to adopt a rotamer which is directed towards the α-helical interface of the coiled coil. Potential models which satisfy this scenario are proposed, and are shown in Fig. 5. Alternatively, the data would also satisfy the scenario where binding of the $\{Au(PEt_3)\}^+$ unit is accompanied by peptide unfolding, resulting in one $\{Au(PEt_3)\}^+$ unit bound through the Cys side chain of an unstructured peptide monomer. However, our goal has been to generate gold(I) phosphine peptide adducts with well defined secondary structure elements which could ultimately be exploited for biomolecular recognition and targeting. This therefore warranted further investigation by circular dichroism (CD), so as to evaluate the secondary structure of the peptide in the absence and presence of increasing concentrations of $[AuCl(PEt_3)]$ at pH 8.

A 30 μM solution of TRIL23C in 10 mM phosphate buffer at pH 8 was monitored by CD and yielded the characteristic coiled coil spectra with negative minima at 208 and 222 nm, see Fig. 6. The 222 nm molar ellipticity (Θ_{222}) value of −34 948 deg dmol^{-1} cm^2 is consistent with a well folded coiled coil structure (>95%). Aliquots of a stock solution of $[AuCl(PEt_3)]$ were titrated into the 30 μM solution of TRIL23C, so that the final concentration of $[AuCl(PEt_3)]$ was 5, 10, 15 and 30 μM, respectively. No significant change was observed in the CD spectra, see Fig. 6, indicating that the secondary α-helical structure is maintained on addition of up to 1 equivalent of $[AuCl(PEt_3)]$ per peptide monomer.

The CD results suggest that on binding $\{Au(PEt_3)\}^+$, the peptide remains well folded, with no noticeable change to the CD spectra. This includes the ratio of $\Theta_{222}/\Theta_{208}$ (1.05), which is indicative of a coiled coil [40,41]. This scenario rules out peptide denaturation on binding $\{Au(PEt_3)\}^+$, but is consistent with $\{Au(PEt_3)\}^+$ bound to the exposed Cys side-chains at the α-helical interface of either a three- or a two-stranded coiled coil (proposed models are shown in Fig. 5), both of which are adopted by the TRI peptide family under certain conditions [14]. The steric bulk of PEt_3 will certainly not be accommodated in the hydrophobic interior of the coiled coil structure, but could be accommodated at the α-helical interface of a coiled coil (Fig. 5). An alternative aggregation state (n) of the gold(I) phosphine coiled coil, $([Au(PEt_3)(TRIL23C\text{-}S)])_n$, cannot be ruled out. Despite this, it is clear these designed peptides retain their secondary structure on binding the $\{Au(PEt_3)\}^+$ unit, and it is this designed secondary structure element which is ultimately going to be important for achieving biomolecular recognition.

2.7. Coiled coils based on natural protein recognition motifs

The complexation of $[AuCl(PEt_3)]$ to TRIL23C is a simple easy to interpret system with which one can probe the binding of the $\{Au(PEt_3)\}^+$ unit through a single Cys side chain of a peptide with defined secondary and tertiary structure in aqueous solution. However, ultimately we are interested in the coordination of $\{Au(PEt_3)\}^+$ to peptides capable of biomolecular recognition as a result of both the primary amino acid sequence and the α-helical secondary structure. We therefore investigated the binding of $[AuCl(PEt_3)]$ to a designed peptide, TRI_{GCN4}, which similar to TRIL23C folds into a three stranded coiled coil with a single Cys residue in the 23 position. However, its

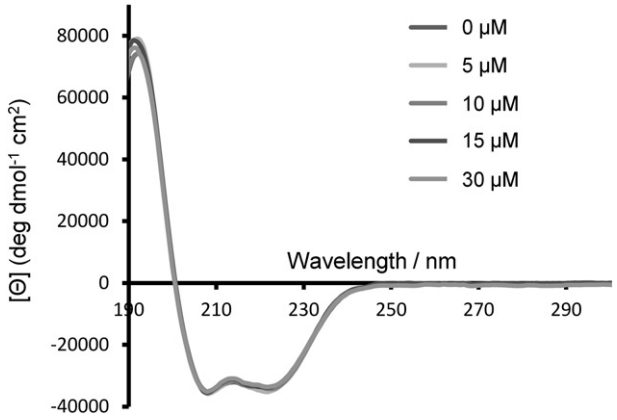

Fig. 6. CD spectra showing the peptide region for a solution of 30 μM TRIL23C in a 0.1 cm quartz cuvette in 10 mM phosphate buffer at pH 8, in the presence of increasing concentrations (5, 10, 15 and 30 μM) $[AuCl(PEt_3)]$. The intensity at 222 nm remains unchanged on increasing the concentration of $[AuCl(PEt_3)]$ and represents a well folded coil (>95%).

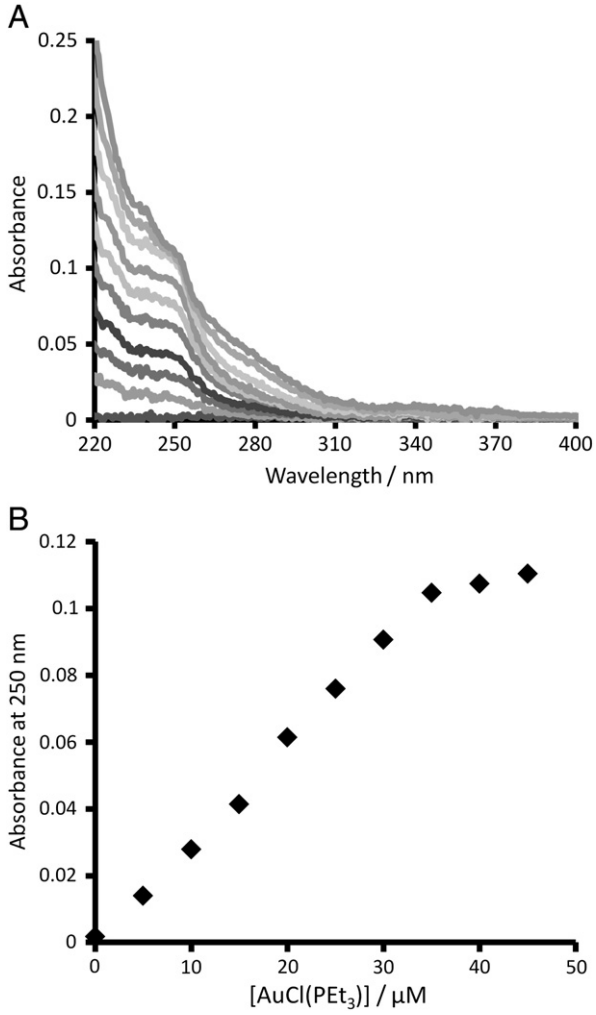

Fig. 7. (A) UV-vis spectra for a titration of [AuCl(PEt_3)] into a solution containing 30 μM TRI_{GCN4} in 10 mM phosphate pH 8. (B) A plot of the absorbance at 250 nm as a function of [AuCl(PEt_3)]/μM, indicating a ca. 1:1 binding ratio.

exterior has been modified to resemble the DNA binding basic domain of the bacterial transcription factor, GCN4.[2]

UV-visible spectroscopy was employed to investigate the reaction of the gold(I) phosphine complex, [AuCl(PEt_3)], based on auranofin, to the synthetic coiled coil, TRI_{GCN4}, based on the transcription factor GCN4. The absorbance at 250 nm was monitored for a titration of increasing concentrations of [AuCl(PEt_3)] into a solution containing 30 μM TRI_{GCN4} in 10 mM phosphate buffer pH 8. This resulted in the formation of near identical spectra to that obtained with TRIL23C, with the absorbance at 250 nm beginning to plateau at a ca. 1:1 ratio of [AuCl(PEt_3)] to TRI_{GCN4}, see Fig. 7.

ESI-MS was used to confirm $\{Au(PEt_3)\}^+$ binding to TRI_{GCN4} and to determine the location of binding. Following the reaction of 100 μM TRI_{GCN4} with 1000 μM [AuCl(PEt_3)] (1:10), abundant multiply charged ions were detected for the peptide ([Au(PEt_3)(TRI_{GCN4}-*S*)]) complex from $[TRI_{GCN4} + \{Au(PEt_3)\} + 2\,H]^{3+}$ - $[TRI_{GCN4} + \{Au(PEt_3)\} + 7\,H]^{8+}$ as shown in Figure S3A. The Maximum Entropy deconvoluted ESI mass spectrum of the {TRIL23C + 10 equivalents [AuCl(PEt_3)]} spectrum is shown in Figure S3B. The spectrum contains adducts, labeled A-C, observed at mass 3577, 3891 and 4206 Da, and are assigned as TRI_{GCN4}, $\{Au(PEt_3)\}TRI_{GCN4}$ and $\{Au(PEt_3)\}_2TRI_{GCN4}$, respectively. The precise site of binding of $\{Au(PEt_3)\}^+$ to TRI_{GCN4} was determined from ETD-generated mass spectra of the 7+ ion, $[TRI_{GCN4} + \{AuPEt_3\} + 6\,H]^{7+}$, which was selected as a precursor ion to undergo fragmentation, see Figure S4. This spectrum provides 80% sequence coverage, however, the precise site of binding of the $\{Au(PEt_3)\}^+$ unit can still be determined to be the Cys residue at position 23 of TRI_{GCN4}.

Both the UV-visible and MS results importantly verify that our approach is not limited to TRIL23C. It can be used to coordinate $\{Au(PEt_3)\}^+$ through the Cys side chain of peptides with primary amino acid sequences and secondary structures, which have been designed for targeting and biomolecular recognition.

3. Conclusions

We report our investigations into complexation of a gold(I) phosphine complex based on the drug auranofin, [AuCl(PEt_3)], with synthetic *de novo* designed peptide structures. These simplified protein folds offer advantages over natural proteins as one can more readily establish structure-function relationships through systematically correlating chemical observations with modifications to the designed sequence. The peptide TRIL23C possesses a single thiol site for gold(I) phosphine coordination, as well as a well folded α-helical coiled coil structure in aqueous solution. It is the well defined secondary and tertiary structure which can ultimately be exploited for biomolecular recognition. UV-visible, ^{31}P NMR and mass spectrometry confirm that coordination readily occurs in aqueous solution in a 1:1 ratio though the single Cys side chain. Binding is pH dependent with basic conditions required for complete coordination. This process is reversible and could potentially offer an interesting trigger or activation mechanism for delivery of the active $\{Au(PEt_3)\}^+$ unit in vivo. The coiled coil structure of our peptide is retained on coordinating the gold(I) phosphine, and possible models consisting of gold(I) bound to the Cys side chain at the α-helical interface of either a two- or a three-stranded coiled coil, have been proposed (Fig. 5). Our findings suggest that this approach can be extended to the design of peptide sequences based on biological recognition motifs of natural proteins, e.g. the bacterial transcription factor GCN4, as redesign of the exterior of TRIL23C based on this sequence, did not alter the gold(I) phosphine coordination chemistry. Ultimately these studies could lead to the development of a new generation of gold(I) phosphine peptide therapeutics.

4. Experimental

4.1. Peptide synthesis and purification

All peptide synthesis reagents were purchased from AGTC bioproducts. Peptides were synthesized on a CEM Liberty 1 automated peptide synthesizer on Rink amide MBHA resin (0.25 mmol scale), using standard Fmoc-amino acid solid-phase peptide synthesis protocols [24]. Iterative coupling and deprotections, separated by washings (30–40 mL DMF), of all Fmoc-L-amino acids were performed using a standard coupling programme (25 W, 75 °C, 300 s), except for the coupling of Fmoc-L-Cys(Trt)-OH (0 W, 50 °C, 120 s followed by 25 W, 50 °C, 240 s) to prevent racemisation, and deprotection programme (35 W, 75 °C, 180 s). The peptide N-terminus was acetylated manually (20% acetic anhydride, 20% DIPEA in DMF), and then cleaved from the resin (90% trifluoroacetic acid (TFA), 5% thioanisole, 2% anisole, 3% 1,2-ethanedithiol for 2 h at 298 K) with simultaneous removal of side-chain protecting groups, to yield the C-terminal amide. Resin was removed by filtration and crude peptides precipitated out of solution on addition of cold diethyl ether. Peptides were purified by preparative reversed phase C18 HPLC with a solvent mixture altered with a linear gradient from 0.05% TFA in water to 0.05% TFA in CH_3CN/H_2O (2:1) over 50 min. Peptides were identified by electrospray ionisation and MALDI-MS. Stock solutions of the apopeptides were prepared in

[2] Sequence Ac-G LKRLRNR LAALESR LRKLERK CKALEEK G-NH_2. Unpublished results A. F. A. Peacock, P. Holmes.

distilled water, and their concentrations determined by calculating the available thiol concentration using the Ellman's test [26] or by measuring the Trp absorbance at 280 nm in the presence of a chemical denaturatant (guanidinium.hydrochloride).

4.2. Synthesis of $HAuCl_4.4H_2O$ [42]

Gold metal (4.50 g) was added to freshly prepared aqua regia (HCl_{Conc}:HNO_{3Conc}; 3:1) (40 ml) and stirred on a hotplate (mild heating) for 15 h or until all the gold had dissolved. The aqua regia was evaporated at 40–45 °C under vacuum to yield a bright yellow residue. This residue was washed with 2×20 ml portions of HCl_{Conc}, and dried at 40–45 °C under vacuum after each wash. The yellow residue of $HAuCl_4.4H_2O$ was thoroughly dried under vacuum and stored under nitrogen. Yield: 9.14 g, 97%.

4.3. Synthesis of [AuCl(PEt_3)] [43]

Excess triethylphosphine (PEt_3) was added drop-wise to a yellow solution of $HAuCl_4.4H_2O$ (0.20 g, 0.49 mmol) in 5 ml ethanol until colourless. The solution was stirred at room temperature for 1 h. The solution was filtered over silica gel (2 cm) and the solvent removed from the filtrate to yield a white precipitate. Yield: 151 mg, 89%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ_H 1.14 (dt; 9 H; J = 18.8, 7.6; $\underline{CH_3}CH_2$), 1.79 (dq; 6 H; J = 10.0, 7.6; $CH_3\underline{CH_2}$); ^{31}P NMR ($CDCl_3$, 162 MHz): δ_P 31.4 (s).

4.4. UV-visible spectroscopy

UV-Visible spectra were recorded in 1 cm pathlength quartz cuvettes at 298 K on a Cary 5000 (TRIL23C) or Cary 50 (TRIL2W and TRI_{GCN4}) spectrometer. Aliquots of a fresh 2.5 mM stock solution of [AuCl(PEt_3)] in (1:1) water:methanol were titrated into a 30 μM solution of peptide monomer in 10 mM phosphate buffer pH 8 and the UV-Vis spectra recorded after 10 min equilibration.

4.5. pH titrations

pH titrations of aqueous solutions of TRIL23C (30 μM) in the presence of 1 equivalent of [AuCl(PEt_3)] were performed at ambient temperature on a Cary 50 UV-visible spectrometer. The pH was measured using a mini-glass combination pH electrode coupled to a Jenway 3510 pH meter, and titrated by adding small aliquots of concentrated solutions of HNO_3 and KOH, respectively. Both forward and reverse titrations from ca. pH 9.5 to 4.5 were recorded. UV-visible spectra were recorded from 200 to 500 nm and the absorbance at 250 nm plotted as a function of pH.

4.6. Circular dichroism spectroscopy

CD spectra were recorded in 1 mm pathlength quartz cuvettes at 298 K on a Jasco J-715 spectropolarimeter. The observed ellipticity in millidegrees was converted into molar ellipticity, (Θ), and is reported in units of deg $dmol^{-1}\,cm^2$. Aliquots of a 2.5 mM stock solution of [AuCl(PEt_3)] in (1:1) water:methanol, were titrated into a 30 μM TRIL23C solution in 10 mM phosphate buffer pH 8 and the CD spectra recorded after 10 min equilibration.

4.7. Mass spectrometry (MS)

Aqueous solutions of peptide (100 μM) and the gold complex [AuCl(PEt_3)] (1000 μM) were prepared and mixed to give a peptide: [AuCl(PEt_3)] molar ratio of 1:10. The samples were incubated at room temperature for 45 min. The mixture was then diluted with 50% aqueous methanol to a concentration of 10 μM prior to MS analysis. ESI-MS was performed on a hybrid quadrupole/ion mobility/oa-ToF mass spectrometer (Synapt-G2, Waters Corporation, Manchester, UK) fitted with electron transfer dissociation (ETD) functionality. The instrument has been described in detail elsewhere [44]. In brief, the instrument comprises three consecutive, gas filled, travelling wave (T-Wave) RF stacked ring ion guides prior to the ToF mass analyser. For ETD type fragmentation, a sub-ambient pressure (~2 mbar) glow discharge anion source [29] was used to fill the Trap (first) T-Wave cell with quadrupole mass selected ETD reagent anions formed from *para*-nitrotoluene (*m/z* 137). During an acquisition, the source polarity and quadrupole set mass are switched to allow multiply charged cations formed from ESI of the peptides to interact with stored reagent anions in the Trap T-Wave. This interaction allows an ion–ion type reaction resulting in ETD product ions which if desired can be separated according to their ion mobilities in the second cell (IMS T-Wave cell). (The mobility section of the instrument was switched off during these studies.) Upon exiting the IMS cell the ions enter the (third) Transfer T-Wave cell which can be used to transfer ions into the ToF or optionally provide supplemental activation (CID) prior to the ToF. For efficient ETD, within the Trap T-Wave cell, the bath gas used was helium set to a pressure of 0.05 mbar. The Transfer T-Wave cell was pressurized to 0.005 mbar with argon. The Trap T-Wave speed and amplitude which influence the ion–ion interaction time as well as the reaction rate were set to 300 m/s and 0.2 V respectively. In collision induced dissociation (CID) mode selected peptide precursor ions were induced to fragment by collisions with Argon. The collision energy was optimized on the precursor ion charge state selected. Data acquisition and processing were carried out using MassLynx (V4.1). Maximum Entropy based deconvolution software was used to establish the approximate mass from which the charge states were derived, for the species investigated. Solutions were infused into the source region of the MS at a flow rate of 5 μL/min.

4.8. NMR spectroscopy

^{1}H and ^{31}P NMR spectra for the characterisation of [AuCl(PEt_3)] in $CDCl_3$ were collected on a Bruker AVANCE (400 MHz ^{1}H and 162 MHz ^{31}P) spectrometer equipped with a 5 mm probe. All ^{31}P NMR spectra of [AuCl(PEt_3)] in the presence of TRIL23C in 10% D_2O were collected at room temperature on a Bruker DRX500 (500 MHz ^{1}H and 202 MHz ^{31}P) spectrometer equipped with a 5 mm probe. Concentrated stock solutions of [AuCl(PEt_3)] (0.5 M) were freshly prepared in DMSO-d_6 and aliquots added to the aqueous 10% D_2O solution in the presence or absence of TRIL23C, so that the final concentration of DMSO-d_6 was less than 1%. All spectra were recorded at 300 K; chemical shifts (δ) are given in parts per million (ppm) downfield of 85% phosphoric acid (H_3PO_4) at zero ppm. Data was processed using Bruker Topspin.

Abbreviations

DMF	dimethylformamide
Fmoc	9-fluorenylmethyloxycarbonyl
NLS	nuclear localisation sequences
CCPs	cell penetrating peptides
ESI	electrospray ionisation
MS	mass spectrometry
CD	circular dichroism
ETD	electron transfer dissociation
CID	collision induced dissociation
PTMs	post translational modifications
TFA	trifluoroacetic acid

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2012.05.010.

References

[1] E.R.T. Tiekink, Inflammopharmacology 16 (2008) 138–142.
[2] C.F. Shaw III, Chem. Rev. 99 (1999) 2589–2600.
[3] C.K. Mirabelli, R.K. Johnson, D.T. Hill, L.F. Faucette, G.R. Girard, G.Y. Kuo, C.M. Sung, S.T. Crooke, J. Med. Chem. 29 (1986) 218–223.
[4] I. Ott, X. Qian, Y. Xu, D.H.W. Vlecken, I.J. Marques, D. Kubuat, J. Will, W.S. Sheldrick, P. Jesse, A. Prokop, C.P. Bagowski, J. Med. Chem. 52 (2009) 763–770.
[5] C.P. Bagowski, Y. You, H. Scheffler, D.H. Vlecken, D.J. Schmitz, I. Ott, Dalton Trans. (2009) 10799–10805.
[6] J. Caddy, U. Hoffmanns, N. Metzler-Nolte, Z. Naturforsch. 62b (2007) 460–466.
[7] J. Lemke, A. Pinto, P. Niehoff, V. Vasylyeva, N. Metzler-Nolte, Dalton Trans. (2009) 7063–7070.
[8] M.T. Coffer, C.F. Shaw III, M.K. Eidsness, J.W. Watkins II, R.C. Elder, Inorg. Chem. 25 (1986) 333–339.
[9] J. Larabee, J.R. Hocker, J.S. Hanas, Chem. Res. Toxicol. 18 (2005) 1943–1954.
[10] M.A. Franzman, A.M. Barrios, Inorg. Chem. 47 (2008) 3928–3930.
[11] F. Mendes, M. Groessl, A.A. Nazarov, Y.O. Tsybin, G. Sava, Isabel Santos, P.J. Dyson, A. Casini, J. Med. Chem. 54 (2011) 2196–2206.
[12] S. Urig, K. Fritz-Wolf, R. Réau, C. Herold-Mende, K. Tóth, E. Davioud-Charvet, K. Becker, Angew. Chem. Int. Ed. 45 (2006) 1881–1886.
[13] J. Zou, P. Taylor, J. Dornan, S.P. Robinson, M.D. Walkinshaw, P.J. Sadler, Angew. Chem. Int. Ed. 39 (2000) 2931–2934.
[14] G.R. Dieckmann, D.K. McRorie, D.L. Tierney, L.M. Utschig, C.P. Singer, T.V. O'Halloran, J.E. Penner-Hahn, W.F. DeGrado, V.L. Pecoraro, J. Am. Chem. Soc. 119 (1997) 6195–6196.
[15] G.R. Dieckmann, D.K. McRorie, J.D. Lear, K.A. Sharp, W.F. DeGrado, V.L. Pecoraro, J. Mol. Biol. 280 (1998) 897–912.
[16] A.F.A. Peacock, O. Iranzo, V.L. Pecoraro, Dalton Trans. 13 (2009) 2271–2280.
[17] B.T. Farrer, C.P. McClure, J.E. Penner-Hahn, V.L. Pecoraro, Inorg. Chem. 39 (2000) 5422–5423.
[18] M. Matzapetakis, D. Ghosh, T.-C. Weng, J.E. Penner-Hahn, V.L. Pecoraro, J. Biol. Inorg. Chem. 11 (2006) 876–890.
[19] A.F.A. Peacock, M. Stachura, L. Hemmingsen, V.L. Pecoraro, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 16566–16571.
[20] V.L. Pecoraro, A.F.A. Peacock, O. Iranzo, M. Luczkowski, Bioinorganic chemistry, in: E.C. Long, M.J. Baldwin (Eds.), ACS Symposium Series 1012, Understanding the Biological Chemistry of Hg(II) Using a De Novo Design Strategy, American Chemical Society, Washington, 2009, pp. 183–197.
[21] A.F.A. Peacock, J.A. Stuckey, V.L. Pecoraro, Angew. Chem. Int. Ed. 48 (2009) 7371–7374.
[22] S. Chakraborty, D.S. Touw, A.F.A. Peacock, J.A. Stuckey, V.L. Pecoraro, J. Am. Chem. Soc. 132 (2010) 13240–13250.
[23] O. Iranzo, D. Ghosh, V.L. Pecoraro, Inorg. Chem. 45 (2006) 9959–9973.
[24] W.C. Chan, P.D. White (Eds.), Fmoc Solid Phase Peptide Synthesis: A Practical Approach, Oxford University Press, New York, 2000.
[25] D.H. Brown, G. McKinlay, W.E. Smith, J. Chem. Soc., Dalton Trans. (1977) 1874–1879.
[26] G.L. Ellman, Arch. Biochem. Biophys. 82 (1959) 70.
[27] R. Rubbiani, I. Kitanovic, H. Alborzinia, S. Can, A. Kitanovic, L.A. Onambele, M. Stefanopoulou, Y. Geldmacher, W.S. Sheldrick, G. Wolber, A. Prokop, S. Wölfl, I. Ott, J. Med. Chem. 53 (2010) 8608–8618.
[28] J.E.P. Syka, J.J. Coon, M.J. Schroeder, J. Shabanowitz, D.F. Hunt, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 9528–9533.
[29] J.P. Williams, J.M. Brown, I. Campuzano, P.J. Sadler, Chem. Commun. 46 (2010) 5458–5460.
[30] H. Deng, G.J. Van Berkel, Anal. Chem. 71 (1999) 4284–4293.
[31] G.J. Van Berkel, G.E. Giles, J.S. Bullock, L.J. Gray, Anal. Chem. 71 (1999) 5288–5296.
[32] J. Xiao, C.F. Shaw III, Inorg. Chem. 31 (1992) 3706–3710.
[33] S.J. Pitteri, P.A. Chrisman, J.M. Hogan, S.A. Mcluckey, Anal. Chem. 77 (2005) 1831–1839.
[34] A.A. Isab, A.L. Hormann, D.T. Hill, D.E. Griswold, M.J. DiMartino, C.F. Shaw III, Inorg. Chem. 28 (1989) 1321–1326.
[35] W. Ashraf, A.A. Isab, J. Coord. Chem. 57 (2004) 337–346.
[36] C.F. Shaw III, M.T. Coffer, J. Klingbeil, C.K. Mirabelli, J. Am. Chem. Soc. 110 (1988) 729–734.
[37] E.M. Kinsch, D. Stephan, Inorg. Chim. Acta 91 (1984) 263–267.
[38] A.A. Isab, P.J. Sadler, J. Chem. Soc., Dalton Trans. (1982) 135–140.
[39] M.T. Coffer, C.F. Shaw III, A.L. Hormann, C.K. Mirabelli, S.T. Crooke, J. Inorg. Biochem. 30 (1987) 177–187.
[40] N.E. Zhou, C.M. Kay, R.S. Hodges, J. Biol. Chem. 267 (1992) 2664–2670.
[41] N. Choy, V. Raussens, V. Narayanaswami, J. Mol. Biol. 334 (2003) 527–539.
[42] A. Haas, J. Helmbrecht, U. Niemann, in: G. Brauer (Ed.), Handbuch der Präparativen Anorganischen Chemie, Verlag Stuttgart, Stuttgart, 1978, p. 1014.
[43] F.G. Mann, A.F. Wells, D. Purdie, J. Chem. Soc. (1937) 1828–1836.
[44] K. Giles, J.P. Williams, I. Campuzano, Rapid Commun. Mass Spectrom. 25 (2011) 1559–1566.