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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)diclororuthenium(II), [cis-Ru(2,2'-bipy)Cl₂] 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)Cl₂] 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)Cl₂] 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.

A solution of 5.0 mM [cis-Ru(2,2'-bipy)Cl₂][H₂O] 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)])₁, 854.4; [cis-Ru(2,2'-bipy)₂(folic acid – H⁺)] following the deprotonation of the folate 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)Cl₂][H₂O] was reacted with stoichiometric folic acid at 65 °C overnight in aqueous solution with no added salts and then the product isolated as the [PF₆]⁻ 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)(H₂O)(OH)]⁺ and [cis-Ru(2,2'-bipy)₂(H₂O)Cl]⁺ (m/z = 449.0, 467.0 respectively) immediately upon solvation. This may preclude folate binding, however, it is worth noting that the unsubstituted [cis-Ru(2,2'-bipy)₂Cl₂] is a neutral species and is less easily detected by this method.

The structure of the isolated product was determined by 1- and 2D, ¹³C and ¹⁹F NMR spectroscopic techniques (APT, HSQC, HMBC, COSY and NOESY.) The ¹³C NMR spectrum was

![Figure 1](image-url) 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)
Figure 2: Section of the homonuclear NOESY spectrum illustrating the five for

Residual water (3.33 ppm) is also evident. See tables S-3, S-4 and S-

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Ruthenium (II) complexes of flavins, pterins and alloazines have been synthesised and studied from an electrochemical point of view.\textsuperscript{22–24} Whilst an adduct between \( [cis\text{-Ru}(2,2\text{'-bipy})_2\text{Cl}_2\text{H}_2\text{O}] \) and folic acid has been reported,\textsuperscript{25} 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,\textsuperscript{25} 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 AS/AR isomers further as the NMR spectra suggested formation of these in a \( \sim 1:1 \) ratio relative to the AR/AS isomers.

Analysis of the isolated, synthetic product formed at 65\textdegree 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 AS/AR: AR/AS and, by comparison to the N10 signal of free folate present, \( \sim 90 \% \) conversion of \( [cis\text{-Ru}(2,2\text{'-bipy})_2\text{Cl}_2\text{H}_2\text{O}] \) to the folate coordinated complex. The physiological relevance of such reactivity was explored further by following a solution of 4.8 mM \( [cis\text{-Ru}(2,2\text{'-bipy})_2\text{Cl}_2\text{H}_2\text{O}] \) and 4.8 mM dihydrofolic acid (DHF) at 37\textdegree C under aqueous conditions by ESI-MS. DHF was observed to bind within 2 days (\( m/z = 428.7; [cis\text{-Ru}(2,2\text{'-bipy})_2\text{DHF}]^{2+} \)) followed by the formation and binding of folic acid in solution (\( m/z = 427.7; [cis\text{-Ru}(2,2\text{'-bipy})_2\text{follic acid}]^{2+}, m/z = 442.1; [folic acid + H^+] \)) after a further 24 hours.

The total concentration of folate species in cells is low, and of these, \( \sim 90 \% \) are polyglutamylated at the glutamate end of the molecule.\textsuperscript{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\textsuperscript{26} can be \( \times 40 \) 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\textsuperscript{27} including those that are being investigated for their cytotoxic properties.\textsuperscript{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. 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 MS spectra. See DOI: 10.1039/b000000x/ experimental procedures, compound characterisation and copies of NMR † Electronic Supplementary Information (ESI) available: Full studentships of Tom Scrase and Simon Page respectively.

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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/

‡ \( \lambda_{\text{max}} = 470 \) nm. Theoretical: C=40.91%, H=3.08%, N=13.46%. Results: C=41.06%, H=3.20%, N=13.36% (2.0806nm); C=40.92%, H=3.15%, N=13.25%. (1.6554nm). \( \text{m/z} = 427.7; [\text{cis-Ru(2,2'-bipy)}][\text{folic acid}]^{2-}\text{m/z} = 854.3; [\text{cis-Ru(2,2'-bipy)}][\text{folic acid-H}^+] \). 1. C. G. Hartinger, N. Metzler-Nolte, and P. J. Dyson, Organometallics, 2012, 31, 5677–5685.


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Folates are potential ligands for ruthenium compounds\textit{ in vivo}.

JPEG format graphics file can be provided when the manuscript is accepted.

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\textit{ in vivo}. This highlights the importance of considering small molecules alongside macromolecules when determining the chemical origins of the cytotoxicity of metallo drug candidates.