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Folates are potential ligands for ruthenium compounds *in vivo*

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Under physiologically relevant conditions, *cis*-bis(2,2'-bipyridine)dichlororuthenium(II), [*cis*-Ru(2,2'-bipy)$_2$Cl$_2$] was observed to bind to folic acid *via* replacement of the two chloride ligands. This binding was shown to be pH dependent and afforded diastereomers, the structures of which were determined by 1- and 2D NMR spectroscopic techniques. We propose that when studying the cytotoxicity of labile ruthenium complexes in cells, folate coordination should be considered.

The orthogonal chemistry of the complexes of heavier transition-metals has received much attention with respect to their potential to yield novel drug candidates.[1,2] Such complexes offer a possible spectrum of activity which extends beyond organic small molecules, simply because of the propensity for metals to form strong coordinate bonds to Lewis bases.[3] Recent advances in the field have seen ruthenium-based compounds emerging as some of the most promising drug candidates.[4–6] Two such compounds, KP1019 and NAMI-A, have now entered clinical trials, passing phase 1 stages.[7–9] However, their precise mode of action and their preferred *in vivo* target or targets have yet to be unequivocally established and this is a barrier to their further development.[10–12] The potential for DNA binding of such compounds has been shown *in vitro*,[13–15] however, there is also evidence for binding to proteins as well as DNA.[16] Indeed, the cytotoxicity of ruthenium complexes may be as a result of binding to multiple targets,[12] including small molecules, such as metabolites and cofactors, which are yet to have been considered. Without a fuller understanding of the interaction of ruthenium compounds with all biomolecules, large and small, a strategic approach to improving metal-based drugs will remain challenging. In this context folates are relevant biomolecules; whilst they are not in high concentration they are ubiquitous cofactors *in vivo* and central to metabolite biosynthesis. Hence, folates are likely to be encountered by any metal complex administered. With several Lewis basic functional groups available, folates offer a range of potential binding motifs to a metal.

We have investigated the products formed between [*cis*-Ru(2,2'-bipy)$_2$Cl$_2$] and folic acid *in vitro*. Whilst ruthenium compounds of clinical interest have one or more labile, monodentate ligands, we expect the interaction of folates with these complexes to yield numerous products such that detailed structural characterisation would be precluded. With only two labile ligands and relatively limited conformational freedom, [*cis*-Ru(2,2'-bipy)$_2$Cl$_2$] offers a ruthenium centre that can accommodate mono or bidentate ligands whilst retaining the chelating bipyridine ligands and hence provides an ideal centre to explore the reaction with folate, including competition with monodentate ligands. The potential for polydentate binding allows for tight, biologically irreversible chelate formation.

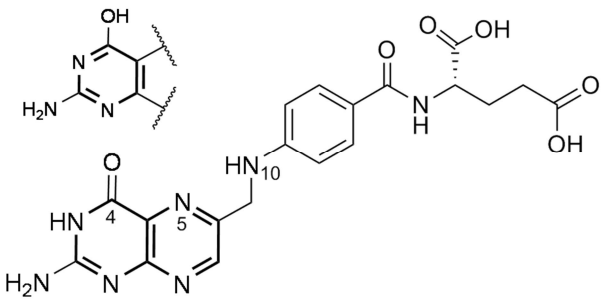

Figure 1 The structure of folic acid highlighting possible chelating sites; nitrogens **N5** and **N10** and the **4** oxygen, which can be considered as either a carbonyl or iminol depending on which tautomeric state is relevant (inset)

A solution of 5.0 mM [*cis*-Ru(2,2'-bipy)$_2$Cl$_2$·2H$_2$O] and 5.0 mM folate was stirred at 37 °C in phosphate buffered saline. MS of the reaction mixture shows no sign of free folic acid with all major ruthenium containing signals correspond to folate bound species within a day (m/z =427.6; [*cis*-Ru(2,2'-bipy)$_2$(folic acid)]$^{2+}$, 854.4; [*cis*-Ru(2,2'-bipy)$_2$(folic acid – H$^+$)]$^+$ following the deprotonation of the folic acid). Attempts to isolate the product of this reaction for further analysis were complicated by the high salt content of the buffer. In order to investigate the folate bound species more fully, [*cis*-Ru(2,2'-bipy)$_2$Cl$_2$·2H$_2$O] was reacted with stoichiometric folic acid at 65 °C overnight in aqueous solution with no added salts and then the product isolated as the [PF$_6$]$^-$ salt in a 59 % yield. The MS data of the compound as synthesised *via* this route was consistent with the folate bound species formed under more physiological conditions. The reaction in water can easily be monitored by ESI-MS revealing ruthenium species such as [*cis*-Ru(2,2'-bipy)$_2$(H$_2$O)(OH)]$^+$ and [*cis*-Ru(2,2'-bipy)$_2$(H$_2$O)Cl]$^+$ (m/z = 449.0, 467.0 respectively) immediately upon solvation. This may precede folate binding, however, it is worth noting that the unsubstituted [*cis*-Ru(2,2'-bipy)$_2$Cl$_2$] is a neutral species and is less easily detected by this method.

The structure of the isolated product was determined by 1- and 2D, ^{13}C and ^{1}H NMR spectroscopic techniques (APT, HSQC, HMBC, COSY and NOESY.) The ^{13}C NMR spectrum was

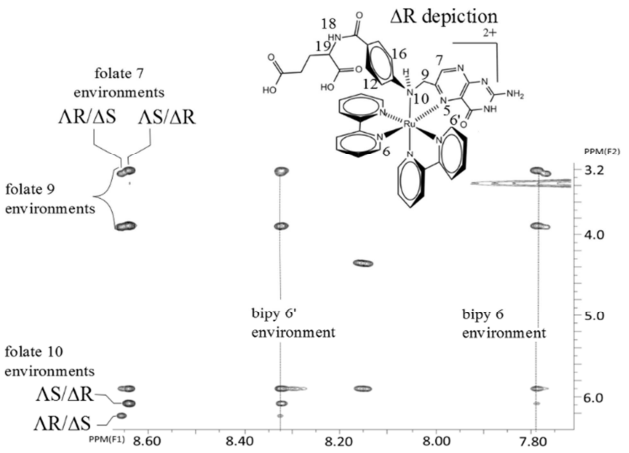

Figure 2: Section of the homonuclear NOESY spectrum illustrating the two clearly distinguishable diastereomers.
In one binding mode (ΛS/ΔR) folate environment 10 shows NOEs to 2,2'-bipyridine environments 6 and 6'. In the other binding mode (ΛR/ΔS) an NOE is only observed between folate environment 10 and 2,2'-bipyridine 6'. The unlabelled cross peaks are due to folate environments 12/16 (5.90 ppm), 18 (8.15 ppm) and 19 (4.34 ppm). Residual water (3.33 ppm) is also evident. See tables S-3, S-4 and S-5 for

collected on a 500 MHz spectrometer and the ^{1}H spectrum and all 2D spectra on a 700 MHz spectrometer (see supplementary information) and the product further characterised by ESI-MS, UV-Vis spectroscopy and elemental analysis.‡ The NMR spectra are consistent with a bidentate binding motif of diastereomers with coordination *via* sites N5 and N10 (see Figure 2), forming a 5 membered ring containing two new ruthenium-nitrogen coordination bonds. The main evidence supporting such N, N coordination includes the observation of two distinguishable species in the NMR spectra, which we assign to the two pairs of diastereomers. The largest shift difference between the two diastereomers is observed at N10 (6.08 and 6.23 ppm) and the intensity of these signals indicate that the diastereomers are present in a ~2:1 ratio (ΛS/ΔR: ΛR/ΔS). In both cases the N10 signals show coupling to two distinct protic environments on the adjacent C9 atom (one diastereomer showing a doublet of doublets and the other a triplet). This is in contrast to a broad singlet observed at 6.90 ppm for the N10 environment of free folic acid. The constrained environment at C9, leading to the loss of degeneracy of the two C9 protons (3.20, 3.87 ppm for ΛS/ΔR isomers; 3.23, 3.90 ppm for ΛR/ΔS isomers), suggests this methylene group is now part of the chelating ring. These shifts also contrast to those of free folic acid where a singlet is observed at 4.48 ppm for the C9 protons. Further key evidence comes from the NOEs highlighted in Figure 2 including the NOEs from bipyridine environments 6 and 6' to folate environments N10 and 12/16. These assignments are consistent with all other spectroscopic assignments made (see supplementary information). This motif of N,N chelation is also consistent with what has been described to be the thermodynamic product of folate metalation with cobalt(II) and nickel(II).[17,18]

The reaction was repeated at 65 °C at pH 2.5 and 6.0 (both citrate buffer) and at pH 9.9 (CAPS buffer) and the species formed followed by ESI-MS in order to follow the formation of the ruthenium species over a range of pH. These three points were chosen to reflect the possible protonation states of the folic acid i.e. predominantly protonated at the pterin moiety and therefore positively charged, neutral, and the carboxylate anion, respectively.[19,20] Coordinate bond formation between the ruthenium complex and folic acid was observed at pH 6.0 and 9.9, however, at pH 2.5 no binding was evident, even after 3 days of heating. This shows that significant protonation of folate atom N5 inhibits binding to the ruthenium centre, consistent with the nitrogen lone pair at this site being integral to chelation.

The proposed binding motif through the N5 and N10 nitrogens was investigated further by reaction of 10-formyl folic acid[21] with [*cis*-Ru(2,2'-bipy)$_2$Cl$_2$.2H$_2$O]. No adduct formation was observed at 37 °C, so the reaction was repeated at 65 °C overnight in aqueous solution but yielded only starting materials. This lends further weight to the importance of N10 in adduct formation.

Ruthenium (II) complexes of flavins, pterins and alloxazines have been synthesised and studied from an electrochemical point of view. [22–24] Whilst an adduct between [*cis*-Ru(2,2'-bipy)$_2$]$^{2+}$ and folic acid has been reported,[23] a limited analysis of the product led to the conclusion that folic acid had chelated to the metal through the O4 and N5 in a motif analogous to flavin coordination. Such binding would produce a single pair of enantiomers (indiscernible by NMR spectroscopy). On replication of the experimental conditions outlined,[23] our subsequent NMR spectral analysis of the complexes isolated once again supports chelation to the ruthenium centre *via* N5 and N10 with the same diastereomeric products being observed. Interestingly, these more energetic conditions appeared to favour the ΛS/ΔR isomers further as the NMR spectra suggested formation of these in a ~4:1 ratio relative to the ΛR/ΔS isomers.

Analysis of the isolated, synthetic product formed at 65°C allowed us to interpret the NMR spectra of the more physiologically relevant mixture. Integration of the N10 proton signals suggest ~2:3 ratio of ΛS/ΔR: ΛR/ΔS and, by comparison to the N10 signal of free folate present, ~90 % conversion of [*cis*-Ru(2,2'-bipy)$_2$Cl$_2$] to the folate coordinated complex. The physiological relevance of such reactivity was explored further by following a solution of 4.8 mM [*cis*-Ru(2,2'-bipy)$_2$Cl$_2$·2H$_2$O] and 4.8 mM dihydrofolate (DHF) at 37 °C under aqueous conditions by ESI-MS. DHF was observed to bind within 2 days (m/z = 428.7; [*cis*-Ru(2,2'-bipy)$_2$(DHF)]$^{2+}$) followed by the formation and binding of folic acid in solution (m/z = 427.7; [*cis*-Ru(2,2'-bipy)$_2$(folic acid)]$^{2+}$, m/z = 442.1; [folic acid + H$^+$]$^+$) after a further 24 hours.

The total concentration of folate species in cells is low, and of these, >90 % are polyglutamylated at the glutamate end of the molecule.[25] As with the 10-formyl folic acid complex above, not all of these folate species have available lone pairs at N5 and N10 for coordination. Nonetheless, the total ruthenium content of cultured cells[26] can be >40 fold higher than folate in molar terms and hence the potential for complexation and the long lifetime of the resulting species will interfere with enzyme binding and the one-carbon carrying role of folates in cells.

The timescale of our results is consistent with the slow ligand exchange rates that are typical of ruthenium-based compounds[27] including those that are being investigated for their cytotoxic properties.[28–30] Indeed it is these slow ligand exchange rates that are likely to be more important than the absolute affinity of folate for the metal centre.

Conclusions

The various fates of organometallic compounds *in vivo* present a challenge in terms of elucidating their mechanisms of cytotoxicity. One current strategy focuses on identifying protein and DNA targets of relatively simple complexes using modern bioanalytical methods.[31,32] The importance of smaller molecules alongside macromolecules should not, however, be overlooked.

We have shown that ruthenium can form a kinetically stable complex with folates under physiologically relevant conditions and have characterised the binding mode as exclusively *via* N5 and N10 coordination. This binding mode is observed both at 37 °C and at raised temperatures and is contrary to a previous proposal based on the similarity of folates to flavins[23] carried out at raised temperatures. Both oxidised and reduced folates can complex to ruthenium. Exposure of labile transition metal complexes to Lewis bases in cells in such great numbers presents a challenge when attempting to deconvolute the key active species, especially when the generation of stereoisomers further complicates any analyses. It is likely that any ruthenium complex with multiple labile sites would similarly form stable complexes with potentially chelating biomolecules such as folates. Given the low concentration of folates in cells, any diverted into a ruthenium complex by the presence of excess ruthenium complexes with labile ligands, will alter the cellular balance of this cofactor. Folate metabolism has long been recognised as a key target for cancer therapy and folate uptake into tumour cells is significantly stimulated.[33–35] Understanding how ruthenium complexes interact with the folate pool may be of significance.

Nonetheless, the tight binding and slow exchange rates of precious metal complexes are attractive properties to incorporate into a medicinal compound in general. To selectively harness the potential of organometallic complexes as metallodrugs, suitable targets need to be established and rationalised and a selective delivery strategy must be adopted to enable specific targeting to molecules of choice.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Full experimental procedures, compound characterisation and copies of NMR and MS spectra. See DOI: 10.1039/b000000x/

‡ λ_{max} = 470 nm. Theoretical: C=40.91%, H=3.08%, N=13.46%. Results: C=41.06%, H=3.20%, N=13.36% (2.0806mg); C=40.92%, H=3.15%, N=13.25% (1.6554mg). m/z = 427.7; [*cis*-Ru(2,2'-bipy)$_2$(folic acid)]$^{2+}$ m/z = 854.3; [*cis*-Ru(2,2'-bipy)$_2$(folic acid-H$^+$)]$^+$.

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A ruthenium (II) complex with labile ligands has been observed to chelate to folates under physiologically relevant conditions. The diastereomeric complexes formed are likely to interfere with the one-carbon carrying role of folates *in vivo*. This highlights the importance of considering small molecules alongside macromolecules when determining the chemical origins of the cytotoxicity of metallodrug candidates.