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## An artificial CO-releasing metalloprotein built by histidine-selective metallation†

Inês S. Albuquerque,<sup>a</sup> Hélia F. Jeremias,<sup>b</sup> Miguel Chaves-Ferreira,<sup>a</sup> Dijana Matak-Vinkovic,<sup>c</sup> Omar Boutureira,<sup>c</sup> Carlos C. Romão<sup>b</sup> and Gonçalo J. L. Bernardes<sup>\*ac</sup>

**We report the design and synthesis of an aquacarbonyl Ru(II) dication *cis*-[Ru(CO)<sub>2</sub>(H<sub>2</sub>O)<sub>4</sub>]<sup>2+</sup> reagent for histidine (His)-selective metallation of interleukin (IL)-8 at site 33. The artificial, non-toxic interleukin (IL)-8-Ru<sup>II</sup>(CO)<sub>2</sub> metalloprotein retained IL-8-dependent neutrophil chemotactic activity and was shown to spontaneously release CO in live cells.**

The chemical re-design of native protein scaffolds as artificial metalloproteins through the attachment of organometallic moieties offers tantalising opportunities not only in (bio)catalysis<sup>1–5</sup> but also for the delivery of organometallic-based drugs.<sup>6</sup> In one example, an artificial metallohydrolase featuring two different metal ions, Zn(II) and Hg(II), which are required for catalysis and structural stability, respectively, was shown to catalyse *p*-nitrophenyl acetate (*p*NPA) hydrolysis with an efficiency comparable to that of human carbonic anhydrase (CA)-II and much higher than similar synthetic complexes.<sup>7</sup> In another example, a bifunctional artificial streptavidin metalloenzyme, displaying both an engineered carboxylate side-chain and a docked biotinylated rhodium(III) complex, enabled catalytic asymmetric C–H activation.<sup>8</sup> In addition to catalysis, artificial metalloproteins have also shown promise for the *in vivo* delivery of organometallic-based drugs. For example, Pt(IV) prodrugs have been designed to specifically bind in a non-covalent manner to human serum albumin (HSA) as a delivery vehicle, enhancing significantly its stability in whole human blood.<sup>9</sup>

Anchoring of a non-native organometallic moiety into a protein template is a challenging task and it is usually achieved using dative, covalent or supramolecular strategies.<sup>4,5</sup> Among these, covalent modification of cysteine (Cys) residues with complexes bearing

reactive electrophilic handles (namely prosthetics) has received considerable attention.<sup>1–5</sup> Cys bioconjugation with  $\alpha$ -haloacetamide functionalised complexes has been used to create, for example, an artificial Diels–Alderase<sup>10</sup> and an artificial metalloenzyme for olefin metathesis.<sup>11</sup> While the covalent conjugation of metal complexes equipped with reactive handles to nucleophilic side chains on proteins has been already demonstrated, the direct metallation of a native or non-native pre-determined site on a protein scaffold by creating a stable side chain–metal bond is less common. In this context, the non-selective metallation of histidine (His), a privileged metallophilic residue, has been exploited in coordination-based protein modification strategies<sup>12</sup> using the nickel(Ni)(II)–His-tag pair<sup>13</sup> as well as in other metallation reactions with cobalt (Co)<sup>14</sup> and ruthenium (Ru) complexes.<sup>15</sup> Unlike these remarkable examples, His-selective metallation of proteins leading to stable, fully functional materials is to the best of our knowledge unprecedented. Alternatively, one could consider employing genetic encoding methods to introduce an unnatural amino acid with a designer side chain for metal binding. This approach has been recently utilized by Roelfes and co-workers to create an artificial metalloprotein for catalytic asymmetric Friedel–Crafts alkylation reactions.<sup>16</sup>

We have previously described the interactions of the Ru carbonyl complex [*fac*-RuCl( $\kappa^2$ -H<sub>2</sub>NCH<sub>2</sub>CO<sub>2</sub>)(CO)<sub>3</sub>] (CORM-3; Fig. 1A) with proteins.<sup>17–19</sup> X-ray crystallography showed the formation of a di-carbonyl Ru protein complex by modification of histidine (His)-15 in hen egg white lysozyme (HEWL) with the fragment *cis*-[Ru(CO)<sub>2</sub>]<sup>2+</sup>.<sup>17</sup> This fragment is likely to arise from the hydrolytic decomposition of CORM-3 in aqueous solution through a water-gas shift reaction (Fig. S1, ESI†).<sup>19,20</sup> In this work, we hypothesised whether the reactivity of the *cis*-[Ru(CO)<sub>2</sub>]<sup>2+</sup> fragment would allow for direct selective His metallation of proteins and thus provide a robust strategy for the construction of synthetic Ru dicarbonyl metalloproteins. We envisioned that the *cis*-[Ru(CO)<sub>2</sub>]<sup>2+</sup> could be generated directly from an aquacarbonyl Ru(II) dication *cis*-[Ru(CO)<sub>2</sub>(H<sub>2</sub>O)<sub>4</sub>]<sup>2+</sup> (Fig. 1A). The expected chemically defined, artificial Ru dicarbonyl metalloproteins are interesting targets as these have been demonstrated to carry and deliver therapeutic active CO carriers *in vivo*.<sup>21</sup>

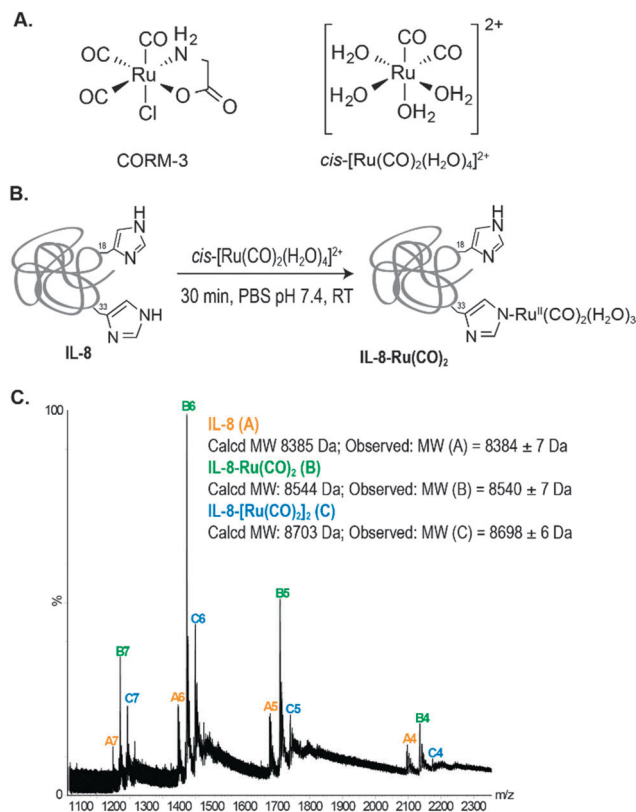
<sup>a</sup> Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal.  
E-mail: gbernardes@medicina.ulisboa.pt

<sup>b</sup> Instituto de Tecnologia Química e Biológica-António Xavier, Universidade Nova de Lisboa, Av da República, 2780-157 Oeiras, Portugal

<sup>c</sup> Department of Chemistry, University of Cambridge, Lensfield Road, CB2 1EW Cambridge, UK. E-mail: gb453@cam.ac.uk; Web: http://gbernardes-lab.com

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**Fig. 1** His-selective metallation of IL-8 with the  $[Ru(CO)_2]^{2+}$  fragment. (A) Structure of CORM-3 and the Ru complex used in this work,  $cis-[Ru(CO)_2(H_2O)_4]^{2+}$ . (B) Metallation of more reactive histidine 33 in IL-8 with the  $cis-[Ru(CO)_2]^{2+}$  fragment. (C) Denatured nanoESI MS of the purified product resulting from the reaction of IL-8 and  $cis-[Ru(CO)_2(H_2O)_4]^{2+}$ . The sample contains mainly IL-8 in complex with one  $cis-[Ru(CO)_2]^{2+}$  fragment (component B) (>80%). In addition, IL-8 only (component A; see also Fig. S2 in the ESI† for denatured nanoESI MS of IL-8) and IL-8 in complex with two  $cis-[Ru(CO)_2]^{2+}$  fragments (component C) could also be detected.

As a protein scaffold we chose the CXC chemokine interleukin-8 (IL-8), which is associated with the promotion of neutrophil chemotaxis and degranulation upon inflammatory stimuli. IL-8 activates multiple intracellular signalling pathways downstream of two G protein-coupled receptors (CXCR1 and CXCR2), and over-expression of IL-8 and/or its receptors has been described in cancer and endothelial cells, infiltrating neutrophils and tumour-associated macrophages, demonstrating the key roles of IL-8 as a regulatory factor within the tumour microenvironment.<sup>22</sup> Structurally, IL-8 features two His residues, His18 and His 33, that in solution display lower  $pK_a$  values (3.7 and 4.9, respectively) than His usually does ( $pK_a \sim 6.0$ ).<sup>23–25</sup> The unique reactivity of each of the His residues present in IL-8 makes it an ideal scaffold to achieve His-selective metallation with the  $cis-[Ru(CO)_2]^{2+}$  fragment.

We began by synthesising the aquacarbonyl Ru(II) dication  $cis-[Ru(CO)_2(H_2O)_4]^{2+}$  complex (Fig. 1). Although our data strongly indicate that the  $cis-[Ru(CO)_2]^{2+}$  fragment is generated *in situ* as the result of the hydrolytic decomposition of CORM-3, we decided to synthesise the complex aquacarbonyl Ru(II) dication  $cis-[Ru(CO)_2(H_2O)_4]^{2+}$  (Fig. 1), which would be a more direct source of the fragment  $cis-[Ru(CO)_2]^{2+}$ . The synthesis and

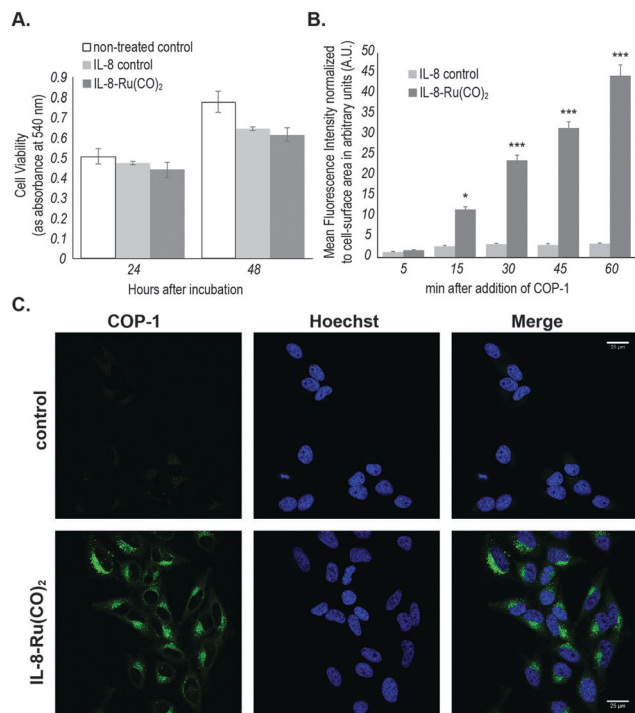
characterization of the tosylate (OTs) salt of this complex has been described by Merbach and co-workers.<sup>26</sup> The reported preparation is, however, lengthy and far from straightforward, so we decided to prepare the  $cis-[Ru(CO)_2]^{2+}$  containing species in aqueous medium, through the reaction of the readily accessible  $[Ru(CO)_2Cl_2]_n$  and Ag(OTs).<sup>27</sup> After removing solid AgCl, the FTIR spectrum (ATR) of the solution showed two CO stretching bands at 2081 and 2009  $cm^{-1}$ . The <sup>13</sup>C NMR spectrum of the residue obtained after evaporation displays a single resonance at  $\delta$  192 ppm (Fig. S3, ESI†). Spectroscopic data are in accordance with what was previously published,<sup>26</sup> and indicate the presence of a single  $cis-[Ru(CO)_2]^{2+}$  species.

With the aquacarbonyl Ru(II) dication  $cis-[Ru(CO)_2(H_2O)_4]^{2+}$  in hand we started the metallation reaction by reacting 50 equivalents of this complex with IL-8 for 30 minutes at room temperature in phosphate buffer solution (PBS) pH 7.4 (Fig. 2B).<sup>‡</sup> The same reaction conditions were also tested using CORM-3 instead of the Ru aquacarbonyl complex. After purification by size exclusion chromatography to remove excess of reagent, denatured nanoESI MS† shows the presence of a major species (>80% conversion), IL-8 in complex with one  $cis-[Ru(CO)_2]^{2+}$  fragment (component B; Fig. 1C). In addition, minor species corresponding to IL-8 only (component A; see also Fig. S4, ESI† for denatured nanoESI MS of IL-8) and IL-8 in complex with two  $cis-[Ru(CO)_2]^{2+}$  fragments (component C) could also be detected. The use of additional equivalents of both reagents and increasing the temperature and reaction time resulted in additional labelling and/or protein loss. This result indicates potential selective labelling of a single His residue but also shows that, in solution, CORM-3 rapidly decomposes to form a highly reactive  $cis-[Ru(CO)_2]^{2+}$  fragment that is captured by exposed, reactive His residues on proteins.

Next, to determine the labelling site, we subjected the IL-8-Ru<sup>II</sup>(CO)<sub>2</sub> conjugate to proteolysis and analysed the digested fragments by nano LC-MS/MS. 19 unique peptides were identified covering 70% of the sequence. The peptide containing amino acids 27–42 [VIESGPHCANTEIIVK] has a  $cis-[Ru(CO)_2]^{2+}$  modification at His33, which was further corroborated in the MS/MS spectrum (Fig. S5, ESI†). No modification could be found at His18, demonstrating that His33 is the primary site of metallation. Interestingly, this result also shows that the His–Ru bond is stable under processing/analysis conditions similar to other His–Ru complexes.<sup>15</sup> The stability of the present site- and residue (His)-selective strategy opens up the development of orthogonal double His modification protocols for the installation of dual labels that will ensure probe homogeneity unlike other nonselective metallation protocols.

Having shown that it is possible to achieve selective metallation of His33 in IL-8, we next studied whether the new artificial chemically defined metalloproteins could spontaneously release one CO ligand from the introduced  $cis-[Ru(CO)_2]^{2+}$  motif. Our group is interested in innovative methods for the *in vivo* delivery of therapeutic CO<sup>6,28</sup> and has recently demonstrated that Ru<sup>II</sup>(CO)<sub>2</sub>-albumin complexes are able to carry and release CO in aqueous solution, live cells and mice.<sup>21</sup> The viability of HeLa cells in the presence of the artificial IL-8-Ru<sup>II</sup>(CO)<sub>2</sub> metalloprotein (Fig. 2A) was



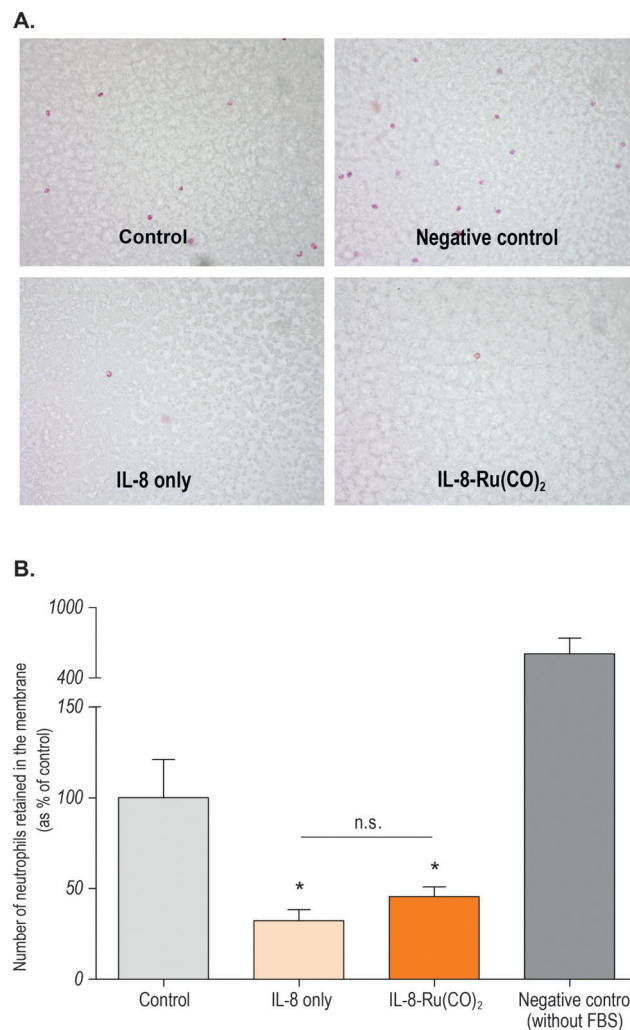


**Fig. 2** CO release in buffered aqueous solution and live HeLa cells from metallated IL-8. (A) Effects of 24 and 48 h of incubation with IL-8-Ru<sup>II</sup>(CO)<sub>2</sub> on the viability of HeLa cells. Cells were incubated with MTT reagent diluted in culture medium for 4 h, medium was removed and the pellet was re-suspended in DMSO. After 20 min, the absorbance was measured at 540 nm (measuring in parallel at 690 nm, to remove interference from phenol red, present in the medium). The results are shown as percentage of control. (B) CO release measurements using COP-1, read from 497 to 558 nm, followed by excitation ( $\lambda_{\text{ex}} = 488 \text{ nm}$ ). Photos were taken at 0, 5, 15, 30, 45 and 60 min after the addition of 1  $\mu\text{M}$  COP-1 in PBS pH 7.4 at 37 °C. Cells were previously incubated with either IL-8 at 25 ng mL<sup>-1</sup> or IL-8-Ru(CO)<sub>2</sub>, at 150  $\mu\text{M}$  before the addition of COP-1. The results are shown as mean fluorescence intensity (arbitrary units) normalized to the cell-surface area. (C) Confocal microscopy images for cellular CO release in HeLa cells treated with IL-8 (control; top panel) or IL-8-Ru(CO)<sub>2</sub> (bottom panel), at 30 min after COP-1 addition. Cells were stained with Hoechst 33342 (blue) and CO-release was measured by COP-1 turn-on reaction (green). Pictures on the right represent a merge of green and blue channels. The scale bar is 25  $\mu\text{m}$ .

verified by MTT assay and confocal microscopy. Furthermore, the selective CO fluorescent turn-on probe (COP-1)<sup>29</sup> was used to check for CO release from IL-8-Ru<sup>II</sup>(CO)<sub>2</sub> in aqueous buffered (PBS) solution at physiological pH 7.4 (PBS pH 7.4). The results reflect a robust fluorescence response of COP-1 in the presence of an IL-8-Ru<sup>II</sup>(CO)<sub>2</sub> solution that increased in a time-dependent manner while COP-1 alone showed very weak fluorescence (Fig. 2B). Using this assay, we could visualize cytosolic CO release (associated with COP-1 staining), which seems to be intensely associated with the perinuclear region (Fig. 2C), in accordance to what we previously reported.<sup>21</sup> Interpreted as a whole, these data further corroborate the benefits associated with the use of protein scaffolds for carrying and delivering CO.

IL-8 is a chemokine produced and secreted by macrophages and other cell-types including epithelial cells. In addition to its role as a promoter of angiogenesis in humans,<sup>22</sup> IL-8 acts

mainly as a chemotactic factor, by inducing migration of neutrophils and other granulocytes towards infection sites. To assess if IL-8 retained its chemotactic activity when conjugated to the *cis*-[Ru(CO)<sub>2</sub>]<sup>2+</sup> motif, a neutrophil migration assay was performed, using human neutrophils isolated from freshly collected blood. Neutrophil migration induced by the artificial metalloprotein IL-8-Ru<sup>II</sup>(CO)<sub>2</sub> was compared against that which



**Fig. 3** *In vitro* neutrophil chemotactic activity of metallated IL-8. (A) Representative photos of Transwell membranes of each condition analysed in the neutrophil migration assays. The top panel includes culture-medium controls ("control" and "negative control", *i.e.* complete culture medium and medium without foetal bovine serum (FBS), respectively), and the bottom panel includes medium supplemented with IL-8 or medium supplemented with IL-8-Ru(CO)<sub>2</sub>. IL-8 was used at physiological concentration (25 ng mL<sup>-1</sup>), and incubated for 3 h. (B) Normalized number of neutrophils that were retained in the Transwell membrane, which did not migrate during the migration assay. Freshly isolated human neutrophils were incubated with either complete medium ("control"), medium without FBS ("negative control"), complete medium with IL-8 ("IL-8 only") or complete medium with IL-8-Ru(CO)<sub>2</sub> ("IL-8-Ru(CO)<sub>2</sub>"), and incubated for 3 h. Bars represent mean  $\pm$  S.E.M. of technical replicates, obtained from two independent donors. Counts were obtained for five fields/insert. Mann-Whitney's U test, two-tailed; \* stands for  $p$ -value < 0.01; n.s. stands for "non-significant".



native IL-8 can induce. In the migration assay, IL-8-Ru<sup>II</sup>(CO)<sub>2</sub> was able to decrease neutrophil retention in the Transwell membrane at similar levels to those induced by IL-8 alone, *i.e.* both unmodified and modified IL-8 increase neutrophil migration (Fig. 3). Previous modification of IL-8 by introduction of the natural posttranslational modification citrullination had led to a considerable reduction in chemotactic activity.<sup>30</sup> Importantly, our data simultaneously demonstrate that both conjugation of IL-8 to the Ru dicarbonyl motif and the CO released from IL-8-Ru(CO)<sub>2</sub> does not disturb IL-8 neutrophil chemotactic activity.

In summary, we have developed a chemical His-selective metallation method that enables the introduction of a Ru dicarbonyl motif at His33 in IL-8. The *cis*-[Ru(CO)<sub>2</sub>]<sup>2+</sup> fragment could be generated directly from the aquacarbonyl Ru(II) dication *cis*-[Ru(CO)<sub>2</sub>(H<sub>2</sub>O)<sub>4</sub>]<sup>2+</sup>. In addition, we were able to show that CO can spontaneously be released from the new artificial metalloprotein, both in aqueous solution and live cells. Importantly, the use of IL-8 as a scaffold for the engineering of a new artificial metalloprotein did not alter the protein's activity leading to a novel, fully functional material. In the case of IL-8 neither the introduction of the Ru moiety nor the CO released impacted IL-8's neutrophil chemotactic activity. Overall we expect that the exquisite His discrimination presented herein will establish the basis to precisely understand the roles of different metallation sites in CO delivery using proteins and hopefully pave the way towards the development of artificial metalloproteins for the safe and controlled delivery of therapeutic CO.

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## Notes and references

‡ General procedure for chemical His metallation of IL-8 with the *cis*-[Ru(CO)<sub>2</sub>]<sup>2+</sup> fragment: typically, a solution of IL-8 was prepared as 1 mg mL<sup>-1</sup> solution in PBS pH 7.4. *cis*-[Ru(CO)<sub>2</sub>(H<sub>2</sub>O)<sub>4</sub>](OTs)<sub>2</sub> or CORM-3 (50 equivalents) was added as a solid to the protein solution (1 mL, *c* = 1.0 mg mL<sup>-1</sup>) in PBS pH 7.4 in a plastic tube and the mixture was vortexed to homogenize. The reaction was maintained for 30 min at room temperature. Purification of the metallated protein was achieved by size exclusion chromatography using a HiTrap desalting column (GE Healthcare) to remove excess reagents. Purified samples were used for mass spectrometry analysis under the conditions described in the ESI.†

1 F. Yu, V. M. Cangelosi, M. L. Zastrow, M. Tegoni, J. S. Plegaria, A. G. Tebo, C. S. Mocny, L. Ruckthong, H. Qayyum and V. L. Pecoraro, *Chem. Rev.*, 2014, **114**, 3495–3578.

2 T. R. Ward, *Acc. Chem. Res.*, 2010, **44**, 47–57.

- 3 J. Bos and G. Roelfes, *Curr. Opin. Chem. Biol.*, 2014, **19**, 135–143.
- 4 M. Dürrenberger and T. R. Ward, *Curr. Opin. Chem. Biol.*, 2014, **19**, 99–106.
- 5 I. D. Petrik, J. Liu and Y. Lu, *Curr. Opin. Chem. Biol.*, 2014, **19**, 67–75.
- 6 S. García-Gallego and G. J. L. Bernardes, *Angew. Chem., Int. Ed.*, 2014, **53**, 9712–9721.
- 7 M. L. Zastrow, F. A. PeacockAnna, J. A. Stuckey and V. L. Pecoraro, *Nat. Chem.*, 2012, **4**, 118–123.
- 8 T. K. Hyster, L. Knörr, T. R. Ward and T. Rovis, *Science*, 2012, **338**, 500–503.
- 9 Y.-R. Zheng, K. Suntharalingam, T. C. Johnstone, H. Yoo, W. Lin, Y. Byun, S. Sadegh-Nasseri, M. G. Pomper, K. C. Lee and S. Lee, *J. Am. Chem. Soc.*, 2014, **136**, 8790–8798.
- 10 J. Bos, F. Fusetti, A. J. M. Driessen and G. Roelfes, *Angew. Chem., Int. Ed.*, 2012, **51**, 7472–7475.
- 11 C. Mayer, D. G. Gillingham, T. R. Ward and D. Hilvert, *Chem. Commun.*, 2011, **47**, 12068–12070.
- 12 S. Uchinomiya, A. Ojida and I. Hamachi, *Inorg. Chem.*, 2013, **53**, 1816–1823.
- 13 T. H. Kim, M. Swierczewska, Y. Oh, A. Kim, D. G. Jo, J. H. Park, Y. Byun, S. Sadegh-Nasseri, M. G. Pomper, K. C. Lee and S. Lee, *Angew. Chem., Int. Ed.*, 2013, **52**, 6880–6884.
- 14 M. C. Heffern, P. T. Velasco, L. M. Matosziuk, J. L. Coomes, C. Karras, M. A. Ratner, W. L. Klein, A. L. Eckermann and T. J. Meade, *ChemBioChem*, 2014, **15**, 1584–1589.
- 15 D. Valensin, P. Anzini, E. Gaggelli, N. Gaggelli, G. Tamasi, R. Cini, G. Gabbiani, E. Michelucci, L. Messori, H. Kozłowski and G. Valensin, *Inorg. Chem.*, 2010, **49**, 4720–4722.
- 16 I. Drienovska, A. Rioz-Martinez, A. Draksharapu and G. Roelfes, *Chem. Sci.*, 2015, **6**, 770–776.
- 17 T. Santos-Silva, A. Mukhopadhyay, J. D. Seixas, G. J. L. Bernardes, C. C. Romão and M. J. Romão, *J. Am. Chem. Soc.*, 2011, **133**, 1192–1195.
- 18 T. Santos-Silva, A. Mukhopadhyay, J. D. Seixas, G. J. L. Bernardes, C. C. Romão and M. J. Romão, *Curr. Med. Chem.*, 2011, **18**, 3361–3366.
- 19 J. D. Seixas, M. F. A. Santos, A. Mukhopadhyay, A. C. Coelho, P. C. M. Reis, L. S. F. Veiros, A. R. Marques, N. Penacho, A. M. L. Gonçalves, M. J. Romão, G. A. J. L. Bernardes, T. Santos-Silva and C. C. Romão, *Dalton Trans.*, 2015, DOI: 10.1039/C4DT02966F.
- 20 W. Hieber and F. Leutert, *Z. Anorg. Allg. Chem.*, 1932, **204**, 145–164.
- 21 M. Chaves-Ferreira, I. S. Albuquerque, D. Matak-Vinkovic, A. C. Coelho, S. M. Carvalho, L. M. Saraiva, C. C. Romão and G. J. L. Bernardes, *Angew. Chem., Int. Ed.*, 2015, **54**, 1172–1175.
- 22 D. J. J. Waugh and C. Wilson, *Clin. Cancer Res.*, 2008, **14**, 6735–6741.
- 23 E. T. Baldwin, I. T. Weber, R. St Charles, J. C. Xuan, E. Appella, M. Yamada, K. Matsushima, B. F. Edwards, G. M. Clore and A. M. Gronenborn, *Proc. Natl. Acad. Sci. U. S. A.*, 1991, **88**, 502–506.
- 24 G. M. Clore, E. Appella, M. Yamada, K. Matsushima and A. M. Gronenborn, *J. Biol. Chem.*, 1989, **264**, 18907–18911.
- 25 G. M. Clore, E. Appella, M. Yamada, K. Matsushima and A. M. Gronenborn, *Biochemistry*, 1990, **29**, 1689–1696.
- 26 U. C. Meier, R. Scopelliti, E. Solari and A. E. Merbach, *Inorg. Chem.*, 2000, **39**, 3816–3822.
- 27 P. A. Anderson, G. B. Deacon, K. H. Haarmann, F. R. Keene, T. J. Meyer, D. A. Reitsma, B. W. Skelton, G. F. Strouse and N. C. Thomas, *Inorg. Chem.*, 1995, **34**, 6145–6157.
- 28 C. C. Romão, W. A. Blättler, J. D. Seixas and G. J. L. Bernardes, *Chem. Soc. Rev.*, 2012, **41**, 3571–3583.
- 29 B. W. Michel, A. R. Lippert and C. J. Chang, *J. Am. Chem. Soc.*, 2012, **134**, 15668–15671.
- 30 P. Proost, T. Loos, A. Mortier, E. Schutyser, M. Gouwy, S. Noppen, C. Dillen, I. Ronsse, R. Conings, S. Struyf, G. Opdenakker, P. C. Maudgal and J. Van Damme, *J. Exp. Med.*, 2008, **205**, 2085–2097.

