Novel microwave synthesis of half-sandwich \([\eta^6-C_6H_6]Ru\) complexes and an evaluation of the biological activity and biochemical reactivity

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We have used a novel microwave-assisted method to synthesize a pair of half-sandwich ruthenium–arene–thiosemicarbazone complexes of the type \([\eta^6-C_6H_6Ru(TSC)Cl]\)PF\textsubscript{6}. The thiosemicarbazone (TSC) ligands are 2-(anthracen-9-ylmethylene)hydrazinecarbothioamide and 2-(anthracen-9-ylmethylene)-N-ethylhydrazinecarbothioamide derived from 9-anthraldehyde. The complexes are moderately strong binders of DNA, with binding constants of \(10^4\) M\(^{-1}\). They are also strong binders of human serum albumin, having binding constants of the order of \(10^5\) M\(^{-1}\). The complexes show some in vitro anticancer activity against human colon cancer cells, Caco-2 and HCT-116, with positive therapeutic indices. They did not show any activity as antibacterial agents against the organisms that were studied. Copyright © 2013 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: thiosemicarbazone; organometallic

Introduction

Despite the fact that since the discovery of the platins (Fig. 1) not a very large number of metal compounds have entered clinical practice, intensive research into the development of such compounds for medicinal purposes has continued. A number of metals are currently being investigated as potential alternatives to platinum as the central atom in complexes used as antitumor agents. Of these metals, ruthenium appears to be the leading candidate with two complexes – NAMI-A (an antimetastatic) and KP1016 or FFC14a (anticancer) – currently in various stages of clinical trials.\textsuperscript{[1,2]} Another class of ruthenium complexes that has elicited strong research interest has been developed by Sadler and co-workers\textsuperscript{[3–5]} as well Dyson and his co-workers.\textsuperscript{[6–8]} These organometallic complexes with the general formula \([\text{arene}Ru(LL)Cl]\)\(^{+}\) are showing strong promise as antitumor (antimetastatic\textsuperscript{[7]} and anticancer\textsuperscript{[3]} ) complexes.

A stumbling block to the development of ruthenium metallo drugs is that the true biological targets of antitumor ruthenium complexes have not been definitively elucidated and may actually vary amongst the different classes of compounds. Certainly, DNA is a part of the molecular target spectrum. However, it has been noted that the biological activity of NAMI-A is not related to its DNA binding ability. In fact, it is believed that proteins associated with metastatic cells might be the true molecular targets.\textsuperscript{[9,10]} KP1016 is also believed to be following a mechanism involving transferrin. We have reported\textsuperscript{[11,12]} that some half-sandwich ruthenium complexes, \([\eta^5-p\text{-cymene}Ru(\text{thiosemicarbazone})Cl]\)\(^{+}\), can inhibit the activity of the topoisomerase II enzyme. Thus it is clear that proteins are potentially high-valued targets for ruthenium drugs.\textsuperscript{[13]} In addition, binding to biomolecules is considered important for both transport and deactivation. In particular, serum proteins such as human serum albumin (HSA) and transferrin play a prominent role in drug pharmacokinetics, as they are usually encountered very soon after drug administration. Therefore, investigating biological interactions is crucial for understanding the behavior and mode of action of potential drugs in biological environments.

We have been investigating the potential of organometallic ruthenium complexes containing bioactive thiosemicarbazone ligands to act as anticancer and antibacterial agents.\textsuperscript{[11,12]} Sadler\textsuperscript{[5]} has pointed out that the nature (particularly the steric nature) of the arene moiety has a significant effect on the anticancer effectiveness of the compounds. In this contribution we therefore follow up our previous studies by reporting on two complexes of the type \([\eta^6-C_6H_6]Ru(\text{anthraldehyde thiosemicarbazone})Cl]\)PF\textsubscript{6} (Fig. 2). Their synthesis, characterization and biophysical reactivity with DNA and small model proteins, along with their anticancer and antibacterial properties, are reported.

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Materials and Methods

Analytical or reagent-grade chemicals were used throughout. All chemicals, including solvents, were obtained from Sigma-Aldrich or other commercial vendors and used as received. Microanalyses (C, H, N) were performed by Columbia Analytical (Tucson, AZ, USA). Proton and carbon NMR spectra were recorded in DMSO-d$_6$ on a Varian Mercury 300 spectrometer operating at room temperature. The residual $^1$H and $^{13}$C present in DMSO-d$_6$ (2.50 and 39.51 ppm respectively) were used as internal references. The splitting of proton resonances in the reported $^1$H NMR spectra is defined as $s$ = singlet, $d$ = doublet, $t$ = triplet, $q$ = quartet and $wm$ = wide multiplet. IR spectra in the range 4000–500 cm$^{-1}$ were obtained using KBr pellets or using the ATR accessory on a Nicolet 6700 FT-IR spectrophotometer. (Peaks are defined as $w$ = weak and $b$ = broad). Cyclic voltammetric (CV) data were collected on a Bioanalytical Systems Inc. Episilon workstation on a C3 cell stand at 296 K. CH$_2$Cl$_2$ solutions (10 M) containing 0.1 M tetrabutylammonium hexafluorophosphate were saturated with nitrogen for 15 min prior to each run. A blanket of nitrogen gas was maintained throughout the measurements. The working electrode was polished before each experiment with alumina slurry. Absorption spectra were recorded on an Agilent 8453A spectrophotometer. Fluorescence spectra were recorded on a Varian Cary Eclipse spectrophotometer. Melting points (triplicate measurements) were determined in open capillaries on a Buchi B-545 melting point apparatus and are uncorrected.

Syntheses

The ligands ATSC (2-(anthracen-9-ylmethylenelydrazinecarbothioamide) and EtATSC (2-(anthracen-9-ylmethylenely)-N-ethylhydrazinecarbothioamide) were synthesized as previously described.[14] The starting metal compound, [(η$^6$-C$_6$H$_6$)RuCl$_2$]$_2$, was synthesized as described in the literature.[15]

Synthesis of metal complexes

$[(η^6$-C$_6$H$_6$)Ru(ATSC)]PF$_6$. 1. In a 35 ml microwave reaction vessel, 100 mg (0.20 mmol) [(C$_6$H$_6$)RuCl$_2$]$_2$, and 112 mg (0.40 mmol) ATSC were suspended in 15 ml methanol. The yellow-brown suspension was degassed with argon for 10 min. After the degassing, the reaction mixture was heated in a microwave reactor according to the following method: temperature = 67°C, power = 225 W, $P_{max}$ = 250 psi, time = 10 min, with high stirring. At the end of the reaction, the reddish solution that was formed was cooled and poured into approximately 10 ml of a cold solution of saturated aqueous KPF$_6$, resulting in an orange suspension. The mixture was filtered, washed with water and ether and air-dried. Yield: yellow-brown solid, 208 mg (82%).

$[(η^6$-C$_6$H$_6$)Ru(EtATSC)]PF$_6$. 2. The IR spectra in the range 4000–500 cm$^{-1}$ were obtained using KBr pellets or using the ATR accessory on a Nicolet 6700 FT-IR spectrophotometer. (Peaks are defined as $w$ = weak and $b$ = broad). Cyclic voltammetric (CV) data were collected on a Bioanalytical Systems Inc. Episilon workstation on a C3 cell stand at 296 K. CH$_2$Cl$_2$ solutions (10 M) containing 0.1 M tetrabutylammonium hexafluorophosphate were saturated with nitrogen for 15 min prior to each run. A blanket of nitrogen gas was maintained throughout the measurements. The working electrode was polished before each experiment with alumina slurry. Absorption spectra were recorded on an Agilent 8453A spectrophotometer. Fluorescence spectra were recorded on a Varian Cary Eclipse spectrophotometer. Melting points (triplicate measurements) were determined in open capillaries on a Buchi B-545 melting point apparatus and are uncorrected.
Half-sandwich ruthenium complexes: biological and biochemical activity

$^{13}$C NMR: 180.41 (–C=S), 149.77 (–C=N) and 126.02–131.27 (benzene and anthracene moieties) ppm. IR (cm$^{-1}$): 3238 (b), 3179 (b), 3090, 1618, 1531, 1523, 1375, 1261, 1065, 1039, 1004, 898, 876, 833, 804, 735, 632. UV-visible (buffer 1, $\lambda_{max}$ (nm) (log $\varepsilon$)): 255 (4.66), 390 (3.79), 287 (shoulder, 4.15).

$\text{[(C}_{6}\text{H}_{6})\text{RuCl(C}_{2}\text{H}_{6})\text{]}PF_{6}$, 2. This complex was synthesized in a similar manner to 1 using $\text{[C}_{6}\text{H}_{6})\text{RuCl}_{2}$ (200 mg, 40 mmol) and EtATSC (246 mg, 0.80 mmol) using the microwave method: temperature = 75°C, power = 225 W, $P_{max}$ = 250 psi, time = 12 min, with high stirring. Yield: yellow-brown solid, 457 mg (86%). Elemental analysis for C$^{24}$H$^{23}$Cl$^{6}$N$^{3}$PRuS; calculated/found: C 43.22/43.22; H 3.48/3.17; N 6.30/6.75. $^1$H NMR: 11.53(s, NH), 9.52 (s, aldehydic CH), 8.70(s, anthracene ring), 8.14 (d, J = 9 Hz, anthracene ring), 7.56–7.66 (m, anthracene ring), 7.37 (s, anthracene ring), 5.95 (s, benzene ring), 4.50 (s, NH–C=C$_2$H$_3$), 3.60 (ethyl CH$_3$) and 1.13 (ethyl CH$_3$) ppm. $^{13}$C NMR: 125.87–131.12 (benzene and anthracene moieties) ppm. IR (cm$^{-1}$): 3294 (w), 3179 (w), 3067, 2997, 2926, 1620, 149.77, 149.77, 139.80, 131.27, 126.02, 114.67, 106.44, 100.44, 880, 832, 734, 634. UV-visible (buffer 1, $\lambda_{max}$ (nm) (log $\varepsilon$)): 255 (4.71), 390 (3.85).

**Cell Culture**

The cell lines used were two human colon cancer cells: HCT116 (human colon carcinoma) and Caco-2 (human epithelial colorectal adenocarcinoma). In addition, normal human colon cells CCD-18Co (human epithelial colorectal adenocarcinoma). The cell lines used were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained at the University of Rhode Island. Caco-2 cells were grown in EMEM medium supplemented with 10% v/v fetal bovine serum, 1% v/v non-essential amino acids, 1% v/v L-glutamine and 1% v/v antibiotic solution and were used from stock solutions were stored at 4°C in a humidified incubator for 3 h. Absorbance at 490 nm (OD$_{490}$) was monitored to drug addition. Test samples and a positive control – etoposide 4 mg mL$^{-1}$ (Sigma) – were solubilized in DMSO by sonication. All samples were diluted with media to the desired treatment concentration and the final DMSO concentration per well did not exceed 0.5%. Control wells were also included on all plates. Following a 24 h, 48 h or 72 h drug incubation period at 37°C with serially diluted test compounds, MTS, in combination with the electron coupling agent phenazine methosulfate, was added to the wells and cells were incubated at 37°C in a humidified incubator for 3 h. Absorbance at 490 nm (OD$_{490}$) was monitored with a spectrophotometer (SpectraMax M2, Molecular Devices Corp., operated by SoftmaxPro v.4.6 software, Sunnyvale, CA, USA) to obtain the number of surviving cells relative to control populations. The results were expressed as the median cytotoxic concentrations (IC$_{50}$ values) and were calculated from six-point dose–response curves using fourfold serial dilutions. Each point on the curve was tested in triplicate. Data were expressed as mean ± SE for three replicates on each cell line.

**Antimicrobial assay**

The in vitro antimicrobial activity of ligands 1 and 2 was investigated as the minimum inhibitory concentration (MIC) against a number of Gram-positive (*Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*) bacteria strains. The bacteria were maintained on nutrient agar and cultured in Mueller Hinton Broth, and compounds were dissolved in DMSO. The MIC was determined from a microdilution method. Approximately 30 ml bacteria culture was incubated overnight at 37°C to produce exponentially growing cells. This was then diluted to yield a suspension containing 1.5 × 10$^6$ CFU ml$^{-1}$ (based on comparison of the turbidity to a 0.5 McFarland standard). Subsequently, 250 μl of this bacteria mixture was then inoculated into a sterile 96-well microplate. Various amounts of the compounds were added to wells to give predetermined concentrations ranging from 2 to 50 μg. The well absorbances (at 600 nm) were recorded on a BioTek Synergy HT microplate reader immediately after inoculation. The microplates were incubated for 24 h at 37°C, and then the absorbance was recorded again after 24 h. The MIC was defined as the lowest concentration of compound that did not produce any visible cell growth or change in absorbance after incubation. Solvent, media and positive growth controls were included on each plate. Ampicillin or streptomycin was used as a standard comparison. Each plate contained three replicates of each concentration and two separate experiments were done.

**DNA Interaction Studies**

All experiments involving the interaction of the complexes with DNA were carried out in Tris buffer (5 mM Tris, 50 mM NaCl, pH 7.20). Stock solutions of ct-DNA was prepared by dissolving commercial nucleic acids in buffer and storing at 4°C for 24 h to attain homogeneity. After dilutions DNA concentration per nucleotide phosphate was determined spectrophotometrically using the molar absorption coefficient of 6600 m$^{-1}$ cm$^{-1}$ at 260 nm.$^{[17]}$ A solution of ct-DNA in the buffer gave a ratio of UV absorbance at 260 and 280 nm of ≥1.8, indicating that the DNA was sufficiently free from protein contamination.$^{[18]}$ The DNA stock solutions were stored at 4°C and used within 4 days after their preparation. Doubly purified water used in all experiment was from a Milli-Q system.
Viscosity measurements

Viscosity studies were done using a Cannon-Manning semi micro-dilution viscometer (type 7S, Cannon Instruments Co., State College, PA, USA) immersed vertically in a thermostated water bath maintained at 30.0 ± 0.1 °C. The viscosity for DNA was measured in the presence and absence of the metal complexes. The DNA concentration was maintained at 100 μM, while the complex concentration was varied from 0 to 60 μM. Data are presented as [(η/η0)1/3 versus 1/R, where R = [DNA/complex], η is the viscosity of DNA in the presence of the complex and η0 is the relative viscosity of DNA alone. Relative viscosity values were calculated from the observed flow time of DNA solution (t) and corrected for the flow time of buffer alone (t0), using the expression η0 = (t – t0)/t0. Flow time was measured with a digital stopwatch, each sample was measured three times and an average flow time was used.

Absorbance titration experiments

Spectroscopic titrations were carried out at room temperature to determine the binding affinity between the complexes and ct-DNA. A constant concentration of the complexes (1.98 × 10⁻⁵ M) in 1 cm quartz cells was treated with aliquots of a stock concentrated solution of the DNA. The absorption of the added DNA was subtracted from the reaction solution. The values of the intrinsic binding constant Kb illustrating the binding strength of the complexes with ct-DNA were determined from the following equation (1):

\[
\frac{[\text{DNA}]}{\varepsilon_a - \varepsilon_f} = \frac{[\text{DNA}]}{\varepsilon_b - \varepsilon_f} + \frac{1}{K_b (\varepsilon_b - \varepsilon_f)}
\]

where εa, εf and εb correspond to the molar absorptivities of the metal complex after each addition of ct-DNA, for the free metal complexes and for the metal complexes in the fully bound form respectively.

Fluorescence titration experiments

In the ethidium bromide (EB) fluorescence displacement experiment, 3 ml of a solution that is 10 μM DNA and 0.33 μM EB (saturated binding levels) in Tris buffer was titrated with concentrated solutions of the complexes, producing solutions with various mole ratios of complex to ct-DNA. After each addition and before measurement, the solution was stirred for 5 min while standing at the appropriate temperature. The fluorescence spectra of the solution were obtained by exciting at 520 nm and measuring the emission spectra from 530 to 700 nm using 5 nm slits. Temperature was controlled using a single-cell Peltier accessory.

Chemical Nuclease Activity

DNA unwinding and cleavage ability of the complexes were evaluated by agarose gel electrophoresis of supercoiled pBR322 DNA. The experiments were done with or without exposure to long-wavelength UV light. Samples of pBR322 DNA (0.1 μg μl⁻¹) were incubated with the complexes (at concentrations ranging from 10 to 200 μM) in Tris buffer (50 mM Tris, 18 mM NaCl, pH 8.2) at 37°C for 30 min in the dark. For the photo-activated experiments, prior to incubation at 37°C, the samples were subjected to 365 nm light for 1 h. In the mechanistic experiments samples containing sodium formate (100 μM), potassium iodide (400 μM) or sodium azide (100 μM) were included in the experiments. The reactions were quenched by addition of 3 μl loading buffer (0.25% bromophenol blue and 15% Ficoll in water). Samples of the reaction mixtures were loaded onto a 1% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.2). The gels were subjected to electrophoresis for 2 h at 70 V, followed by staining with 0.5 μg ml⁻¹ ethidium bromide for 30 min. The bands on the gel were visualized under UV light and photographed using a GEL Logic 440 imaging system with Kodak molecular imaging software.

Reaction with Human Serum Albumin (HSA)

For fluorescence titration, a similar procedure to the EB displacement experiments was done. Solutions of HSA (fatty acid free) were prepared in Tris buffer (50 mM Tris, pH 7.40, 100 mM NaCl) and stored in the dark at 4°C. The protein concentration was determined spectrophotometrically using the molar absorptivity of 3.6 × 10⁴ M⁻¹ cm⁻¹ at 280 nm.[20] In the experiments, a 3.0 ml solution of HSA (5 μM) was placed in a quartz cuvette and titrated with various amounts of a concentrated solution of the complex, producing solutions with varied mole ratios of complex to HSA. The complex concentration ranged from 3 to 30 μM. The fluorescence spectra of the solutions were obtained by exciting at 295 nm and measuring the emission spectra from 300 to 500 nm.

Binding site competitive experiments

Site marker studies between HSA and the metal complexes were accomplished by fluorescence titration. Equimolar amounts (5 × 10⁻⁶ M) of HSA and either flufenamic acid or phenylbutazone were incubated for 30 min at room temperature. The mixtures were subsequently titrated with the metal complexes and the decrease in fluorescence of the solution was monitored. The instrumental conditions used for the HSA reactions were used for these experiments as well.

Discussion

Synthesis and Characterization

The thiosemicarbazones used in this study have been previously reported by us.[14] The complexes (1 and 2) were synthesized according to a novel microwave-enhanced reaction of [Ru(C⁶H₆)Cl₂] with 2 equiv. of the thiosemicarbazone in methanol followed by addition of an aqueous solution of potassium hexafluorophosphate. The yellow-brown solids that were obtained were quite soluble in DMSO but less so in alcohols. Based on elemental analysis and spectroscopic data, we propose that the complexes are best formulated as [((η⁶-C₆H₆)Ru(TSC)Cl)(TSC)Cl]PF₆ (Fig. 2).

The electrochemical (cyclic voltammetry) behavior of the complexes has been studied in DMSO at a scan rate of 100 mV s⁻¹, using tetrabutylammonium hexafluorophosphate as the supporting electrolyte. The complexes showed similar features in the investigated sweep range (0–2.0 V). There is one irreversible oxidation wave at 1.18 V (Fig. S1, supporting information) corresponding to the Ru(II)/Ru(III) couple. This peak becomes less pronounced as scan rate is increased and moves to 1.11 V concurrently. This Ru-centered oxidation is irreversible even up to a scan rate of 500 mV s⁻¹. This pattern of behavior is similar to what we have observed for similar complexes bearing a p-cymene arene cap. For such complexes Epa values range from 1.02 to 1.11 V.
The thiosemicarbazone ligand offers two potential ligating forms (via tautomerism) and we propose that it coordinates as the thione tautomer. This idea is suggested by the infrared spectroscopic data for the complexes. The amine region shows two (for 1) and three (for 2) broad and weak peaks; these of course correspond to the amine and hydrazinic hydrogens. The hydrazinic hydrogen would have been absent had the thiol tautomer (as the ionic form) been the coordinated species. These peaks are shifted from the positions in the free ligands. The coordination through the azomethine nitrogen and the thione sulfur is also obvious in the infrared spectra. The ν(C=NC) bands of thiosemicarbazones are sensitive to metal chelation, providing evidence for metal coordination, and the peak due to this chromophore shifts by about 70 cm⁻¹ (to higher energies relative to the free ligand). The C=S peaks also change on coordination. The 10 cm⁻¹ shift (to lower wavenumbers) of the peak attributable to the thioamide IV ν(C=S) band on complexation is usually interpreted as evidence for coordination through the neutral ligand.[21]

The ¹H NMR spectra of the complexes are essentially a combination of the signals from the ligands plus those from the benzene moiety. In the spectra all indications are that the ligands remain neutral, as evidenced by the presence of the hydrazinic protons near 11.49 ppm for 1 and 11.53 ppm for 2. The signals from the anthracene moiety are common for both complexes. There are singlets at 7.37 ppm and 8.70 ppm and a doublet centered at 8.14 ppm (J = 9 Hz). There is also a multiplet at 7.56–7.66 ppm. The aldehydic hydrogen is seen at 9.44 ppm for 1 and 9.52 ppm for 2. None of these peaks are significantly different from the free ligands. The –NH₂ protons in 1 and the NH (amine) proton in 2 show up at 4.67 ppm and 4.50 ppm, respectively. In the case of 1 this is significantly different from the free ligand. The ethyl group has signals at 1.13 ppm (–CH₃) and 3.60 ppm (–CH₂). In the NMR spectra there is also a singlet at 5.94 ppm. This is attributed to the benzene ring and its position is typical for complexes like these.

Cytotoxicity Studies

The in vitro antiproliferative activities of complexes 1 and 2 (as measured by the IC₅₀) were evaluated against a panel of human tumor cell lines: HCT-116 (colon carcinoma) and Caco-2 (epithelial colorectal adenocarcinoma), and a non-malignant cell line, CCD-18Co (colon fibroblasts). This was achieved by means of a colorimetric (MTS) assay which measures mitochondrial dehydrogenase activity as an indication of cell viability. The effects of the compounds on the viability of these cells were evaluated after an exposure period of 24 h, 48 h and 72 h. Etoposide, a potent anti-neoplastic drug, was used as a standard comparison treatment. Both complexes showed activity and their corresponding IC₅₀ values, corresponding to inhibition of cancer cell growth at the 50% level, are listed in Table 1. Generally, the longer the exposure time, the more cytotoxic were the complexes, with 72 h exposure being as much as two times more effective compared to 24 h exposure. Both 1 and 2 show good cytotoxic profiles, with IC₅₀ values ranging from 23 to 104 μM (after 72 h). Between cell lines there are no discernible trends. The Caco-2 cells were more sensitive to complex 1 than were the HCT-116 cells, by a factor of almost two. On the other hand, for complex 2 the pattern was reversed, with the HCT-116 cells being more sensitive to the compound than the Caco-2 cells. In general, however, complex 2 displayed higher antiproliferative activities than 1;

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>HCT-116</th>
<th>Ti</th>
<th>Caco-2</th>
<th>Ti</th>
<th>CCD-18Co</th>
<th>Ti</th>
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<tbody>
<tr>
<td>24 h</td>
<td>142 ± 5</td>
<td>1.58</td>
<td>92.7 ± 10.4</td>
<td>2.46</td>
<td>784.6 ± 32</td>
<td>2.12</td>
</tr>
<tr>
<td>48 h</td>
<td>117 ± 3</td>
<td>1.15</td>
<td>71.6 ± 12.2</td>
<td>1.14</td>
<td>62.6 ± 4.9</td>
<td>1.25</td>
</tr>
<tr>
<td>72 h</td>
<td>104 ± 3</td>
<td>1.97</td>
<td>59.0 ± 2.3</td>
<td>1.80</td>
<td>384.4 ± 4.9</td>
<td>1.60</td>
</tr>
<tr>
<td>Etoposide (72 h)</td>
<td>18.3 ± 1.4</td>
<td>2.30</td>
<td>16.5 ± 2.0</td>
<td>2.56</td>
<td>4.2 ± 1.0</td>
<td>4.26</td>
</tr>
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**Table 1.** Anti-proliferative activity represented by IC₅₀ values (μM) of complexes 1 and 2 in a panel of three human cell lines – 2 tumorigenic (HCT-116 and Caco-2) and one normal (CCD-18Co).
IC$_{50}$ values were 4.5 times less for the HCT-116 cells and 2 times less for the Caco-2 cells. When compared to the standard etoposide treatment neither complex was as effective. An important point to make, however, relates to the therapeutic indices for the test compounds. The therapeutic index (TI) is a number representing the ratio of the IC$_{50}$ values against the CCD-18Co cells to that for the cancer cells. The larger this number is, the more selective are the compounds for the cancer cells over the normal cells. Consider the 72 h exposure as seen in Table 1. Despite the fact that complex 2 was more active than 1 against the Caco-2 cells, it has a TI that is two times less than that of complex 1. In addition, etoposide is 3.5 times more active than 1 against the Caco-2 cells, but the complex has the better therapeutic index: 3.47 versus 2.56. Thus, while it might appear that these complexes are not fantastic in their activity, there is enough evidence to suggest that further study is warranted. As a final point to note, considering the structural nature of the thiosemicarbazone moiety, there may be some effect of the alkyl substituent located on the amine nitrogen on the cytotoxicity of the complexes, with the ethyl group appearing to enhance antiproliferative activity. In similar complexes, but with an extended aromatic group as the arene face cap, it has been suggested that the arene facilitates strong interaction with DNA and that DNA binding plays a role in the mechanism of cytotoxicity.[5]

**Reaction of Complexes with DNA**

**Electronic absorption spectroscopy**

Metal complexes binding to DNA can do so via a variety of mechanisms including intercalation between the base pairs, groove binding and electrostatic binding. Electronic absorption spectroscopy is a common and effective method to examine the binding interactions (modes and extent) of metal complexes with DNA.[22-24] Given the structure of the complexes under investigation with the flat, extended aromatic moiety on the thiosemicarbazone ligand, we speculate that the compounds should be capable of binding to DNA through intercalation. Complexes which adopt this method of binding generally have electronic absorption bands that are red-shifted relative to the free complex and also display hypochromism. It is accepted that this is due to stacking interactions of the aromatic groups with the DNA base pairs, resulting in a change in conformation.

Absorption titration experiments of the DNA base pairs, resulting in a change in conformation. this is due to stacking interactions of the aromatic groups with

In this equation $r$ is the number of moles of complex bound per mole of DNA. $C_f$ is the molar concentration of the free DNA, $n$ is the number of binding sites and $K$ is the intrinsic binding constant. For a given plot, the intercept represents the ratio of the IC$_{50}$ values against the CCD-18Co cells. The values are given in Table 2. From the table it is seen that the binding constants are of the order 10$^{-4}$ M$^{-1}$, which characterizes them as weak to moderate intercalators. Given the magnitude of these values, it is even possible that the cationic nature of the complexes could provide for electrostatic interactions.

**Fluorescence titration**

The fluorescence titration experiment has also been widely used to characterize the interaction of metal complexes with DNA by following the changes in the emission intensities of solutions of the complexes upon addition of DNA. Both complexes exhibit moderate luminescence in Tris buffer, with a maximum wavelength near 452 nm upon excitation at 256 nm. The results from the fluorescence titration of the complexes with ct-DNA are shown in Fig. 4. It is observed that the emission from the complexes was quenched on addition of the nucleic acid. The binding constants for these reactions were calculated using the Scatchard equation (2):

$$\frac{r}{C_f} = nK - rK$$

In this equation $r$ is the number of moles of complex bound per mole of DNA, $C_f$ is the molar concentration of the free DNA, $n$ is the number of binding sites and $K$ is the intrinsic binding constant.
constant. Table 2 shows that the binding constant for 1 is slightly smaller than for 2, despite the fact that its fluorescence emission is better quenched \( (K_{SV} = 2.26 \times 10^4 \text{M}^{-1}) \) compared to \( 1.99 \times 10^5 \text{M}^{-1} \) for 2. \( K_{SV} \) is the Stern–Volmer quenching constant, obtained by treating the titration data according to the Stern–Volmer equation (3):

\[
\frac{F_0}{F} = 1 + K_{SV}[Ru] = 1 + K_{SV} [Ru]_0
\]

In this equation \( F_0 \) and \( F \) are the fluorescence intensities of the reaction solution in the absence and presence of the metal compound.

**Ethidium bromide competition titration**

With the aim of further shedding light on the possibility of intercalative binding, we carried out a fluorescence competition experiment. Here a solution of ct-DNA mixed with ethidium bromide, a recognized intercalator, is titrated with the complexes. It was observed that the fluorescence of the DNA–ethidium bromide solution could be effectively quenched by both complexes (Fig. S2, supporting information). A quantitative analysis of the data, to assess the strength of the binding through the apparent binding constant, according to equation (4) produced results given in Table 2:

\[
K_{app} = \frac{K_{EB}[EB]}{[Ru]_{50\%}}
\]

In this equation \( K_{EB} \) is the binding constant for ethidium bromide, taken as \( 1.0 \times 10^7 \text{M}^{-1} \), and \([Ru]_{50\%}\) is the concentration of the complex that causes a 50% reduction of the initial fluorescence. These results are consistent with absorption data obtained from the absorption titrations to the extent that complex 1 binds more strongly to DNA than complex 2.

**Viscometric studies**

We have studied the interaction of the complexes with DNA by viscometry. The classical DNA intercalators will lengthen the strands, resulting in an increase in the viscosity of the DNA solutions. On the other hand, complexes that bind exclusively in the DNA grooves, or by a partial or non-classical intercalation of the compound (under the same conditions), can reduce its effective length, and consequently its viscosity, by bending the DNA helix. The experimental data (Fig. S3, supporting information) showed that the viscosity of the DNA solutions did change over the studied range of metal concentrations. There is an increase in the viscosity of the solutions, followed by a decrease at higher concentrations. This would suggest that the complexes interact with DNA via weak or partial intercalation.

**Chemical Nuclease Activity**

The chemical nuclease characteristics of the complexes were investigated in the cleavage of supercoiled pBR322 plasmid DNA in a medium of Tris–boric acid–EDTA (TBE) buffer (pH 8.2) at 37°C under dark conditions as well as under ultraviolet irradiation at 365 nm by gel electrophoresis. Under the dark experimental conditions, it was observed that when the concentration of the complexes was increased while keeping that of the DNA constant, higher concentrations of the complexes can cause cleavage of the DNA, as evidenced by the appearance of the nicked-circular (NC) form of the DNA in the gel lanes (Fig. 5, 1D and 2D). Based on the intensities of the bands in the lane there does not appear to be any significant difference in the cleavage ability of the complexes. Given the close structural similarity between the complexes, this result is not surprising. When the reaction solutions were exposed to UV radiation, significant DNA cleavage is observed even at the lower end (5 \( \mu \text{M} \)) of the complex concentration range (Fig. 5, 1L and 2L). The extent of cleavage increases with increasing concentration of 1 or 2. Thus it is clear that the complexes can be transformed into better nucleases by UV light.

To assess the role of reactive oxygen species (ROS) in the mechanism of the photo-induced cleavage, a couple of mechanismic probes were employed in the gel electrophoresis experiments. These included adding NaN₃, which is a known singlet

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**Figure 4.** Emission spectral changes of complex 2 on titration with ct-DNA. \([Ru] = 100 \mu \text{M}, [DNA] = 0–50 \mu \text{M} \) at 30°C. Arrow indicates the change in intensity upon increasing DNA concentration. Inset: Scatchard plot of the data for both complexes.

**Figure 5.** Agarose gel electrophoresis diagram for the cleavage of pBR322 DNA by 1 and 2 at ambient temperature in the dark (D) and upon irradiation with 365 nm light (L) under aerobic conditions. Irradiation time was 1 h and incubation time was 1 h.
oxygen scavenger, and HCOONa and KI, which are hydroxyl radical scavengers. The results show that even at the relatively high concentration on 100 μM none of these substances could prevent the cleavage of DNA when irradiated with UV light (Fig. S4, supporting information). This suggests that the common ROS are not involved in the mechanism for the photo-cleavage of DNA by 1 or 2.

Interaction with Human Serum Albumin

Given the importance of HSA in moderating drug behavior, we investigated the interaction of the complexes with HSA. This was done by monitoring the decrease in the fluorescence intensity of HSA solutions on addition of 1 and 2. HSA is a single-chain protein composed of three homologous domains (I–III), with each domain comprised of two subdomains (A and B).[25] Of particular note is the presence of a single tryptophan residue, Trp214. This residue is believed to be responsible for the majority of the intrinsic fluorescence of the protein. HSA has a strong fluorescence emission with a peak near 350 nm upon excitation at 295 nm. The emission is sensitive to the changes in the local environment of the tryptophan and so can be attenuated by binding of a small molecule at or near this residue.

Figure 6 shows the changes in fluorescence intensity of an HSA solution as increasing amounts of 1 are added. There is a significant (78%) reduction in the fluorescence from the initial amount with a blue shift to 329 nm (Δλ = 8 nm). These changes indicate that the conformation of the protein is affected by binding to 1. The binding may be quantitated using the Stern–Volmer equation (2). As seen in Fig. 6, the plot is linear, as expected, and it gives a calculated quenching constant of (1.54 ± 0.86) × 10^5 M⁻¹. For complex 2, the Stern–Volmer plot shows a distinct upward curvature. For protein solutions having homogeneous emission, as would be expected from HSA with its single tryptophan residue, upward curvature has been observed with diverse quenchers.[26] In fact, the fluorescence of most proteins is likely heterogeneous[27] and in the case of HSA one cannot completely eliminate tyrosine emission. Table 3 shows the binding constants obtained from a Scatchard analysis (which is still a popular way of representing data) (Fig. S5, supporting information). This analysis is based on equation (1). This analysis yielded binding constants of the order of 10^5 M⁻¹, indicating strong binding.

The blue shift that occurred during the fluorescence titration suggests that the environment of the tryptophan residue became more nonpolar. When the tryptophan is in a polar environment the emission maximum is near 350 nm. It is closer to 325 nm when the environment is more non-polar. Such a transformation would require significant reorganization of the protein, possibly beyond the usual stochastic structural fluctuations. This would likely involve the expulsion of water molecules; the non-polar pocket is created and the system becomes more random.

Site marker experiments

HSA is known to contain two primary binding regions for various ligands, generally referred to as site I, located in the subdomain IIIA, and site II, which is situated in subdomain IIIB.[28] These hydrophobic pockets are of similar size and display similar chemistry. The sole tryptophan residue mentioned above is located in site I. This site binds small drugs such as warfarin and phenylbutazone. The binding of warfarin at this site is thought to include both hydrophobic and electrostatic interactions. Given that both complexes have hydrophobic groups, are cationic and have been seen to quench the fluorescence of HSA, we sought to

![Figure 6. Fluorescence emission of HSA in the presence of increasing amounts of 1. [HSA] = 3.0 μM; [Ru] = 0–30 μM. Temperature = 30 °C. Inset: Stern–Volmer plot of the data.](image)

| Table 3. Binding constants for the reactions of the complexes with HSA and HSA with the site markers. |
|----------------------------------|----------------------------------|
|                                  | Binding constant × 10^5 M⁻¹       |
|                                  | 1                                |
| HSA                             | 2.05 ± 0.05                      |
| HSA + flufenamic acid           | 7.73 ± 0.36                      |
| HSA + phenylbutazone            | 8.54 ± 0.45                      |
|                                  | 2                                |
| HSA                             | 1.39 ± 0.05                      |
| HSA + flufenamic acid           | 1.25 ± 0.04                      |
| HSA + phenylbutazone            | 9.12 ± 0.31                      |

![Figure 7. Mass spectra of the resulting solution from a 24 h incubation of the complexes with proteins. U = ubiquitin; L = hen egg white lysozyme; C = horse cytochrome c; black (bottom spectra): complex 1; red (top) spectra): complex 2.](image)
determine if there was a clear preference for one binding site over the other. We carried out site marker experiments in which 1 and 2 were titrated into solutions of HSA and phenylbutazone or HSA and flufenamic acid (which is a characteristic site II marker). In both experiments, addition of the complexes to an equimolar solution of the HSA + the site marker resulted in a decrease in the fluorescence of the albumin solution, suggesting that both site markers could displaced from their binding sites on the protein. There was no obvious difference in the extent of the decrease between the two complexes. However one can consider the binding constants calculated from these experiments (Table 3) (Fig. S6, supporting information). For 1 the decrease in binding constants (from the free HSA) was significant but almost the same (62% and 58%) for flufenamic acid and phenylbutazone, respectively. It is difficult to make a suggestion of preference for either site I or site II. Complex 2, on the other hand, seems to show a clear preference for site I. The binding constant from the flufenamic acid experiment is practically the same as for the free HSA (a 10% decrease). However, for phenylbutazone there is a 34% decrease in the binding constant. As mentioned above, given the structural and electronic nature of the complexes these results seem plausible. It is clear, however, that the traditional criteria for ligands binding to the two sites, i.e. heterocyclic anions for site I and aromatic carboxylates for site II, is not easily applicable to fairly big inorganic coordination compounds.

**Interaction with proteins**

The complexes were interacted with three model proteins: ubiquitin, hen egg white lysozyme (HEWL) and horse cytochrome c. The reactions were followed by absorbance spectroscopy and mass spectrometry. Solutions of the complexes mixed with the proteins were incubated for 24 h before extensive ultrafiltration to remove unbound complex. The filtered solutions were then subjected to matrix-assisted laser desorption/ionization mass spectrometry. The data obtained suggested that the proteins can be ruthenated with two ruthenium species attached to the protein (Fig. 7). This species appears common for both metal complexes and the three proteins. One adduct appears to contain a Ru–Cl unit (137 m/z above the native protein peak, [protein–Ru–Cl]) and the other contain an Ru–Cl–H$_2$O (155 m/z above the protein peak, [protein–Ru–Cl–H$_2$O]). One important point to make is that the metallated adducts do not contain the arene or the thiosemicarbazone ligand. This idea is not that uncommon as other workers have made similar observations.

Electronic absorption measurements also show that the complexes can interact with these proteins. The main peaks of the complexes show significant hypochromism when they are incubated with the proteins and a small but discernible blue shift (2 nm) is seen for 2 (Fig. 8 and Fig. S7, supporting information). Neither ubiquitin nor HEWL has any absorption above 280 nm, so changes to the spectra observed may be attributed to changes affecting the complexes. For ubiquitin it should be noted that the last spectrum shown in Fig. 8 is similar (but without the blue shift) to that seen for the hydrolysis reaction of the complex in the same buffer (data not shown). This hydrolysis reaction is presumably the substitution of the chloride ligand by water. Thus it is quite difficult to separate the hydrolysis reaction from the protein binding reaction. For cytochrome c there is clear perturbation of the protein bands near 316 nm and 549 nm as the complexes have no absorption in this region.

**Conclusions**

We have made, using a novel microwave-assisted synthetic procedure, two new organometallic complexes and have investigated their biological activity. The complexes exhibit no antibacterial activity against the studied pathogens but show encouraging anticancer activity with positive therapeutic indices. They have also been shown to be capable of interacting with various proteins, which lends promise to more detailed study, particularly by mass spectrometry.

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