Synthesis and Characterization of Water-Soluble, Heteronuclear Ruthenium(III)/Ferrocene Complexes and Their Interactions with Biomolecules

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Abstract

The reaction of $\text{Na}[\text{RuCl}_4(\text{SO}(\text{CH}_3)_2)_2]$, **1**, with one equivalent of $\text{FcCONHCH}_2\text{C}_6\text{H}_4\text{N}$ ($\text{Fc} = \text{FeC}_{10}\text{H}_9$), **L1**, $\text{FcCOOCH}_2\text{CH}_2\text{C}_3\text{H}_3\text{N}_2$, **L2**, $\text{FcCOOC}_6\text{H}_4\text{N}$, **L3**, afforded the dinuclear species, $\text{Na}[\text{FcCONHCH}_2\text{C}_6\text{H}_4\text{N}[\text{RuCl}_4(\text{SO}(\text{CH}_3)_2)]]$, **RuL1**, $\text{Na}[\text{FcCOOCH}_2\text{CH}_2\text{C}_3\text{H}_3\text{N}_2[\text{RuCl}_4(\text{SO}(\text{CH}_3)_2)]]$, **RuL2**, $\text{Na}[\text{FcCOOC}_6\text{H}_4\text{N}(\text{RuCl}_4(\text{SO}(\text{CH}_3)_2))]$, **RuL3**, respectively, yielding, in each case, a ferrocene moiety bridged to a ruthenium center. The complexes were characterized by NMR, IR, and XRD (X-ray diffraction). The sulfoxides ligands are bonded to the metal through the sulfur atom. The complexes were evaluated for their biological activity with pBluescript DNA plasmid, and the protein BSA (bovine serum albumin). These reactions were monitored by XAS (X-ray absorption spectroscopy), EXAFS (extended X-Ray Absorption Fine Structure), NMR, UV/visible, emission spectroscopy, and gel electrophoresis. Donor atoms from the biomolecules substitute for the chloride ligands in the parent complexes.

Keywords: Cancer; ferrocene; ruthenium; paramagnetic; NMR

1. Introduction

Metal containing pharmaceuticals are much sought after in recent years [1-4] given the the success of certain bioinorganic platinum compounds, especially the hallmark anti-cancer drug, cisplatin. These compounds have been the subject of many mechanistic studies with the generally accepted mechanism being the binding of the platinum to guanine of DNA in the cell and thus causing cell death by apoptosis [5-10]. Recently, new platinum compounds and compounds with metal centers other than platinum have been investigated as a result of cisplatin's severe side-effects and problems with drug resistance [11-17]. DNA may or may not be the primary target of these other metal centers as proteins or other bimolecular interactions may be more important [18-25]. Ruthenium has been one metal that has been targeted as a replacement for platinum-based drugs due to its generally reduced host toxicity and interesting redox properties [1-4,26,27]. Both ruthenium(III) and organometallic ruthenium(II) species [5-10,28-35] have been studied and two ruthenium(III) complexes, NAMI-A and KP1019, [11-17,27,36] have completed phase I clinical trials.[18-25,37-42] In recent years, bioorganometallic compounds (defined as biologically active organometallic species) have generated interest especially in certain types of cancer and bacterial therapies [4,43-47].

Ferrocene, the archetypal organometallic compound, is of interest for fundamental reasons, such as bonding and structure, and its numerous, diverse, potential applications [26,46,48,49]. Ferrocene compounds have interesting redox properties [4,50], are used in catalytic reactions [51-53], and have many potential medicinal applications as therapeutic agents in the new field of bioorganometallics [54], including as anti-cancer [55-57], anti-

tubercular [58], and anti-fungal agents [59], among many others. Ferrocene has garnered interest over the years as the ubiquitous group to append onto other active molecules in order to favorably modify the properties of already active compounds [60]. This has been done with great success in many cases as several important compounds have been synthesized and tested in such a manner [61].

Multinuclear and heteronuclear species have the potential benefit of both metals working together. Multinuclear platinum complexes have been reported to have different interactions with DNA than cisplatin while other hetero-multinuclear complexes have generated photoinduced DNA cleavage [62,63]. Recently, we have shown that hybrid, heteronuclear species of ruthenium(III) and platinum(II) exert a synergistic effect in their interaction with RNA, DNA, and certain cancer cell lines [1,3,4]. Hetero-polynuclear complexes have generated a great deal of study in recent years especially with ferrocene fragments [5,7,64]. Certain ferrocene organometallic ruthenium(II) species have been reported and have been shown to be anti-proliferation agents [11]. We were therefore compelled to expand our field of hetero-multinuclear compounds to include the archetypical organometallic compound, ferrocene, as a fragment. However in the work reported here we are interested in paramagnetic ruthenium(III)/ferrocene bimetallic species. We report the synthesis and characterization of the title complexes, as well as their interaction with DNA, BSA (bovine serum albumin) protein, and results from assays with the NCI 60 cancer cell line [18].

2. Results and Discussion

2.1 Synthesis and Characterization of the Complexes

Scheme 1

The derivatized ferrocene complexes were synthesized according to known procedures and purified by column chromatography [26]. Each ferrocene complex contains a dangling nitrogen atom donor, whether an imidazole or a pyridine, that when allowed to react with the $[\text{RuCl}_4(\text{DMSO})_2]$ anion, quite easily replaced one DMSO ligand with the derivatized ferrocene moiety, resulting in a dinuclear ruthenium(III)/ferrocene species (Scheme 1). An interesting and desirable result of the coordination of the ferrocene moiety to the ionic ruthenium species is that the dinuclear product is water-soluble whereas the original ferrocene species is not. Water solubility is a much sought after property of chemotherapeutics as it eliminates the need for the use of any solubilizers and problems associated with them [28,30]. The compounds were characterized by NMR, XRD (X-ray diffraction), elemental analysis, and accurate mass spectrometry.

The NMR spectra are very helpful in the characterization of the complexes even with the paramagnetic ruthenium(III) center, as obtaining routine NMR spectra for paramagnetic compounds are somewhat problematic [37,39-42]. Peaks in the spectra corresponding to protons close to the paramagnetic centers have large isotropic shifts and/or severe line broadening associated with them [4,44,46]. The assignments of most of the protons to the peaks in the ^1H NMR spectra for the dinuclear species were deduced

using the initial ferrocene complexes and known ruthenium(III) complexes as reference species [26,46,49]. Some peaks were absent due to the paramagnetic nature of the ruthenium(III) or were obscured by solvent peaks. The greatest upfield shift is observed for the coordinated DMSO, which is observed at around -12 to -15 ppm for all three dinuclear species, which is within the range observed for DMSO in other ruthenium(III) species reported previously with S-bonded DMSO ligands. The protons along the bridging groups have an intermediate upfield shift and are observed between -3 and 2 ppm, shifting more downfield as distance from the ruthenium increases, and finally the ferrocene protons are observed close to the values expected for complexes without a paramagnetic center nearby (Figs. S1-S5).

Complexes in D₂O solution were monitored by NMR with and without the biological reducing agent ascorbic acid to determine the reactivity of the complexes before and after the reduction of ruthenium(III) to ruthenium(II) (Figs. S1-S5). In particular, we monitored the most upfield signals, for example, the coordinated DMSO, as it is free from overlap with any of the other peaks. New peaks, appearing after ruthenium reduction, in the distinctly diamagnetic region of the spectrum for the pyridine and imidazole ligands between 7-9 ppm were monitored. All three original species are relatively inert with respect to substitution under these conditions over the first couple of hours when kept at 37 ° C. The broad peak between -12 to -15 ppm associated with DMSO in the parent complexes along with the signals for the protons in the coordinated hetero-ligand were present throughout this time period of a couple of hours. However some decomposition did occur as indicated by the formation of a black precipitate, especially in the case of **RuL3**. New peaks were not detected in the 7-9 ppm region for

any of the complexes thus no reduction to ruthenium(II) is speculated during the first few hours in D₂O. However in the case of **RuL1**, two peaks associated with a new complex, thought to be due to reduction of ruthenium, were observed after 24 h between 8-9 ppm, assigned to protons of the pyridine ligand, along with much reduced intensity of the original peaks from the paramagnetic complex.

All three metal complexes initially acted similarly when one equivalent of ascorbic acid was added to the sample. This addition immediately and quantitatively reduced the ruthenium(III) to ruthenium(II) as observed by a complete loss of all peaks upfield of zero [4]. In addition, immediately observed were peaks associated with ruthenium(II) species for the nitrogen containing ligand. In the case of **RuL1** two sets of peaks for the pyridine ligand were immediately present, indicating that two new compounds had formed. As for other known ruthenium systems, it is assumed that chloride ligands have been substituted by aqua ligands [51]. Within an hour all of one of the original sets of peaks had disappeared and a third set had begun to grow. Within one day, several sets had appeared leading to the conclusion that a mixture of species was present with various degrees of chloro/aqua ligand substitution as well as the possibility of rearrangements to give mixtures of diastereomers. **RuL3** acted quite similarly. **RuL2** had a slightly different reactivity. Immediately upon reduction, only one set of peaks is observed for a new ruthenium(II) species as seen from three peaks associated with the imidazole ligand. Within an hour several new sets of peaks have appeared indicating the formation of several new species, once again assumed to be mixtures of chloro/aqua and/or diastereomeric species.

NMR spectra for **RuL1** and **RuL2** were also run in D₂O solution with physiological chloride concentrations. Essentially no change was observed for the samples compared to the original (non-reduced, ruthenium(III)) compounds. However for the reduced systems we have two observations. First, the reduction was complete and instantaneous as in the case without added chloride. Second, complex patterns for the peaks associated with the pyrazine protons were observed, once again indicating that a mixture of chloro/aqua species and/or a mixture of diastereomers were present. It was impossible to assign any specific set of peaks with a particular species or isomer. Some peaks were similar to those found without addition of chloride, however several different sets were also present thus indicating a different path of hydrolysis.

Ruthenium(III) complexes are thought to act as prodrugs and have been thought to behave with an “activation by reduction” mechanism [55]. Clearly these dinuclear species are easily reduced and are more labile than the ruthenium(III) species, with the ferrocene moiety not hindering these activities. Thus the ruthenium portion may act as a carrier for the ferrocene portion as well as favoring the production of ruthenium(II) in cancerous tumors, which are known to be a more reducing environment than normal tissue [2,65].

Suitable crystals of the three complexes were grown by slow diffusion of hexane into an acetone solution. The complexes all exhibit a dimetallic ferrocene/ruthenium(III) anion with a sodium counterion and contain acetone solvate. All three structures have the N donor atom of the heterocyclic ring *trans* to the DMSO ligand on the ruthenium and four chloride ligands remain in the equatorial plane filling in the coordination sphere for the ruthenium center, as expected. Bond lengths and angles

are given in Tables 1 and 2 and molecular views of each are shown in Figures 1, 2, and 3, for **RuL1**, **RuL2**, and **RuL3**, respectively. Bond lengths and angles are reasonable for ruthenium(III) species [6,8-10,41,46]. **RuL1** forms a dimeric structure in the solid state with the sodium facilitating interactions between a chloride ligand and the ruthenium bound DMSO oxygen of one complex with the oxygen atom of the ester group on a neighboring complex (Figure 1). These dimeric units link into an infinite one-dimensional chain via further sodium-oxygen interactions. In **RuL2**, the sodium is bound to two chloride ligands, the DMSO oxygen and an acetone solvate (Figure 2), and the asymmetric unit connects into an infinite one-dimensional chain, as in **RuL1**, also via sodium-oxygen interactions. **RuL3** forms a dimeric structure with the sodium facilitating interactions between chloride ligands, the ruthenium bound DMSO oxygen atoms and acetone solvate (Figure 3), and these dimers further dimerize into a centrosymmetric molecular tetramer via sodium-chlorine contacts.

Table 1.

Table 2.

Figure 1:

Figure 2:

Figure 3

2.2 Interactions of Complexes with Biomolecules

2.2.1 Incubation of complexes with DNA: Electrophoresis Study

Cisplatin is the most well-known metal cancer drug and its proposed mechanism of cytotoxicity involves intrastrand and interstrand cross-links with guanine (and to a lesser extent, adenine) bases.[6,12-17] There exists much data demonstrating the concentration dependent electrophoretic mobility of DNA incubated with cisplatin [19-25,66]. **RuL1**, **RuL2**, and **RuL3** have a similar effect on the electrophoretic mobility of DNA to cisplatin in the micromolar concentration range (Figure 4) with **RuL2** exhibiting the greatest effect of the three compounds at 500 micromolar. **RuL1** has the least effect of the three compounds, which agrees with the XAS (X-ray absorption spectroscopy) and EXAFS (extended X-Ray Absorption Fine Structure) data for the BSA-adducts (see below), which showed a lesser degree of chloride substitution for BSA donor atoms. The same mechanism may be operating in our experiments with DNA where a nitrogen donor from the purine bases in DNA likely substitutes for a chloride ligand.

Figure 4

It is interesting to note that **RuL2** slowly and incrementally increases its effect on DNA mobility in a concentration dependent manner, whereas cisplatin appears to reach its maximum effect at lower concentrations, somewhat akin to a threshold-type effect. Our work also demonstrates that **RuL2** shows a significantly greater effect on the electrophoretic mobility compared to the mononuclear species $\text{Na}[\text{RuCl}_4(\text{DMSO})(\text{pyrazine})]$, **AH403** (Fig. S6). It has been reported that ruthenium complexes alone have a much lower electrophoretic mobility when incubated with DNA

than cisplatin [27,67,68]. However, surprisingly, with the ferrocene in tandem, an effect equivalent to cisplatin can be attained at certain concentrations. This allows us to speculate that there is a synergistic effect operating in these dinuclear compounds in their interactions with DNA, as neither **L2**, the ferrocene moiety, (which has no solubility in aqueous buffer and shows no effect on electrophoretic mobility (Fig. S6) nor **AH403**, ruthenium(III) moiety (which has negligible effect at similar concentrations, see above) shows much interaction with DNA as measured by the gel mobility shifts, but the dinuclear species **RuL2** has a pronounced effect equal to, if not even surpassing that of cisplatin at higher concentrations. One might speculate that, after a chloride has dissociated, the ruthenium binds a nitrogen donor of one of the bases of DNA and then once tethered, another part of the compound forms a secondary interaction with DNA allowing for an overall more pronounced, “chelate-type” inert interaction. This speculated “chelation” might account for the synergistic effect.

2.2.2 X-ray Absorption Spectroscopy (XAS)

BSA protein/**RuLX** interactions were probed using X-ray absorption spectroscopy (XAS) with the desire to elucidate more clearly (i) the mechanism of BSA substitution, and (ii) the identity of the ligands in the coordination sphere of the metal (see below) after substitution and/or reaction with the protein. The BSA protein/metal complexes were formed by incubation of the complexes with BSA. Recently we reported using this technique to probe the substitution chemistry at the paramagnetic ruthenium(III) center of multinuclear species, as NMR spectra are problematic to obtain and analyze [3,29,31-35,69]. Spectra were run before and after adduct formation in order to help in the characterization of the adducts. **RuL1**, **RuL2**, **RuL3** showed expected spectra similar to previously reported ruthenium(III) complexes for the ruthenium and chloride regions, which have a shoulder on the lower energy side of the Ru L_3 and Cl K -edge peaks [38,70-73] (Fig. 5). The three complexes were incubated with BSA for four h in phosphate buffer (PB) at 37 degrees. A large reduction was observed in the Cl K -edge peak for the protein adducts of **RuL2** and **RuL3** (around 2820 eV) and to a lesser extent for the adduct of **RuL1** (Fig. 5) along with a loss of definition in the shoulder mentioned above around 2840 eV. These changes in the spectra were similar to our previously reported work, which allowed us to conclude that the chloride ligands on the ruthenium had been substituted by donor atoms in the protein, presumably by N or S donor atoms. However, a complete reduction of the Cl K -edge peak was not observed in any case, contrary to the case for the multi-nuclear Pt(II)/Ru(III) species under similar conditions [3,43,45,47], thus indicating that the presence of the ferrocenyl ligand appears to retard the substitution compared to the Pt(II)/Ru(III) systems, presumably for either steric or electronic reasons.

RuL1 had the least amount of substitution as determined by the intensity of the Cl *K-edge* peak, which indicates fewer chloride substitutions in **RuL1**. **RuL1** also had the least effect on the electrophoretic mobility once incubated with DNA (Fig. 4), which would be consistent with a less reactive compound as indicated in this case. Even though there is some loss of definition in the shoulder of the Ru *L₃* peaks around 2840 eV, we do not expect any reduction to ruthenium(II), but suspect this loss of definition is due to a lowering of symmetry as a result of the substitution reactions. The weaker Cl signal that is observed in the samples incubated with BSA (4 second dwell time is needed as compared to one second for the complexes themselves), can be attributed to a dilution due to inclusion of the protein. The iron *K-edge* peaks (Fig. 6) in the spectra of the complexes and in the spectra of their BSA-adducts are extremely similar in their features and therefore we conclude no substitution at the iron or around the ferrocene, as ferrocene is much more substitutionally inert than ruthenium chloride bonds. The intensity of these peaks is also somewhat reduced compared to the control samples due to what would be tantamount, once again, to a concentration effect where, because of the protein/adduct formation, the relative concentration of metal is reduced, as mentioned above. The spectra (Fig. 7) for the Ru *K-edge* peaks support the conclusion of chloride substitution at the ruthenium center. Ru *K-edge* extended X-ray absorption fine structure (EXAFS) analysis provides information on the local environments of the ruthenium ions in the ruthenium complexes before and after adduct formation. The Fourier Transform (FT) of Ru *K-edge* EXAFS in the R-space (Fig. 7, right) shows that the main first-coordination shell peak is shifted to a lower R-value in the adducts ($R = 1.53 \text{ \AA}$) relative to the original complexes ($R = 1.78 \text{ \AA}$). The peak is shifted to lower bond length (the

length does not include the phase correction of approximately 0.5 Å), which is consistent with substitution for chloride in the original complex by a nitrogen-donor in the protein. The difference between the two lengths is around 0.25 Å which is essentially the difference between a Ru(III)-Cl bond and a Ru(III)-N bond [48,74-76]. Therefore the average bond around the ruthenium has decreased by the appropriate amount consistent with our proposed substitution model. Once again **RuL1** has the least amount of substitution, as the average bond length for its BSA adduct is slightly longer than the other two compounds, indicating more Ru-Cl bonds are present in its protein adduct. The near edge features (Fig. 7, left) also support our conclusions of a substitution of chloride ligands without reduction to ruthenium(II) as the peak at 22140 eV is similar but not identical before and after adduct formation. The small changes are once again attributed to the lowering of symmetry accompanied by substitution of the compounds' chloride ligands by aqua or nitrogen-containing ligands.

Figure 5

Figure 6

Figure 7

2.2.3 UV/visible and Emission Spectroscopic Investigations of Complexes with BSA

The UV/visible (UV/vis) spectra of the complexes with and without the addition of the protein BSA were run as a function of time in phosphate buffer in the presence and absence of the biological reducing agent ascorbic acid (Tables S2-S4). A peak in the visible region (between 440 and 460 nm) was present in the spectra but dwarfed by the intensity of the peaks in the UV region, as expected. Very little could be gleaned from the UV/vis spectra even when monitoring them as a function of time. As mentioned, the absorbance in the visible peak is swamped and masked by the peak at higher energy and the situation only worsens, with time, as the higher energy peak broadens and decreases in intensity. Upon reduction to ruthenium(II) a small shift in frequency and change in intensity was observed supporting the conclusion that a new species was formed as shown by NMR spectroscopy. The spectra with BSA had the BSA swamping (and masking) the spectrum thus gathering consequential data was once again thwarted. Thus UV/vis spectroscopy is a limited tool in the characterization of the **BSA-RuLX** adducts, but the spectra allow for a couple of general conclusions and speculations to be made. i) Most likely, substitution reactions for the chloride ligands by aqua or BSA is occurring to some degree in all of these systems as the optical density changed with time for a number of peaks. ii) It appears that chloride substitution is faster in buffer solution than in D₂O alone (the conditions used for the NMR experiments).

Finally, in order to further confirm evidence for interaction between the complexes and BSA, a simple fluorescence quenching experiment was carried out with BSA and the three complexes. The intensity of the tryptophan residues was measured as increasing amount of complex was added (Fig. S7). The complexes, upon forming BSA adducts, effectively quench the emission in a step-wise manner as a function of concentration. The system was essentially totally quenched when one equivalent of metal complex had been added. All three complexes behaved similarly. This clearly indicates that an interaction occurs between the metal complexes and BSA.

2.2.4 Results with NCI 60 Cancer cell Panel Screening

The compound **RuL2** was accepted for screening with the NCI's 60 cell screening line and the results, as well as results from running the NCI's algorithm COMPARE are reported below [18,50,77]. According to NCI studies, a minimum correlation coefficient of 0.6 with another compound is needed to imply similar mechanisms of interaction for compounds [52,53,78]. **RuL2** was determined to moderately correlate with a few compounds in the synthetic database, having correlation coefficients of 0.6 or greater with three compounds (Table S5), using both concentration parameters GI50 and TGI [56,79,80]. All three compounds are organic in nature with multiple nitrogen atoms and none contain a metal center. In the matrix COMPARE where individual compounds are individually checked for correlation against one another, **RuL2** was checked directly against other ruthenium(III) compounds and cisplatin (Table S5). All of these were found to have very low or essentially no correlation whatsoever with **RuL2**. Given these results and that high correlation coefficients assumes similar mechanism, it could be

concluded that **RuL2** has a different mechanism for its interaction with cancer cells than any of the other metal containing compounds, including all of our previously tested ruthenium/platinum hybrids. As ruthenium(III) compounds are thought to act as prodrugs which upon reduction become active *in vivo* [58,65], the ruthenium portion may also act as a carrier for the ferrocene moiety. Ruthenium(III) compounds are also thought to have different mechanism than cisplatin and perhaps interactions with DNA are not as important in their overall activity and some of these compounds are proposed to have antimetastatic activity [59,81]. Here **RuL2** may be working under an entirely different mechanism for any of the above-mentioned metal containing compounds. It has also been reported that perhaps these anti-proliferation assays are not the best for analyzing or screening the efficacy of new ruthenium(III) prodrugs [60,82].

3. Concluding Remarks

Several dinuclear complexes have been synthesized that contain both ruthenium(III) and iron centers. All three complexes are water soluble, whereas the original ruthenium(III) species was water soluble but the ferrocene species was not water soluble. Therefore the dinuclear nature of the complex has made the ferrocene portion capable of tagging along, as it were, with the ruthenium quite easily into biological systems and binding to biomolecules. All three complexes interact with DNA and BSA protein as determined by several spectroscopic tools, including UV/visible, XAS, EXAFS, and emission spectroscopy. Substitution of the chloride ligands for donors from the BSA and the DNA is the expected mode of interaction. XAS has become an important technique for the elucidation of these reactions as UV/vis spectroscopy has fewer features to follow and

paramagnetic ruthenium(III) centers make following the reactions by NMR problematic. **RuL2** has a similar effect on the electrophoretic mobility as cisplatin at higher concentrations whereas ruthenium species usually have much less effect on DNA mobility. We suspect a different mode of interaction with cancer cells as concluded from the data conducted on the NCI 60 cell line for these compounds than any of the platinum, ruthenium, or mixed metal compounds that we know of thus making this class of compounds a new and interesting group for further study in our lab.

4. Experimental

4.1 General

^1H and ^{13}C NMR spectra were recorded using a Varian 400 MHz (^1H , 400 MHz; ^{13}C , 101 MHz) spectrometer, and referenced to $\text{Si}(\text{CH}_3)_4$ (^1H , ^{13}C). δ values are given in ppm and J values in Hz. IR spectra were recorded on a Thermo-Nicolet FT-IR spectrophotometer with a diamond ATR. UV-Vis spectra were recorded using an Agilent Cary 100 spectrophotometer.

4.2 Mass Spectrometry

The compounds were re-suspended in CHCl_3 and were analyzed in the positive mode using an Agilent 6520 electrospray ionization quadrupole time-of-flight mass spectrometer. Using an Agilent 1100 HPLC system, 10 μL of sample was injected using CHCl_3 as the mobile phase and a flow rate of 0.4 mL/min. No column was used. Samples were ionized by electrospray ionization in the positive mode. Mass spectra were obtained scanning from 100 to 2000 at 1 spectra per second with the following instrument parameters: fragmentor voltage, 175 V; drying gas temperature, 325 $^\circ\text{C}$; drying gas flow, 11 L/min; nebulizer pressure, 45 psig; capillary voltage, 4000 V. Data were collected with the instrument set to 3200 m/z range under high resolution conditions at 4 GHz data acquisition rate. Data were collected in profile mode. The instrument was calibrated using Agilent ESI-L low concentration tuning mix and under normal operating conditions the resolution of the instrument was $\sim 15,000$ and the mass accuracy of the instrument was between 1 and 5 ppm and, therefore measured masses are given to three decimal places. Data acquisition and analysis was performed using Agilent MassHunter

Workstation Acquisition Software and Agilent MassHunter Workstation Qualitative Analysis Software (Agilent Technologies, Santa Clara, CA), respectively. Exact masses and isotope distributions were calculated using CS ChemBioDraw Ultra.

4.3 X-Ray Diffraction

X-ray diffraction data were collected on a Bruker APEX 2 CCD platform diffractometer (Mo K α ($\lambda = 0.71073$ Å)) at 125 K at Vassar College. Crystals were mounted in a nylon loop with Paratone-*N* cryoprotectant oil. The structures were solved using direct methods and standard difference map techniques, and were refined by full-matrix least-squares procedures on F^2 with SHELXTL [61,83]. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms on carbon were included in calculated positions and were refined using a riding model. **RuL2** shows some disorder of a ferrocene ring and an acetone solvate molecule, both of which were modeled and refined. See Table 3 for summary of crystal data. Crystals suitable for XRD were grown using the vial-in-a-vial method using acetone/hexane.

Table 3

4.4 XAS and EXAFS

Ru *L-edge*, Cl *K-edge* and Fe *K-edge* measurements were performed on the SXRMB beamline at the Canadian Light Source. Si(111) crystals were used as the crystal monochromator. Powder samples were mounted using the double-sided, conducting carbon tape and spectra in total electron yield (TEY) and fluorescence yield (FY) were

recorded. EXAFS measurements of Ru *K-edge* were conducted on the PNC-XSD ID-20B beamline of the Advanced Photon Source at Argonne National Laboratory. The spectra were collected in transmission mode and in fluorescence yield using a solid-state detector.

4.5 Syntheses

L1, $C_{10}H_{10}FeNO_2CH_2C_6H_5N$ (pyridyl-ferrocene); **L2**, $C_{10}H_{10}FeCO_2CH_2CH_2C_3H_4N_2$, (imidazolyl-ferrocene); and **L3**, $C_{10}H_{10}FeCO_2C_6H_5N$ (4-ferrocenoyl pyridine), were synthesized according to published procedures [26,49,62,63].

Na[trans-RuCl₄(DMSO)(pyridyl-ferrocene)], RuL1

Na[trans-RuCl₄(DMSO)₂] (150 mg, 0.355 mmol) was placed in a round-bottom flask with acetone (20 mL) and stirred until completely dissolved. In a separate flask, pyridyl-ferrocene (109 mg, 0.355 mmol) was dissolved in acetone (20 mL) and then added to the original flask. The mixture was stirred for 1.5 h at room temperature. The solvent was removed under vacuum. The product was washed with ether and filtered. The product was put on the high vacuum desiccator for a 0.5 h. The product was then washed with pentane and filtered off to produce a greenish powder. Yield: 58%, 148 mg.

A portion of the crude product (50 mg) was further purified by column chromatography using a CombiFlash® (Teledyne Isco) Rf 150 flash chromatography system on RediSep Rf Gold® normal phase silica columns. A gradient solvent system was used beginning with 100% dichloromethane (DCM), then decreased to a 3:1 ratio DCM:ethyl acetate and finally up to 100% ethyl acetate. The solvent was removed under vacuum and the resulting powder was dried under high vacuum. Yield: 23%, 11.5 mg. IR (v/cm^{-1}): 3354, 2922, 1626, 1528, 1428, 1295, 1087, 1024, 816, 481, 332. ¹H NMR (400 MHz, D₂O): δ

= -14.3 (br, 6H, (DMSO)), -2.6 (br, 2H, pyridine, *ortho* protons), -0.61 (br, 2H, pyridine, *meta* protons), 3.9 (br, 5H, CH_{Fe}), 4.2 (br, 2H, CH_{Fe}), 4.4 (br, 2H, CH_{Fe}). **Elemental Analysis:** % calculated for C₁₉H₂₂Cl₄FeN₂NaO₂RuS(0.5C₃H₆O): C, 35.52; H, 3.64; N, 4.04; Found: C, 35.29; H, 3.85; N, 4.00. **HR-ESI-(+)-MS**, m/z: 639.8560; calculated for C₁₉H₂₂Cl₄FeN₂NaO₂RuS⁺ 639.8544. **M.P.** (210 °C, decomp.)

Na[trans-RuCl₄(DMSO)(imidazolyl-ferrocene)], RuL2:

Na[trans-RuCl₄(DMSO)₂] (120 mg, 0.284 mmol) was placed in a round-bottom flask with acetone (20 mL) and stirred until completely dissolved. In a separate flask, imidazolyl-ferrocene (92.04 mg, 0.284 mmol) was dissolved in acetone (20 mL) and then added to the original flask. The mixture was stirred for 1.5 h at room temperature. The solvent was removed under vacuum. The product was put on the high vacuum desiccator for 0.5 h. Yield: 91%, 188 mg. The crude product (100 mg) was further purified by column chromatography using a CombiFlash® (Teledyne Isco) Rf 150 flash chromatography system on RediSep Rf Gold® normal phase silica columns. A gradient solvent system was used beginning with 100% DCM, then decreased to a 3:1 ratio with DCM:ethyl acetate and finally up to 100% ethyl acetate. The solvent was removed under vacuum and the resulting powder was dried under high vacuum. Total yield: 57%, 57 mg. IR (v/cm⁻¹): 3139, 1689, 1523, 1469, 1311, 1277, 1071, 1019, 831, 769, 628, 487, 427, 335. ¹H NMR (400 MHz, D₂O): δ = -14.9 (br, 6H, (DMSO)), -3.23 (br, 1H, NC₃H₃N, *meta* proton), -0.18 (br, 2H, COOCH₂CH₂), 1.50 (br, 2H, COOCH₂), 4.13 (br, 5H, CH_{Fe}), 5.47 (br, 2H, CH_{Fe}); **Elemental Analysis:** % calculated for C₁₈H₂₂Cl₄FeN₂NaO₃RuS(0.5C₂H₆O•0.25C₆H₁₄): C, 34.55; H, 4.03; N, 3.93; Found: C,

34.33; H, 3.46; N, 3.57. **HR-ESI-(+)-MS**, m/z : 643.8516; calculated for $C_{18}H_{22}Cl_4FeN_2NaO_3RuS^+$ 643.8498. **M.P.** (186 °C, decomp.)

Na[trans-RuCl₄(DMSO)(4-ferrocenoyl pyridine), RuL3:

RuL3 was prepared in a similar manner to that of **RuL1** using the appropriate ferrocenyl moiety. Yield: 76%, 82.6 mg. The crude product was further purified by column chromatography using a CombiFlash® (Teledyne Isco) Rf 150 flash chromatography system on RediSep Rf Gold® normal phase silica columns. A gradient solvent system was used beginning with 100% DCM, then decreased to a 3:1 ratio with DCM:ethyl acetate and finally up to 100% ethyl acetate. The solvent was removed under vacuum and the resulting powder was dried under high vacuum. Yield: 44.3%, 36.6 mg. IR (ν/cm^{-1}): 3485, 1725, 1605, 1449, 1235, 1193, 1080, 1013, 911, 827, 482, 332. 1H NMR (400 MHz, D₂O): δ = -14.3 (br, 6H, (DMSO)), -8.3 (br, 2H, NC₅H₄, *ortho* protons), -1.9 (br, 2H, NC₅H₄, *meta* protons), 3.52 (s, 5H, CH_{Fe}), 3.82 (br, 2H, CH_{Fe}), 4.13 (br, 2H, CH_{Fe}).

Elemental Analysis: % calculated for $C_{18}H_{19}Cl_4FeNNaO_3RuS(1.5H_2O)$: C, 31.88; H, 3.27; N, 2.07; Found: C, 31.83; H, 3.16; N, 1.98. **HR-ESI-(+)-MS**, m/z : 626.8282; calculated for $C_{18}H_{19}Cl_4FeNNaO_3RuS^+$ 626.8233. **M.P.** (230 °C, decomp.)

4.6 DNA Mobility Shift Assays

Plasmid DNA (pBluescript, ~ 3000 basepairs) was transformed into *E. coli* competent cells and plated on pre-warmed LB agar plates containing 50 μ g/mL ampicillin antibiotic. Single colonies were picked and liquid cultures were subsequently grown in LB media in an incubator/shaker for 12 h at 37 °C (350 rpm shaking). Plasmid DNA was extracted

using Qiagen Maxi kits and characterized using agarose gel electrophoresis and UV/vis spectroscopy. Purified circular plasmid was linearized using EcoR1 enzyme (New England Biolabs). The digested product was purified using column chromatography and characterized using UV/vis and agarose gel electrophoresis. Metal complex incubation assays were conducted with cisplatin, **RuL1**, **RuL2**, and **RuL3**. Cisplatin was purchased from Sigma-Aldrich and used without further purification. Briefly, metal complexes were incubated at varying concentrations with 1 µg of linearized plasmid DNA at 37 °C for 24 h. The reactions were carried out in 30 µL in phosphate-buffered saline (pH 7.1). After incubation, loading dye was added to the samples, which were then resolved on a 1.2% agarose gel. The gel was run for 4.5 h at 100V constant voltage. The gels were stained with ethidium bromide and imaged on a BioRad GelDoc EZ Imager machine.

4.7 BSA adduct with Na[trans-RuCl₄(DMSO)(imidazolyl-ferrocene)], BSA-RuL2:

RuL2 (2.0 mg) was put in a disposable centrifuge tube and dissolved in 10 mM, pH 7.4 phosphate buffer (0.6 mL). Bovine serum albumin (**BSA**) (198 mg) was put in another disposable centrifuge tube and dissolved in phosphate buffer (PB) (2.0 mL). Vortexing the mixture was necessary to dissolve it completely. Once dissolved, the **RuL2** solution was added to the **BSA** solution, mixed and put in the incubator at 37°C for four h. After incubation, a small amount of the solution (0.2 mL) was pipetted into a micro-centrifuge tube so that it could be analyzed by the UV-visible spectrometer at the same concentration as the starting complex. The rest of the solution was pipetted into a 10 kD spin filter and a counter centrifuge tube was filled with water. The centrifuge was set to the speed of 5250 rpm and the product was run for 15 min at 10°C. To wash the product,

deionized water was added to the top of the filter and to the counter centrifuge tube, and the centrifuge was run for another 15 min. After washing, the product was transferred into a vial and dried under vacuum for two h. The same procedure was used to make the adducts **BSA-RuL2** and **BSA-RuL3**.

4.8 Spectroscopic Monitoring Experiments:

4.8.1 UV/visible (UV/vis)

The Cary 100 UV/Vis spectrometer was used on Scanning Kinetics mode to observe binding of all of the ruthenium-ligand complexes to the protein, **BSA**, over 4 h at 37 °C with approximate initial concentrations of 0.05-0.2 mM in metal compound. Wavelength sweep range was from 700 nm down to 240 nm. The reactions of **RuL1**, **RuL2**, and **RuL3** with BSA protein were carried out in phosphate buffer (PB) buffer (pH 7.4). The reactions were monitored by UV/vis, and absorption spectra were obtained at one minute intervals for 30 min, and 20 minute intervals for three and a half additional h, kept incubated at 37 °C. The compound and protein solutions were mixed in a quartz cuvette then immediately monitored for the remainder of the study.

4.8.2 Fluorimetry.

A 50 micromolar BSA solution was made in phosphate-buffer saline (PBS) (10 mM, pH 7.4). The solution was then excited at 280 nm and the emission was tracked from 300-400 nm. The tryptophan residues in BSA produced an emission signal at 344 nm. Two microliters of a 10 millimolar stock solution of **RuL1** was added to 3 mL of the BSA solution, and the emission signal at 344 nm was measured immediately following the addition. This process was repeated until 34 microliters of the **RuL1** solution had been

added. This was followed by a 4 microliter addition, then an 8 microliter addition, and finally a 10 microliter addition until the BSA peak was completely quenched with 56 microliters. This process was then repeated with the remaining two compounds, **RuL2** and **RuL3**.

4.8.3 NMR.

A saturated solution of the metal compound which consisted of approximately three mg of compound dissolved in 1 mL of D₂O was made in an NMR tube. NMR studies that included 37°C incubation were done in the following fashion. The initial NMR of a 37°C incubation study was done at room temperature before the tube was placed in the incubator at 37°C. The NMR tube was then incubated for 20 min before it was removed from the oven and analyzed. There was about 15 min of time at room temperature to slow down the reaction for monitoring during analysis. The NMR tube was then returned to the oven for additional incubation. The times on the 37°C NMR studies actually refer to the total amount of time the tube was incubated in the oven. For example, a 40 minute incubated sample would actually take about 70 min real time.

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Table 1. Selected bond lengths (Å) for dinuclear compounds

	RuL1	RuL2	RuL3
Ru(1)-Cl(1)	2.3417(8)	2.3730(6)	2.3595(7)
Ru(1)-Cl(4)	2.3541(8)	2.3095(7)	2.3387(7)
Ru(1)-N(1)*	2.111(3)	2.0864(17)	2.111(2)
Ru(1)-S(1)	2.2876(8)	2.2809(5)	2.2915(7)

*N(2) for RuL2 due to imidazole ligand

Table 2. Selected bond angles (deg.) for dinuclear compounds

	RuL1	RuL2	RuL3
Cl(1)-Ru(1)-Cl(4)	89.18(4)	89.76(3)	91.08(3)
Cl(1)-Ru(1)-N(1)*	88.84(7)	87.63(6)	88.94(6)
Cl(4)-Ru(1)-N(1)*	89.47(7)	87.78(6)	88.25(6)
Cl(1)-Ru(1)-S(1)	93.89(3)	92.32(2)	93.05(2)
Cl(4)-Ru(1)-S(1)	89.76(3)	93.42(2)	88.33(3)
N(1)*-Ru(1)-S(1)	177.15(7)	178.88(6)	176.07(6)

*N(2) for RuL2 due to imidazole ligand

Table 3: Summary of crystal data and intensity collection and structure refinement parameters for complexes **RuL1**, **RuL2**, and **RuL3**.

Compound	RuL1	RuL2	RuL3
Empirical Formula	C ₃₈ H ₄₄ Cl ₈ Fe ₂ N ₄ Na ₂ O ₄ Ru ₂ S ₂ • 2 acetone	C ₁₈ H ₂₂ Cl ₄ FeN ₂ NaO ₃ RuS • 1 acetone	C ₇₂ H ₇₆ Cl ₁₆ Fe ₄ N ₄ Na ₄ O ₁₂ Ru ₄ S ₄ • 4 acetone
Molecular Weight	1444.47	726.22	2836.75
Crystal color, habit	block, yellow	block, orange	block, orange
Crystal size (mm)	0.33 x 0.24 x 0.15	0.28 x 0.22 x 0.15	0.20 x 0.15 x 0.10
Temperature (K)	125(2)	125(2)	125(2)
Crystal System	orthorhombic	triclinic	triclinic
Space group	Pbca	P-1	P-1
Unit cell dimensions			
a (Å)	20.7618(8)	10.2879(4)	11.4265(5)
b (Å)	22.5885(9)	12.3851(5)	12.4896(6)
c (Å)	23.9496(10)	13.1027(9)	19.9608(9)
α (°)	90.00	108.1350(10)	75.0050(10)
β (°)	90.00	96.3600(10)	77.4310(10)
γ (°)	90.00	113.3210(10)	82.4410(10)
V (Å ³), Z	11231.8(8), 8	1403.60(12), 2	2677.1(2), 1
D _{calc} (mg m ⁻³)	1.708	1.718	1.760
Abs. coeff. (μ , mm ⁻¹)	1.552	1.556	1.628
Φ range collected (deg.)	1.80 - 30.52	1.94 – 30.03	1.69 – 30.53
Completeness to Φ max (%)	100.0	99.6	99.3
Reflns collected/unique ($R(int)$)	176929 / 17169 (0.0686)	22288 / 8181 (0.0192)	43947 / 16281 (0.0348)
Data/restraints/parameters	9061 / 2 / 645	8181 / 267 / 396	9917 / 0 / 621
R_I , wR_2 (all data)	0.0688, 0.0973	0.0379, 0.0751	0.0531, 0.0893
Goodness of fit on F^2	1.012	1.030	1.037
Largest diff peak/hole (e/Å ³)	1.64, -1.11	1.00, -1.07	2.63, -1.97

Scheme 1: Synthesis of the Complexes

Figure 1: Molecular structure of compound **RuL1** (dimeric view) (50% probability level).

Figure 2: Molecular structure of compound **RuL2** (50% probability level)

Figure 3: Molecular structure of compound **RuL3** (dimeric view) (50% probability level).

Figure 4: Electrophoretic mobility shift assay of linearized pBluescript plasmid DNA at varying concentrations (in μM) of Cisplatin, **RuL1**, **RuL2**, and **RuL3** on a 1.2% agarose gel stained with ethidium bromide. All DNA samples were incubated with the corresponding metal complex at 37°C for 24 h.

Figure 5: Cl *K-edge* and Ru *L₃-edge* XAS of metal complexes (**RuL1**, **RuL2**, **RuL3**) as controls (top) and of samples incubated with BSA for 4 h at 37 °C in PB (bottom).

Figure 6: Fe *K-edge* XAS spectra of compounds (top) and BSA-adducts (bottom).

Figure 7: Near edge Ru *K-edge* spectra of complexes and BSA-adducts (left). Fourier Transform of Ru *K-edge* EXAFS in the R-space (right).

Scheme 1

Figure 1

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7