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Organometallic Nucleoside Analogues: Effect of the Metallocene Metal Atom on Cancer Cell Line Toxicity

Media K. Ismail, Katie A Armstrong, Samantha L Hodder, Sarah L. Horswell, Louise Male, Huy V. Nguyen, Edward A. Wilkinson, Nikolas J. Hodges,* and James H. R. Tucker.*

A new chiral organometallic nucleoside analogue containing ruthenocene is reported, in which alklythymine and alklyhydroxyl groups are attached in adjacent positions on one cyclopentadienyl ring. The synthetic procedures for this metallocene derivative and two control compounds are described, along with their characterisation that include cyclic voltammetry and X-ray crystallography. Their biological activities in a human pancreatic cancer cell line (MIA-Pa-Ca-2) were significantly lower than those for three previously reported analogous ferrocene compounds, indicating that the choice of metallocene metal atom (Fe or Ru) plays a pivotal role in determining the anticancer properties of these nucleoside analogues, which in turn suggests a different mode of action from that of a conventional nucleoside analogue.

Introduction

A topical area within the field of metal-based anticancer drug research involves examining the effect of incorporating organometallic moieties into known organic drugs and related biological molecules. Ferrocene is a popular choice in this respect as a so-called bioisosteric group because of its stability and well-understood reactivity and electrochemistry. Its incorporation into the breast cancer drug tamoxifen to form the ferrocifen family of compounds has revealed potent activities in different cell lines to those of the parent compound. This suggests novel modes of action related to the redox properties of the ferrocene unit, which may help combat drug resistance in the clinic.

Nucleoside and nucleobase analogues are an important class of chemotherapeutic agent, with 5-fluorouracil (5-FU) and gemcitabine (Gem) two examples of leading drugs on the market. This presents a similar opportunity to decorate and derivatise the nucleobase moieties of DNA/RNA with organometallic groups to give a range of new biologically active and medicinally relevant compounds. As part of our work in this area, we previously reported the thymidine analogue 1-(SR1)Fe in which the five-membered Cp ring of ferrocene replaced the five-membered sugar ring of the nucleoside (Fig. 1). This compound demonstrated excellent anticancer activities in a range of human cancer cell lines, with both the hydroxyl linker and the nucleobase moiety required for optimal cytotoxicity. A subsequent structure activity relationship (SAR) study found a correlation between the IC50 values in cancer cells and the length of the hydroxyalkyl linker in these so-called ferronucleosides. In continuing with this line of enquiry, we next decided to consider the role played by the metal atom in the lead compound 1-(SR1)Fe, the subject of this report. In metallocene-based drug discovery, changing the metal from iron to ruthenium is a worthwhile endeavour, given the stability of ruthenocene and its amenability to functionalisation. Furthermore, and of particular relevance from an SAR point of view, ruthenocenes have different redox properties to ferrocenes, having more positive oxidation potentials and less reversible electrochemistry. It follows that any difference in biological activity between the two metallocenes could indicate a role for redox processes in the mode of action. Indeed, work on ruthenocifen derivatives has indicated different anticancer activities to the ferrofens, with their biological behaviour more similar to the parent organic compound tamoxifen. However despite these findings, other reports on the biological activities of ruthenocene compounds or organoruthenium nucleobase derivatives are relatively rare. Here we report on the synthesis and anticancer properties of 1-(SR1)Ru, the direct ruthenocene analogue of 1-(SR1)Fe, and two related control compounds (Fig. 2). Our findings do indeed suggest an important role for the metal atom in controlling the anticancer activities of these metallocene-containing nucleoside analogues.

![Fig. 1 Structures of the nucleoside thymidine (left), a generic nucleoside analogue (middle), and ferronucleoside drug candidate 1-(SR1)Fe (right).](image-url)
Results and Discussion

Synthesis

We considered that the synthesis of 1-(S,R)-Ru and the two control compounds 2-(S) and 3 would allow a direct comparison with the three analogous ferrocene compounds previously reported and also enable similar synthetic routes to be followed. In the case of the main target 1-(S,R)-Ru, this meant building the compound up from the known acetoxy derivative 4-(S) (Scheme 1), which itself was prepared via a two-step route from acetyliruthenocene (see ESI). This was then treated with NHMe$_2$ to give the ruthenocene version of Ugi’s amine 5-(R) whose chiral purity was found to be greater than 98%, as evidenced by chiral HPLC (see ESI). Its X-ray structure was determined for the first time from crystals grown from a solution of racemate at 0 °C (Fig. 2). The next step was to introduce planar chirality through the diastereoselective synthesis of 6-(R,S) via treatment with n-BuLi in diethyl ether and then quenching with iodine in THF. This compound was then converted to the acetoxy derivative 7-(R,S) by heating at 50 °C for two hours in acetic anhydride. A short reaction time and a relatively low temperature were used to avoid elimination of the amine group to give the alkene. The arm was then extended to three carbon atoms by reacting with freshly prepared 1-ethoxyvinyl(trimethylsilyl) ketone to give the ethyl ester product 8-(R,S). The ester was reduced to the corresponding alcohol 9-(R,S) using the mild reducing agent DIBAL-H, before being protected with the TBDPS group to give compound 10-(S,S). This compound was then formylated in dry ether in two steps in situ by reacting with n-BuLi in a lithium-halogen exchange followed by addition of DMF to give compound 11-(S,S). A Wittig reaction on the aldehyde added another carbon atom to give the alkene 12-(S,R), which was then converted to the primary alcohol 13-(S,R) by hydroboration-oxidation with BH$_3$·THF. Finally, a Mitsunobu coupling reaction on the alcohol 13-(S,R) with benzoyl-protected thymine gave the fully protected product, which was treated first with TBAF to remove the silyl group and then with ammonia in methanol to remove the benzoyl group, giving the target compound 1-(S,R)-Ru. HPLC analysis on this compound confirmed its formation in high chiral purity (>98%, see ESI). Crystals suitable for X-ray crystallography were grown from an acetone solution of the racemate at 0 °C (Fig. 2). An internal O-H…O H-bond is formed between the hydroxyl hydrogen atom and one carbonyl oxygen atom on the thymine base. Intramolecular hydrogen bonding has previously been observed within other bioorganometallic compounds.

Fig. 2 Structures of the main target compound 1-(S,R)-Ru (middle) and the two control compounds 2-(S) and 3 (left and right) respectively.

Scheme 1 Synthesis route for the target compound 1-(S,R)-Ru from synthon 4-(R).

Fig. 2 Crystal structure of one of the two crystallographically-independent molecules of 1-(S,R)-Ru with ellipsoids drawn at 50% probability level. The structure also contains two independent molecules of acetone, which have been omitted for clarity. Intramolecular hydrogen bonding is shown using a dotted line.
The synthesis of control compounds 2-(S) and 3 also started from compound 4-(R), and proceeded through the routes depicted in Scheme 2. The chiral alcohol was obtained via a linker extension reaction using 1-ethoxyvinylxylo trimethylsilane, followed by reduction of the ester 14-(S) with LiAlH₄. Achiral 3 was obtained in four steps, first involving elimination of the acetoxy group to give the vinyl ruthenocene 15. Crystals of this compound suitable for X-ray diffraction were successfully grown from a solution of DCM layered with hexane (see ESI). A hydroboration-oxidation reaction then yielded the anti-Markovnikov product 16 containing the desired hydroxyethyl linker. The X-ray structure of this compound was also obtained from crystals grown using the same conditions (see ESI). The route was completed using the same methodology described earlier via a Mitsunobu coupling reaction with the protected thymine base to give the protected product 17, which was then deprotected with ammonia to give the target compound 3. Crystals of the latter were grown by slow evaporation from a solution of ethyl acetate layered with hexane. The resulting X-ray structure, showing the correct bond connectivity, is depicted in Fig. 4.

The electrochemistry of ruthenocene is more complicated than that of ferrocene. The 17-electron ruthenocenium cation is considerably more unstable and reactive than its ferrocene counterpart with Ru(VI) products formed from both the chemical\(^{15,16}\) and electrochemical\(^{11,16,17}\) oxidation of ruthenocene. The appearance of ruthenocene cyclic voltammograms (CVs) and those of its derivatives are highly dependent on the type of solvent and electrolyte,\(^{18,19,20}\) in non-coordinating boron-containing electrolytes that do not form ion pairs, the cation has been reported to form two different dimers in a temperature-dependent ratio.\(^{20,21}\) In coordinating solvents and in the presence of more conventional electrolytes,\(^{18,19,22}\) the behaviour is different again. For example, a two-electron oxidation has been reported in acetonitrile using [NBu₄][PF₆] as a supporting electrolyte,\(^{18,23}\) with the cation binding to acetonitrile to form [MeCN-RuCp]₂⁺, which then undergoes further oxidation to give [MeCN-RuCp]⁴⁺ followed by a double reduction back to ruthenocene. Given these previous findings, it was decided to conduct CV experiments on the three target compounds 1-(S,R₉)-Ru, 2-(S) and 3 in acetonitrile in the presence of [NBu₄][PF₆] and compare them with 1-(S,R₉)-Fe under the same conditions. The experiments were performed in the presence of decamethylferrocene (dmfc) as an internal reference, as reported previously.\(^{10}\)

All three ruthenocene compounds showed a similar EC (electrochemical-chemical) oxidation process at a positive potential value, with no return wave observed under the conditions used. Voltammograms for 1-(S,R₉)-Ru at different scan rates are displayed in Fig. 5, with those for 2-(S) and 3 presented in the ESI. The \(E_{pa}\) data for all three compounds are presented in Table 1, along with the corresponding value for the ferrocene analogue 1-(S,R₉)-Fe, which is considerably more negative (\(E_{pa} = 455\) mV, \(E_{pa} = 424\) mV\(^{10}\)).

![Fig. 3 Crystal structure of 3 with ellipsoids drawn at the 50% probability level.](Image)
This difference in value reflects the large difference in the relative stabilities between the oxidized and reduced forms of the two metallocenes, with the ferrocene derivative clearly being thermodynamically much easier to oxidise than its ruthenocene counterpart. The ferrocene derivative also shows reversible electrochemistry. The increase in $E_{pa}$ for compounds 2-(S) and 3 compared with 1-(S,R,R$_E$)-Ru can be explained by a greater inductive effect (+I) as the number of electron donating groups on the Cp ring increases, giving more stability to the charged ruthenocenium ion. The same trend is observed for the analogous ferrocene control compounds of 2-(S) and 3, which have $E_{pa}$ values of 497 mV and 540 mV respectively vs. dmfc (see ESI).

Table 1  Peak potential ($E_{pa}$ in mV) and IC$_{50}$ values (in µM) in MIA-Pa-Ca-2 pancreatic cancer cells of ferrocene compound 1-(S,R,R$_E$)-Fe and ruthenium compounds 1-(S,R,R$_E$)-Ru, 2-(S) and 3.

| Compound             | 1-(S,R,R$_E$)-Fe | 1-(S,R,R$_E$)-Ru | 2-(S) | 3       |
|----------------------|-----------------|------------------|------|--------|
| $E_{pa}$($^\circ$)   | 455             | 932              | 948  | 968    |
| IC$_{50}$($^\circ$)  | 9.3 (7.3-12.1)  | 46.0 (37.7-56.6) | >80  | >80    |

(a) Mean value over scan rates 10-100 mV/s versus the $E_{pa}$ of decamethylferrocene, at 1 mM concentration in dry acetonitrile containing 0.1 M TBAF$_6$. Confidence limit = ±5 mV.
(b) The mean from three independent biological experiments (n = 3), calculated using a variable slope 4 parameter model in Prism V8. Values in parentheses are the 95% CI. IC$_{50}$ of cisplatin = 8.3 (5.1-17.0) µM.

Biological studies

The three ruthenocene compounds were next tested for cytotoxic activity in the pancreatic ductal adenocarcinoma cell line MIA-Pa-Ca-2 and compared with the ferrocene counterpart 1-(S,R,R$_E$)-Fe as well as with cisplatin. Assays were performed after 4 days incubation time using crystal violet staining. Cell viabilities, expressed as a percentage of a negative control, were plotted against concentration (µM) as shown in Fig. 6, with the resulting IC$_{50}$ values presented in Table 1. As found previously for other cancer cell lines, the IC$_{50}$ value for the ferrocene derivative 1-(S,R,R$_E$)-Fe sits in the low micromolar range, with a value similar to that of cisplatin. However, the five-fold reduction in the toxicity for the ruthenocene analogue clearly shows that the identity of the metal ion has a significant impact on cytotoxicity. It is worth noting that the control compounds 2-(S) and 3 were even less toxic than 1-(S,R,R$_E$)-Ru, with IC$_{50}$ values of >80 µM. This agrees with our previous findings on analogous and related ferrocene compounds, in that those metallocenes that are more electron rich, for example by having two groups attached to one cyclopentadienyl ring, are more cytotoxic. Indeed the previously published ferrocene analogues of 2-(S) and 3, which display more positive $E_{pa}$ values, are less toxic than 1-(S,R,R$_E$)-Fe. Overall the trend in the biological data supports the hypothesis that there is a significant relationship between the redox properties of the metallocene units in this series and cancer cell line toxicity.

Conclusion

A ruthenocene-containing nucleoside analogue and two control compounds have been synthesised and fully characterized by a combination of spectroscopic, X-ray crystallography and electrochemical measurements. Their oxidation potentials were affected by the type and number of the linker groups attached to the ruthenocene unit. All three compounds gave very low biological activities in MIA-Pa-Ca-2 pancreatic ductal adenocarcinoma cells, with IC$_{50}$ values for the two mono-functionalised controls higher than that for the bis-functionalised target compound. The main finding of this study is the five-fold difference in cytotoxicity between 1-(S,R,R$_E$)-Ru and its ferrocene counterpart 1-(S,R,R$_E$)-Fe. Given their otherwise identical chemical structures and stereochemistries, this difference can confidently be attributed to the change in metal atom from iron.
to ruthenium. While such a change would make little difference to a metalloenzyme’s size or lipophilicity, it clearly does affect its redox properties. The ferroenzymes in this series demonstrate more reversible electrochemistry than their ruthenocene counterparts, with their oxidised forms accessible at significantly lower potentials. These differences in electrochemical behaviour signify an important role for the iron atom in determining the anticancer activity of the lead compound 1-(S,S,O)-Fe. This in turn suggests a mode of action different from that of a conventional nucleoside analogue, one that points more towards intra-cellular redox-triggered and ROS-mediated pathways leading to cell death. This line of enquiry is currently under investigation in our laboratory.

**Experimental**

**Synthesis**

(R)-1-α-N,N-Dimethylaminoethylruthenocene, 5-(R)

1-α-Acetoxyethylruthenocene

(R)-1-α-Acetoxyethylruthenocene (4-R) (see ESI) (0.1 g, 3.15 mmol) was dissolved in THF (10 mL), and the solution was cooled to 0 °C. After stirring for 10 mins, iodine (0.23 g, 0.91 mmol), in THF (10 mL), was added over 10 mins. The reaction was then quenched at 0 °C with sodium thiosulfate (10 mL, 0.1586 g, 0.3365 mmol) was dissolved in EtO (5 mL) at room temperature. n-BuLi (0.3 mL, 1.7 M, 2 equiv) was added and the mixture stirred overnight. The mixture was then cooled to −78 °C, and iodine (0.23 g, 0.91 mmol), in THF (10 mL), was added over 10 mins. The mixture was then stirred at −78 °C for 90 mins before being warmed to room temperature and stirred for an additional 90 mins. The reaction was then quenched with sodium thiosulfate (10 mL, 15% w/v). After dilution with EtO (15 mL), the layers were separated, and the aqueous layer was washed further with EtO (3 × 5 mL). The combined organic fractions were dried over MgSO₄. The solvent was removed in vacuo before purification via flash column chromatography (10% EtO in hexane) to yield the title compound as a pale-yellow solid (0.0263 g, 38%). δH (400 MHz, CDCl₃) 4.63 – 4.57 (m, 2H), 4.57-4.54 (m, 1H), 4.53 (s, 6H), 3.62 – 3.54 (m, 1H), 2.31 (s, 6H), 1.41 (d, J = 6.9 Hz, 3H), 3C (101 MHz, CDCl₃) 72.4 (CP), 71.1 (CP), 70.2 (CP), 70.1 (CP), 69.5 (CP), 59.6 (CH), 40.4 (CH₂), 17.5 (α-CH). HRMS (ES) (m/z) calcd for C₉H₁₆O₂RuNa 304.0639, found 304.0636. Vₐₙₙ/cm³ 3200(C-H), 2923 (CH₃), 2851 (CH₃), 1453 (CH), 1370 (CH₂), 1100(C-N), 806 (C-C), 720 (CH₂-Ar).

(R,S,R)-1-(α-N,N-Dimethylaminoethyl)-2-iodoruthenocene, 6-(R,S,R)

The Ugi amine 5-(R) (0.1 g, 0.33 mmol) was dissolved in EtO (5 mL) at room temperature. n-BuLi (0.3 mL, 1.7 M, 2 equiv) was added and the mixture stirred overnight. The mixture was then cooled to −78 °C, and iodine (0.23 g, 0.91 mmol), in THF (10 mL), was added over 10 mins. The mixture was then stirred at −78 °C for 90 mins before being warmed to room temperature and stirred for an additional 90 mins. The reaction was then quenched with sodium thiosulfate (10 mL, 15% w/v). After dilution with EtO (15 mL), the layers were separated, and the aqueous layer was washed further with EtO (3 × 5 mL). The combined organic fractions were dried over MgSO₄. The solvent was removed in vacuo before purification via flash column chromatography (10% EtO in hexane) to yield the title compound as a pale-yellow solid (0.0263 g, 38%). δH (400 MHz, CDCl₃) 4.87 (dd, J = 2.3, 1.1 Hz, 1H), 4.68 (d, J = 2.5 Hz, 1H), 4.55 (t, J = 2.4 Hz, 1H), 4.52 (s, 5H), 2.02 (s, 3H), 1.51 (d, J = 6.5 Hz, 3H), 5C (101 MHz, CDCl₃) 170.3 (C=O), 91.9 (ipso Cp), 73.8 (CP), 71.7 (CP), 69.4 (CH), 68.9 (CP), 39.9 (ipso Cp), 21.2 (CH₃), 19.4 (CH₂). HRMS (ES) (m/z) calcd for C₁₉H₂₃O₂NaRu 466.9058 found 466.9054. Vₐₙₙ/cm³ 3098 (=C-H), 2979 (CH₃), 2823 (CH₃), 1727 (C=O), 1449 (CH₃), 1367 (CH₂), 1229 (C-O), 1044, 1018, 806 (CH=CH), 752 (CH₂-Ar). [α]D = +25(±2) (c = 0.25 in CHCl₃).

(S,S,O)-1-(α-Methyl(2-ethylpropanoate))-2-iodoruthenocene, 7-(S,S,O)

Compound 6-(R,S,R) (1 g, 2.3 mmol) and acetic anhydride (8.3 mL, 81 mmol) were heated at 50 °C for 2 hrs. Acetic anhydride was removed under vacuum (0.1 mmHg) and the residue purified with flash column chromatography (10% EtO and 4%TEA in hexane) to yield a light yellow oily product (0.9 g, 86%). δH (400 MHz, CDCl₃) 5.67 (q, J = 6.5 Hz, 1H), 4.87 (dd, J = 2.3, 1.1 Hz, 1H), 4.68 (d, J = 2.5 Hz, 1H), 4.55 (t, J = 2.4 Hz, 1H), 4.52 (s, 5H), 2.02 (s, 3H), 1.51 (d, J = 6.5 Hz, 3H), 5C (101 MHz, CDCl₃) 170.3 (C=O), 91.9 (ipso Cp), 73.8 (CP), 71.7 (CP), 69.4 (CH), 68.9 (CP), 39.9 (ipso Cp), 21.2 (CH₃), 19.4 (CH₂). HRMS (ES) (m/z) calcd for C₂₀H₃₁O₂NaRu 525.9265, found 542.9269. Vₐₙₙ/cm³ 3287 br (OH), 3094 (=C-H Fe), 2955 (CH₃), 2924 (CH₂), 2852 (CH₃), 1524, 1458 (CH₃), 1374 (CH₂), 1054, 997 (C=O), 805 (C=C). [α]D = +10(±3) (c = 0.2 in CHCl₃). m.p: 98–100 °C.
(S,S,S)-1-[α-Methyl-(3-(tert-butyldiphenylsiloxy)propyl)]-2-iodoforocene, 10-(S,S,S) Compounds 9-(S,S,S) (0.2 g, 0.47 mmol) was dissolved in DCM (15 mL) at room temperature. TEA (0.098 mL, 0.699 mmol), tert-butyldiphenylsilyl chloride (0.18 mL, 0.699 mmol), and DMAP (catalytic amount) were then added to the mixture solution. The reaction was then stirred overnight at room temperature before quenching with water (5 mL). The organic layer was separated, and the aqueous layer was washed with EtO (2 × 10 mL). The combined organic fractions were dried over Na₂SO₄, the solvent removed in vacuo, and the residue purified via flash column chromatography (10% Et₂O in hexane) to yield a yellow oil (0.3 g, 88%).

δH max 7.68 – 7.62 (m, 4H), 7.43 – 7.34 (m, 6H), 4.52 (s, 5H), 4.51 – 4.50 (m, J = 1.0 Hz, 1H), 4.41 (dd, J = 2.2, 1.1 Hz, 1H), 1.79 – 1.70 (m, 6H), 1.56 – 1.54 (m, 1H), 1.41 (t, J = 6.9 Hz, 3H), 0.95 (s, 9H). δC max 190.1 (C=O), 135.7 (Ar), 133.8 (ipso Ar), 129.6 (Ar), 127.7 (Ar), 101.8 (ipso Cp), 82.6 (ipso Cp), 72.7 (Cp), 72.4 (Cp), 72.4 (Cp), 67.6 (Cp), 62.6 (Cp), 52.9 (CH), 41.5 (CH), 37.5 (CH), 26.9 (CH), 20.9 (CH), 19.3 (ipso t-Bu). HRMS (m/z) c алкa C₇H₅O₂Ru 302 584, found 303 586.

(5,S,S)-1-[α-Methyl-(3-(tert-butyldiphenylsiloxy)propyl)]-2-vinylruthenocene, 12-S,R,R

Triphenylmethylyphosphine bromide (0.268 g, 0.708 mmol), potassium tert-butoxide (0.08 g, 0.78 mmol), and dibenzo-18-crown-6-ether (catalytic amount) were dissolved in dry THF (20 mL) under argon. The mixture was stirred for 30 mins and then 11-(S,S,S) (0.269 g, 0.472 mmol) in dry THF (5 mL) was added to the mixture. The reaction was then stirred overnight at room temperature before quenching with water (5 mL) and extracting with EtO (2 × 10 mL). The combined organic fractions were dried over Na₂SO₄, the solvent removed in vacuo, and the residue purified via flash column chromatography using hexane to yield a yellow oil product (0.215 g, 80%). δH max 7.68 – 7.62 (m, 4H), 7.42 – 7.34 (m, 6H), 6.48 (dd, J = 17.4, 10.8 Hz, 1H), 5.28 (dd, J = 17.4, 1.7 Hz, 1H), 4.89 (dd, J = 10.8, 1.7 Hz, 1H), 4.81 (t, J = 1.7 Hz, 1H), 4.45 (s, 5H), 4.43 (s, J = 3.9 Hz, 1H), 3.65 – 3.60 (m, 1H), 2.70 – 2.63 (m, 1H), 1.79 – 1.70 (m, 1H), 1.58 – 1.54 (m, 1H), 1.12 (d, J = 6.9 Hz, 3H), 1.04 (s, 9H, Me). δC (101 MHz, CDCl₃) 135.6 (Ar), 134.1 (ipso Ar), 132.7 (Ar), 129.5 (Ar), 127.6 (Ar), 111.2 (CH₃), 88.7 (ipso Cp), 71.4 (Cp), 68.7 (Cp), 68.5 (Cp), 66.9 (Cp), 62.2 (CH₂), 42.5 (CH₂), 27.5 (CH₂), 20.9 (CH₂), 19.2 (ipso t-Bu). HRMS (m/z) calk для C₃H₅RuO₂ 597.1829, found 597.1815.

(S,R,R)-1-[α-Methyl-(3-[(hydroxy)ethyl]propyl)]-2-[(thyminyl)ethyl]ruthenocene, 1-(S,R,R) Ru

Compound 12-(S,R,R) (0.215 g, 0.379 mmol) was dissolved in dry THF (15 mL). B₃H₄THF (1 M, 1 mL, 1.084 mmol) was then added dropwise at room temperature and the mixture stirred for 2 hrs. ETOH (1.4 mL), Na₂SO₃ solution (3M, 1.4 mL, 3.79 mol), and H₂O₂ (30 wt% in water, 0.73 mL, 8.338 mmol) were then added successively, and the mixture was stirred for 1 h at room temperature. The reaction mixture was then added to DCM (20 mL), washed with brine (10 mL), and then dried over Na₂SO₄. The solvent was removed in vacuo and the residue purified via flash column chromatography (10% EtOAc in hexane) to yield a yellow oil product (0.17 g, 77%). δH max 7.67 – 7.62 (m, 6H), 7.43 – 7.34 (m, 6H), 4.52 (s, 5H), 4.51 – 4.50 (m, J = 1.0 Hz, 1H), 4.41 (dd, J = 2.2, 1.1 Hz, 1H), 4.37 (t, J = 2.3 Hz, 1H), 3.71 – 3.56 (m, 4H), 2.53 – 2.31 (m, 1H), 1.76 – 1.70 (m, 1H), 1.56 – 1.47 (m, 1H), 1.07 (d, J = 6.9 Hz, 3H), 1.05 (s, 9H). δC (101 MHz, CDCl₃) 135.6 (Cp), 134.0 (ipso Ar), 129.6 (CH₄Ar), 127.7 (Ar), 99.8 (ipso Cp), 87.4 (ipso Cp), 71.2 (Cp), 70.4 (Cp), 68.0 (Cp), 62.9 (CH₂), 62.6 (CH₂), 42.0 (CH₂), 28.7 (CH₂), 27.2 (CH₂), 26.9 (CH₂) 21.4 (CH₂), 19.2 (ipso t-Bu). HRMS (m/z) calk для C₇H₅RuO₂ 609.1759, found 609.1739. Vmax/cm⁻¹ 3420 (OH), 3071 (≠CH فc), 2955 (CH₃), 2927 (CH₃), 1589, 1470 (CH₂), 1427 (CH₂), 1388 (CH₂), 1361, 1109, 997 (C-O), 822, 738 (C-H Ar), 702 (≠CH-fc). δπ = +52 (±3) (c = 0.2 in CHCl₃).
TBAF (5 mL) for 2 hrs. The solvent was removed, and the residue was then redissolved in ammonia in methanol (7N, 2 mL) and stirred at room temperature for 30 mins. The solvent was evaporated, and the crude product was purified via flash column chromatography (5% MeOH in DCM) to give the product as a white solid (105 mg, 74%).

$\delta$H (400 MHz, CDCl$_3$) 9.33 (s, 1H), 7.04 (s, 1H), 4.50 (s, 5H), 4.45 (dd, $J = 2.2$, 1.2 Hz, 2H), 4.41 (t, $J = 2.3$ Hz, 1H), 3.86 – 3.77 (m, 1H), 3.74 – 3.62 (m, 3H), 2.68 – 2.54 (m, 3H), 2.40 (s, br 1H), 1.92 (s, 3H), 1.78 – 1.73 (m, 1H), 1.65 – 1.57 (m, 1H), 1.18 (d, $J = 6.9$ Hz, 3H). $\delta$C (101 MHz, CDCl$_3$) 164.2 (C=O), 151.1 (C=O), 140.4 (CH thymine), 111.0 (ipso thymine), 95.3 (ipso Cp), 80.7 (ipso Cp), 69.3 (Cp), 67.6 (Cp), 65.9 (Cp), 60.2 (Cp), 49.8 (CH$_2$), 43.2 (CH$_2$), 27.9 (CH$_3$), 27.1 (CH), 19.2 (CH$_3$ thymine), 12.3 (CH). HRMS (ES) (m/z) calcd for C$_{10}$H$_{15}$N$_2$O$_2$Ru 332.0029 found 332.0027. V$_{max}$ cm$^{-1}$ 1645, 1519, 1404, 1383, 1210, 1110, 857, 795, 734, 712, 432, 298, 155.

$\alpha$ = +10.5 (±2) (c = 0.1 in CHCl$_3$).

1-vinyln ruthenoc ene, 15
1-methox ylvinylruthenocene 4-(R) (0.23 g, 0.95 mmol, 1.0 eq) and LiBr (0.8 g, 9.5 mmol, 10 eq) was dissolved in DMF (7 mL) under an atmosphere of argon. The reaction was heated gradually to 80°C and stirred for 30 mins. The reaction was quenched with water (20 mL), extracted with DCM, and dried over MgSO$_4$. The solvent was removed in vacuo and the crude product purified by flash column chromatography on neutralized silica gel using 15% Et$_2$O and 3% TEA in hexane. The solvent was removed in vacuo to give the titled compound as a yellow solid (0.15 g, 61%). $\delta$H (300 MHz, CDCl$_3$) 6.33 (dd, $J = 17.5$, 10.7 Hz, 1H), 5.28 (dd, $J = 17.5$, 1.5 Hz, 1H), 4.89 (dd, $J = 10.7$, 1.5 Hz, 1H), 4.77 (t, $J = 1.7$ Hz, 1H), 4.56 (s, 5H), 4.51 (s, 3H), 3.67 (t, $J = 1.6$ Hz, 2H), 2.46 (s, br 1H), 2.44 (t, $J = 6.0$ Hz, 2H). $\delta$C (101 MHz, CDCl$_3$) 133.8 (CH vinyl), 110.1 (CH$_2$), 87.6 (ipso Cp), 71.1 (Cp), 70.5 (Cp), 69.0 (Cp). HRMS (m/z) Calcd for C$_{11}$H$_{15}$Ru 257.9982, found 257.9979. V$_{max}$ cm$^{-1}$ 3094 (C=H), 2958, 2915, 2848 (CH$_3$), 1734, 1461, 1367 (C=C), 805, 753 (C=H).

$\alpha$ = +10.5 (±2) (c = 0.1 in CHCl$_3$).

1-(3H-oxypyridin-2-yl) ruthenoc ene, 2(S)
Compound 15 (0.23 g, 0.84 mmol, 1.0 eq) was dissolved in dry THF (10 mL) under an atmosphere of argon. BH$_4$THF (1 M in THF) (1.3 mL, 1.25 mmol, 1 eq) was added dropwise, and the resulting solution was stirred for 2 hrs. $\text{EtOH}$ (1.3 mL) was added dropwise, followed by NaOH solution (3 M in H$_2$O) (1.3 mL) and H$_2$O$_2$ (30% wt in water, 1.3 mL) and the solution stirred for 2 hrs. The reaction was quenched with $\text{H}_2$O, extracted with DCM and then dried over MgSO$_4$. The solvent was removed in vacuo and the crude product purified by flash column chromatography on silica gel using an eluent of 20% EtOAc in hexane. The solvent was removed in vacuo to give the title compound as a yellow oil (0.61 g, 63%). $\delta$H (400 MHz, CDCl$_3$) 4.52 (s, 5H), 4.43 (t, $J = 1.7$ Hz, 2H), 2.68 – 2.54 (m, 3H), 2.40 (s, br 1H), 1.92 (s, 3H), 1.78 – 1.73 (m, 1H), 1.65 – 1.57 (m, 1H), 1.18 (d, $J = 6.9$ Hz, 3H). $\delta$C (101 MHz, CDCl$_3$) 172.76 (C=O), 98.3 (ipso Cp), 70.5 (Cp), 70.1 (Cp), 69.5 (Cp), 68.9 (Cp), 60.3 (CH$_3$), 43.7 (C=H), 29.6 (CH), 21.5 (CH$_3$), 14.3 (CH$_2$). HRMS (ES) (m/z) calcd for C$_{10}$H$_{14}$O$_2$Ru 346.0507, found 346.0508. V$_{max}$ cm$^{-1}$ 3095 (CH$_3$), 2964, 2929 (CH$_3$), 1731 (C=O), 1369 (CH$_3$), 1030 (C=O), 817 (=C=H). $\alpha$ = +10.5 (±2) (c = 0.1 in CHCl$_3$).

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(ipso-Cp), 70.9 (Cp), 70.7 (Cp), 70.0 (Cp), 50.8 (CH₂), 28.8 (CH₂), 12.2 (CH₃). HRMS (m/z) calcld for C₂₃H₂₃N₂O₂Ru⁴⁺Na⁺ 407.0309, found 407.0311. V_{calc/cm²} 3210 (NH), 2098 (=C-H Fc), 2954 (CH₂), 2921 (CH₃), 2850 (CH₂), 1685, 1672 (C=O), 1462, 1385 (CH₃), 1353 (C-N), 803, 759 (C=C).

Electrochemistry

Cyclic voltammetry measurements were performed using 1.0 mM solutions in dry and de-oxygenated MeCN with 0.1 M solution of the electrolyte hexafluorophosphate TBAPF₆ (Fluka ≥ 99%) using 1.0 mM of dmfc as an internal reference. Data were measured with a BioAnalytical Systems Inc. (West Lafayette, IN) EC Epsilon potentiostat and with a C3 cell stand with a three-electrode unit. The cyclic voltammograms were recorded under argon at room temperature using a three-electrode cell. The electrodes were obtained from IJ Cambria (Llanelli, Wales). A platinum wire was used as a counter electrode (C.E), a glassy carbon electrode with 3 mm diameter was used as a working electrode (W.E) and an Ag/AgCl|3 M KCl electrode was used as a reference electrode (R.E.) and connected to the cell via a frit. Cleaning of all glassware was achieved by soaking overnight in 1:1 ammonia (35%) and hydrogen peroxide (30%), followed by multiple rinsing with ultrapure water (from a Millipore tandem Elx-A10 system, resistivity > 18 MΩ cm, TOC < 5 ppb). The glassware was then left overnight in ultrapure water, then rinsed again and dried in an oven prior to use. The electrodes were cleaned as follows before their use: the R.E was cleaned with dry acetonitrile and the C.E. was flame annealed. The W.E was cleaned by polishing with aqueous slurries of successively finer grades of alumina (1.0 µm, 0.3 µm and 0.05 µm) and then rinsed with ultrapure water and MeCN, dried with a flow of argon, then kept in ankyte solution.

X-ray crystallography

Crystal structure determination of 1-(5-R₆)Ru: C₂₃H₂₃N₂O₂Ru (M = 496.56 g/mol): triclinic, space group P-1 (no. 2), a = 12.1133(5) Å, b = 12.9420(7) Å, c = 15.0302(8) Å, α = 70.115(5)°, β = 84.171(4)°, γ = 84.068(4)°, V = 2198.4(2) Å³, Z = 4, T = 100.01(10) K, μ(CuKα) = 6.008 mm⁻¹, Dcalc = 1.500 g/cm³, 15164 reflections measured (7.284° ≤ 2θ ≤ 136.502°), 8029 unique (Rint = 0.0314, Rsigma = 0.0440) which were used in all calculations. The final R₁ was 0.0498 (I > 2σ(I)) and wR₂ was 0.0967 (all data). The crystal was a non-merohedral twin with the two domains related by 180° about the reciprocal direction [0 0 1] with the refined percentage ratio 54.2(3)%.

Crystal structure determination of 5-(R₆): C₁₄H₁₄NRu (M = 302.37 g/mol): monoclinic, space group P2₁/c (no. 14), a = 7.6382(3) Å, b = 20.2298(9) Å, c = 8.0749(3) Å, β = 90.0174(9)°, V = 1231.27(9) Å³, Z = 4, T = 100.01(10) K, μ(CuKα) = 10.053 mm⁻¹, Dcalc = 1.631 g/cm³, 4460 reflections measured (8.742° ≤ 2θ ≤ 138.252°), 2291 unique (Rint = 0.0261, Rsigma = 0.0371) which were used in all calculations. The final R₁ was 0.0280 (I > 2σ(I)) and wR₂ was 0.0670 (all data).

Crystal structure determination of 15: C₁₅H₂₈Ru (M = 257.29 g/mol): monoclinic, space group P2₁/c (no. 14), a = 11.9888(12) Å, b = 5.6864(5) Å, c = 14.2237(16) Å, β = 100.876(9)°, V = 952.26(16) Å³, Z = 4, T = 100(2) K, μ(MoKα) = 1.590 mm⁻¹, Dcalc = 1.795 g/cm³, 3039 reflections measured (5.834° ≤ 2θ ≤ 55.172°), 3039 unique (Rsigma = 0.0354) which were used in all calculations. The final R₁ was 0.0732 (I > 2σ(I)) and wR₂ was 0.2153 (all data). The crystal was a non-merohedral twin with the two domains related by 180° about the reciprocal direction [1 0 0] with the refined percentage ratio 54.2(3)%: 45.8(3).

Crystal structure determination of 16: C₁₆H₂₉ORu (M = 275.30 g/mol): trigonal, space group P-3 (no. 147), a = 30.3090(5) Å, c = 5.97018(11) Å, V = 4747.12(18) Å³, Z = 18, T = 100.00(10) K, μ(CuKα) = 11.714 mm⁻¹, Dcalc = 1.733 g/cm³, 21374 reflections measured (5.834° ≤ 2θ ≤ 144.214°), 6213 unique (Rint = 0.0387, Rsigma = 0.0342) which were used in all calculations. The final R₁ was 0.0445 (I > 2σ(I)) and wR₂ was 0.0953 (all data). The structure contains three crystallographically-independent molecules. The hydrogen atoms bonded to O(1), O(101) and O(201) were located in the electron density and their positions and thermal parameters were freely refined.

CCDC 1953303 – CCDC 1953308 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Biological studies

MIAPaCa2 (85062806) pancreatic ductal adenoma cancer cells were purchased from the European Collection of Authenticated Cell Cultures. Cell culture media and supplements were purchased from Gibco (Thermo Scientific), all plasticware was purchased from Greiner Bio-One. Cells were maintained at 37 °C in a 5% CO₂ humidified incubator and grown in T25 tissue culture flasks in DMEM supplemented with 10% (v/v) foetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM l-glutamine. Cells were sub-cultured twice weekly before confluency using a standard trypsin-EDTA protocol. Cell cultures were confirmed free from contamination.
Mycoplasma sp. contamination using the EZ-PCR mycoplasma detection kit according to the manufacturer’s instructions.

Crystal violet assay: Cells were sub-cultured into 96-well plates at a density of 6250 cells per well in 100 µL of complete DMEM and left overnight to allow the cells to attach. The next day culture media was removed and replaced with fresh media containing test compounds (0–80 µM) dissolved in DMEM with a final concentration of 0.5% w/v DMSO prepared from 50 mM stock solutions, except cisplatin which was prepared as a 2 mM stock solution in phosphate buffered saline (PBS). All cultures were incubated for 72 hrs prior to commencement of the crystal violet assay as described below. The old media was removed, cells washed with 100 µL of PBS, then 100 µL 4% v/v paraformaldehyde was added. After 15 min this was removed and 100 µL of crystal violet solution (0.5% w/v in 10% v/v ethanol) added and plates incubated for 20 min. Next the crystal violet solution was removed, and plates washed with PBS (4 x 100 µL) before being allowed to air dry for 20 min at room temperature. Finally, samples were solubilized using 10 % w/v acetic acid before measuring the absorbance at 590 nm using a well-plate reader.

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Footnotes:

† The X-Ray structures of the chiral compounds were determined from crystals grown from solutions of racemic mixtures (see the ESI for more details).

‡ The enantiomer 1-{(R,S)},Ru, isolated from a racemic batch of the target compound [see ESI], was found to be even less toxic, with an IC₅₀ of >80 µM, the highest concentration investigated. This data suggests that stereochemistry also plays a significant role in determining anticancer activity of these ferronucleosides. This aspect is currently being investigated further and will be included in a future report.
Organometallic Nucleoside Analogues: Effect of the Metallocene Metal Atom on Cancer Cell Line Toxicity

Changing the metal atom within a metallocene nucleoside analogue from iron to ruthenium results in a five-fold reduction in biological activity in a pancreatic cancer cell line.