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Anticancer activity of multinuclear arene ruthenium complexes coordinated to dendritic polypyridyl scaffolds

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ABSTRACT

The rational development of multinuclear arene ruthenium complexes (arene = *p*-cymene, hexamethylbenzene) from generation 1 ($\mathbf{G^1}$) and generation 2 ($\mathbf{G^2}$) of 4-iminopyridyl based poly(propyleneimine) dendrimer scaffolds of the type, DAB-$(NH_2)_n$ (n = 4 or 8, DAB = diaminobutane) has been accomplished in order to exploit the 'enhanced permeability and retention' (EPR) effect that allows large molecules to selectively enter cancer cells. Four compounds were synthesised, i.e. [{(*p*-cymene)$RuCl_2$}$_4\mathbf{G^1}$] (**1**), [{(hexamethylbenzene)$RuCl_2$}$_4\mathbf{G^1}$] (**2**), [{(*p*-cymene)$RuCl_2$}$_8\mathbf{G^2}$] (**3**), and [{(hexamethylbenzene)$RuCl_2$}$_8\mathbf{G^2}$] (**4**), by first reacting DAB-$(NH_2)_n$ with 4-pyridinecarboxaldehyde and subsequently metallating the iminopyridyl dendrimers with [(*p*-cymene)$RuCl_2]_2$ or [(hexamethylbenzene)$RuCl_2]_2$. The related mononuclear complexes [(*p*-cymene)$RuCl_2$(L)] (**5**) and [(hexamethylbenzene)$RuCl_2$(L)] (**6**) were obtained in a similar manner from *N*-(pyridin-4-ylmethylene)propan-1-amine (L). The molecular structure of **5** has been determined by X-ray diffraction analysis and the *in vitro* anticancer activities of the mono-, tetra- and octanuclear complexes **1–6** studied on the A2780 human ovarian carcinoma cell line showing a close correlation between the size of the compound and cytotoxicity.

1. Introduction

The use of transition metal complexes as potential therapeutics and in diagnostic medicine is of considerable importance and a number of excellent reviews and books are available [1]. The field stemmed from the discovery of the anticancer activity of the complex *cis*-diamminedichloroplatinum(II), cisplatin, one of the most widely used anticancer drugs effective against testicular cancer and other tumours [2]. Second generation alternatives to cisplatin, viz. carboplatin and oxaliplatin, are also effective chemotherapeutic agents for certain cancers [3]. Nevertheless, despite the successes of cisplatin and related platinum antitumour agents, research into non-platinum anticancer agents has evolved due to problems associated with platinum-based chemotherapies. Notably, the application of cisplatin in the clinic is marred by side-effects and drug resistance. Perhaps the most distressing side-effect is that cisplatin-induced frameshift [4] and base substitution [5] mutations may be responsible for secondary malignancies observed decades after patients have been treated and cured with this drug. In order to overcome the limitations of platinum based therapies compounds containing titanium [6], gold [7] and ruthenium [8] centres have been established and some have already entered early clinical trials. From these studies ruthenium compounds have been shown to display less general toxicity than their platinum counterparts [9], and like platinum compounds are also able to interact with DNA and proteins [10]. The majority of ruthenium compounds evaluated for anticancer activity are coordination compounds with the ruthenium in the +3 oxidation state. It has been proposed that in this oxidation state ruthenium is less active and is reduced *in vivo* to more active ruthenium(II) complexes, a process favoured in the hypoxic environment of a tumour [11]. However, it should be noted that ruthenium(II) compounds also exhibit a low general toxicity and since cancer cells can also become oxidized at certain stages of their growth cycle oxidation of the ruthenium cannot be excluded [12]. Since π-coordinated arenes are known to stabilize ruthenium in its +2 oxidation state, the potential of arene ruthenium(II) complexes as anticancer agents, and their associated aqueous chemistry, is being increasingly investigated. The first complex evaluated of this kind was [Ru(benzene)(metronidazole)Cl_2] which presented a higher activity compared to the antitumour drug metronidazole itself [13]. More recently the hydrophilic phosphine-containing [Ru(*p*-cymene)(pta)Cl_2] (pta = 1,3,5-triaza-7-phosphatricyclo [3.3.1.1] decane), phosphite-containing [Ru(*p*-cymene)(P-sugar)Cl_2] [14], and

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diamine-containing [Ru(arene)(YZ)(Cl)][PF_6] (arene = *p*-cymene or biphenyl; YZ = chelating diamine) [15] compounds have been investigated for anticancer activity. A few recent studies describing polynuclear organometallic Ru complexes with anticancer activity have been reported. A few dinuclear arene Ru complexes that exhibit a cytotoxic effect against cancer cells are known. Complexes with sulfoxide [16] and ethylendiamine [17] ligands were found to be relatively inactive *in vitro*, whereas pyrone-derived dinuclear complexes have demonstrated *in vitro* anticancer activity in the low μM range [18–20]. The *in vitro* anticancer activity of the latter compounds are determined to some extent by their lipophilicity [20], and their mononuclear analogues are not active in cell cultures [21]. In addition, dinuclear complexes and tri- and tetranuclear clusters such as $[H_3Ru_3(benzene)(hexamethylbenzene)_2O]^+$ [22] and $[H_4Ru_4(benzene)_4]^{2+}$ [23] have been studied *in vitro* or *in vivo* for their activity, and some show promise against cisplatin-resistant cell lines. These latter compounds are relatively large and it is not clear how they enter cancer cells. In this respect, however tumours can be specifically targeted by exploiting the 'enhanced permeability and retention' (EPR) effect. The EPR effect is a phenomenon in which macromolecules can accumulate at the tumour site due to an increase in blood vessel permeability within diseased tissues compared to normal tissues [24]. The normal endothelial layer surrounding the blood vessels feeding healthy tissues restricts the size of molecules that can diffuse from the blood. In contrast, the endothelial layer of blood vessels in diseased tissues is more porous providing access to the surrounding tissue. Furthermore, diseased tissue does not usually have a lymphatic drainage system so once macromolecules have entered the tissue they are retained. Indeed, a tetraruthenium cluster was even found to be highly active against the polio virus without damaging the host cells, thereby offering the potential of developing highly selective drugs [23].

In order to exploit size selective uptake of drugs into tumour cells effectively, large compounds are required, and in recent years, dendrimers have found potential as molecular tools in biological applications [25], especially as nano-carriers [26], diagnostic agents [27] and as chemotherapeutics [28]. Moreover, another advantage of dendrimers is their multivalency, which leads to increased interaction between a dendrimer-drug conjugate and a target bearing multiple receptors, further improving the selectivity to cancer cells. With the aim of reducing the inherent problems related to cisplatin such as poor water solubility, high toxicity and side effects, the combination of platinum-based drugs with dendritic systems is very appealing. However, transition metal complexes with anticancer activity, based on a dendritic scaffold are quite rare. Duncan et al. reported the conjugation of cisplatin to a polyamidoamine (PAMAM) dendrimer, modified on the periphery with sodium carboxylate functionalities [29]. The dendrimer-platinate is water soluble and displayed antitumour activity when administered intravenously against a B16F10 melanoma, whereas cisplatin was inactive. Using the commercially available butanediamine poly(propyleneimine) dendrimer ($DAB(PA)_4$) which provides four peripheral amine groups a series of tetranuclear platinum compounds were synthesised. The cationic compound $[DAB(PA)_4\text{-}\{Pt(NH_3)_2Cl\}_4]^{4+}$ was designed to overcome cisplatin resistance [30], while the neutral derivative $DAB(PA)_4\text{-}\{Pt(dmso)(L)\}_4$ (L = *meso*-1,2-bis(4-fluorophenyl)ethylenediamine) was prepared to increase selectivity for breast cancer cells [31]. With the established anticancer activity of arene ruthenium complexes and the current interest in dendrimers for biological applications, we decided to explore the synthesis of multinuclear ruthenium complexes based on a poly(propyleneimine) scaffold and investigate their cytotoxicity against A2780 human ovarian cancer cell line. As far as we are aware these studies are the first to target arene ruthenium compounds using this approach although tumour targeting of arene ruthenium compounds via covalent attachment to recombinant human serum albumin via a pH cleavable linker has been reported [32].

2. Results and discussion

2.1. Synthesis and characterisation of iminopyridyl-functionalised dendritic ligands G^1 and G^2

A first- (G^1) and second-generation (G^2) iminopyridyl dendrimer based on a poly(propyleneimine) scaffold was synthesised via a standard Schiff-base reaction. The 4-pyridylimine-functionalised dendritic ligands (G^1–G^2) were synthesised by the reaction of 4-pyridinecarboxaldehyde with DAB-$(NH_2)_n$ (n = 4, 8 for G^1 and G^2, respectively) (Fig. 1). The dendritic ligands are isolated as orange-yellow oils, in moderate yields. They are soluble in dichloromethane, chloroform, methanol, diethyl ether and tetrahydrofuran.

The Schiff base condensation reaction is confirmed by appearance of the peak in the range between 8.17 and 8.23 ppm assigned to the imine protons for G^1 and G^2. Two distinct doublets ($^3J \sim 6.00$ Hz) are observed for the aromatic protons on the 4-pyridyl rings. $^{13}C\{^1H\}$ NMR spectra for both ligands (G^1–G^2) show similar spectra. Aliphatic carbons were seen in the region of 25–60 ppm and aromatic carbons in the region of 120–150 ppm for both generations. As expected for both ligands the imine-carbon was the most deshielded signal at 159 ppm. For both G^1 and G^2, the IR spectra show two absorption bands of strong intensity at ~1647, 1599 cm^{-1} and are assigned to the $(C{=}N)_{imine}$ and $(C{=}N)_{aromatic}$ vibrations respectively.

2.2. Synthesis and characterisation of ruthenium complexes 1–6

The dinuclear arene ruthenium complexes $[(arene)RuCl_2]_2$ (arene = *p*-cymene, hexamethylbenzene) react with the dendritic ligands G^1 and G^2 by stirring at room temperature in dichloromethane to yield the neutral tetranuclear (**1–2**) and octanuclear (**3–4**) ruthenium metallodendrimers (Fig. 2). The yellow-orange solids (**1–4**) are isolated as air-stable solids in high yields (79–98%). The complexes are soluble in most organic solvents such as dichloromethane, chloroform, ethanol, dimethylsulfoxide, acetone, acetonitrile, diethyl ether and tetrahydrofuran.

The ^1H NMR spectra of **1–4** shows broadened peaks upon complexation of the multinuclear ruthenium moieties. The aliphatic protons of the dendritic core and side arms occur at similar shifts to those of the dendritic ligand precursors. Evidence for the coordination of the ruthenium metal to the aromatic nitrogen atom can be seen by a shift in the doublet (assigned to aromatic protons on the carbon adjacent to pyridyl nitrogen atom) from 8.66 to 9.10 ppm. A shift in the signal is due to the electron-withdrawing effects of the coordinating metal, resulting in the signals being shifted downfield. There is also no distinct shift in the imine proton suggesting no coordination at this position. The ^1H NMR spectrum for the second generation complexes (**3–4**) showed similar shifts to the first generation. This alludes to coordination at the pyridyl nitrogen only and not the imine nitrogen. This is further confirmed by IR spectroscopic studies. Infrared spectroscopic studies show a shift in the $(C{=}N)_{aromatic}$ peak from a lower wavenumber to a higher wavenumber at around 1615 cm^{-1}. The $(C{=}N)_{imine}$ absorption band remains unchanged at around 1646 cm^{-1}. These shifts were as a direct result of the synergic effect. The second generation dendritic complexes show similar trends. The ruthenium functionalised dendrimers (**1–4**) were precipitated with the inclusion of solvent, trapped between the dendritic arms. The elemental analysis data correlates with the inclusion of 2 molecules and 4 molecules of dichloromethane for **1** and **3**, respectively.

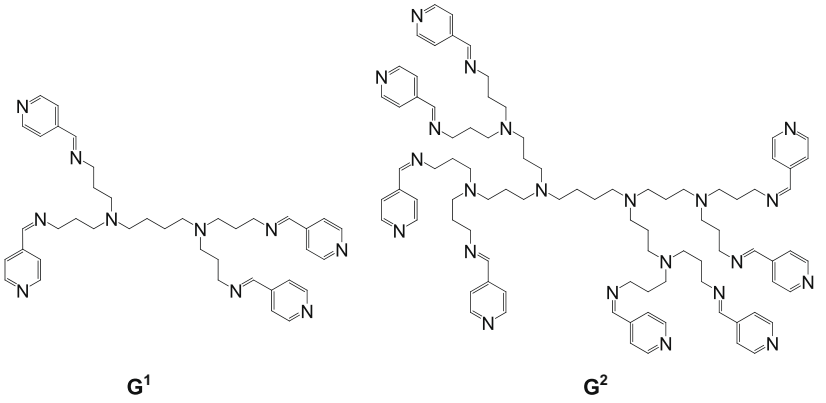


Fig. 1. First- and second-generation of iminopyridyl dendritic ligands G^1 and G^2.

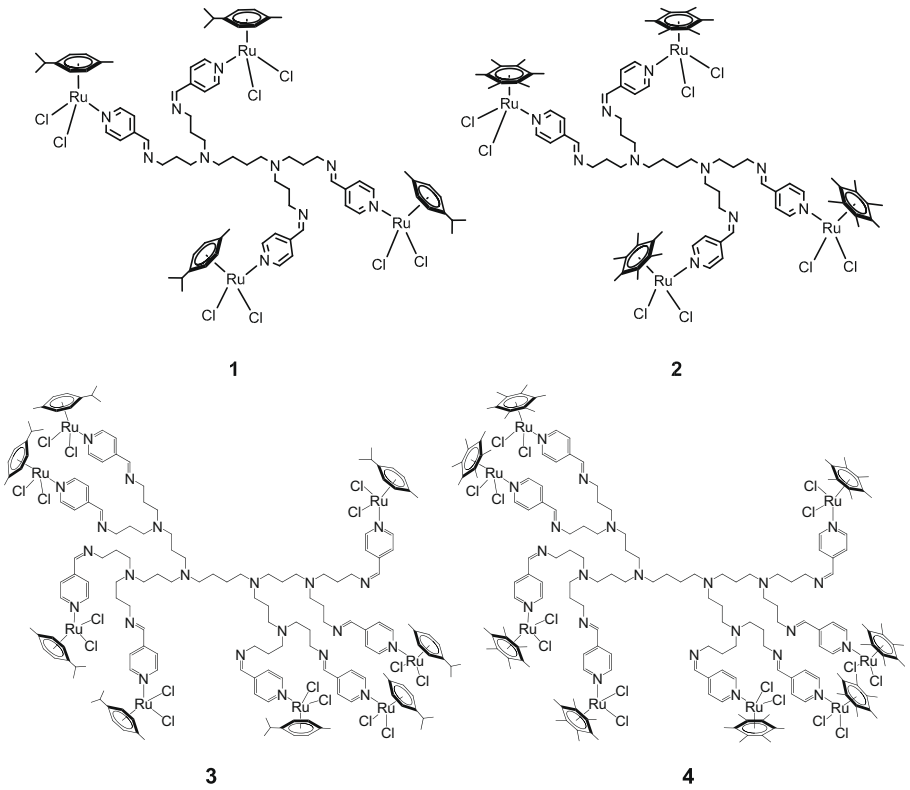
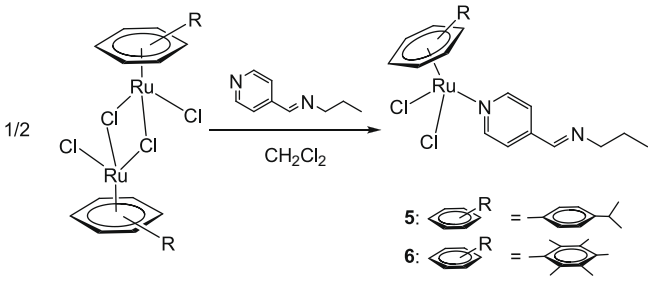


Fig. 2. Tetra- and octanuclear arene ruthenium dendritic systems **1–4**.

For comparison, the analogous mononuclear ruthenium complexes (**5–6**) were synthesised. These were prepared by reacting the ligand, *N*-(pyridin-4-ylmethylene)propan-1-amine (L), with the dinuclear arene ruthenium complexes $[(arene)RuCl_2]_2$ (arene = *p*-cymene, hexamethylbenzene) in dichloromethane at room temperature, see Scheme 1.

The mononuclear ruthenium complexes (**5–6**) are isolated as yellow-orange solids in high yields. They are air-stable and soluble in dichloromethane, chloroform, ethanol, dimethylsulfoxide, acetone, acetonitrile, diethyl ether and tetrahydrofuran. The aromatic protons adjacent to the pyridyl nitrogen atom appear more downfield than for the uncoordinated ligand (8.66–9.10 ppm), while the imine protons remain at the same position as for the dendritic ligand (~8.2 ppm). A shift in the $(C{=}N)_{aromatic}$ absorption band in the IR spectrum of **5** and **6** is seen from around 1550–1615 cm^{-1}. The $(C{=}N)_{imine}$ absorption band around 1647 cm^{-1} remains constant and confirms that no coordination occurred at this site. The coordination of the ruthenium metal occurred to the aromatic nitrogen atom was further confirmed by the X-ray structure analysis of complex **5**.



Scheme 1. Synthesis of the mononuclear arene ruthenium complexes **5** and **6**.

2.3. X-ray diffraction study of complex **5**

X-ray quality crystals for complex **5** were obtained by slow diffusion of hexane into a concentrated dichloromethane solution of **5**. The molecular structure of **5** shows that, like other arene ruthenium complexes [33], the metal centre adopts a piano-stool, pseudo-tetrahedral geometry, with ruthenium coordinated by the arene ligand, two chlorides and the iminopyridyl ligand. An ORTEP drawing of **5** is shown in Fig. 3 together with selected bond lengths and angles.

The distance between the ruthenium atom and the centre of the C_6H_4 aromatic ring of the *p*-cymene ligand is 1.670(4) Å. The Ru–N and Ru–Cl bond distances in **5** are comparable to those reported in the pyridine (py) derivatives [(1,3,5-trimethylbenzene)$RuCl_2$(py)] [34] and [(hexamethylbenzene)$RuCl_2$(py)] [35]. Similarly, the Cl–Ru–N and Cl–Ru–Cl bond angles of complex **5** [84.8 (2) and 87.0(2)°] are similar to those in [(1,3,5-trimethylbenzene)-$RuCl_2$(py)] and [(hexamethylbenzene)$RuCl_2$(py)]. As suggested previously, only the aromatic nitrogen atom is found to be coordinated to the ruthenium atom, thus validating the coordination mode proposed in complexes **1**–**6**.

2.4. Anticancer activity of complexes **1**–**6**

The ability of **1–6** to inhibit cancer cell growth was evaluated *in vitro* using the biological MTT assay which measures mitochondrial dehydrogenase activity as an indication of cell viability using the A2780 ovarian cancer cell line. The compounds were incubated at various concentrations (in triplicate) in the A2780 cells and cell viability was measured after an incubation period of 72 hours. Each experiment was conducted in duplicate and the IC_{50} values (inhibition of cancer cell growth at the 50% level) listed in Table 1 are calculated as an average over the two experiments.

All compounds display moderate anti-proliferative activity in the A2780 cell line. While the IC_{50} values determined are higher than that of cisplatin, the most active compounds, **3** and **4**, are relatively low for ruthenium compounds. These are the first examples of ruthenium-arene-dichloro complexes with an imino-pyridine ligand to be tested for *in vitro* activity. However, analogous hexamethylbenzene and *p*-cymene cyanopyridine complexes have previously been reported to show significant unwinding of supercoiled DNA and also inhibit haem polymerase activity. In contrast, [(*p*-cymene)Ru(pyridine)Cl_2] was found to show negligible activity in the TS/A cell line [36].

Table 1
IC_{50} values of cisplatin and complexes **1 – 6** on A2780 human ovarian cancer cell line.[a]

Compounds	**1**	**2**	**3**	**4**	**5**	**6**	cisplatin
IC_{50} (μM)	43	40	21	20	98	94	1.6

[a] Maximum error is < ±5 μM.

There is a clear trend between the size of the dendritic compound and cytotoxicity, i.e. the monoruthenium compounds have modest cytotoxicity whereas [{(*p*-cymene)$RuCl_2$}$_8$**G^2**] **3** and [{(hexamethylbenzene)$RuCl_2$}$_8$**G^2**] **4** are cytotoxic. Based on this observation, the biological properties of **3** and **4** are worth studying further as they may be able to target cancerous tissues more effectively than smaller compounds by exploiting the enhanced permeability and retention effect, a property that needs to be evaluated *in vivo*. Moreover, the activity shown here for **3** and **4** is not too dissimilar to that of the multinuclear arene ruthenium adduct of recombinant human serum albumin [26], but the facile synthesis and considerably lower cost of the dendrimer system, cannot be overlooked.

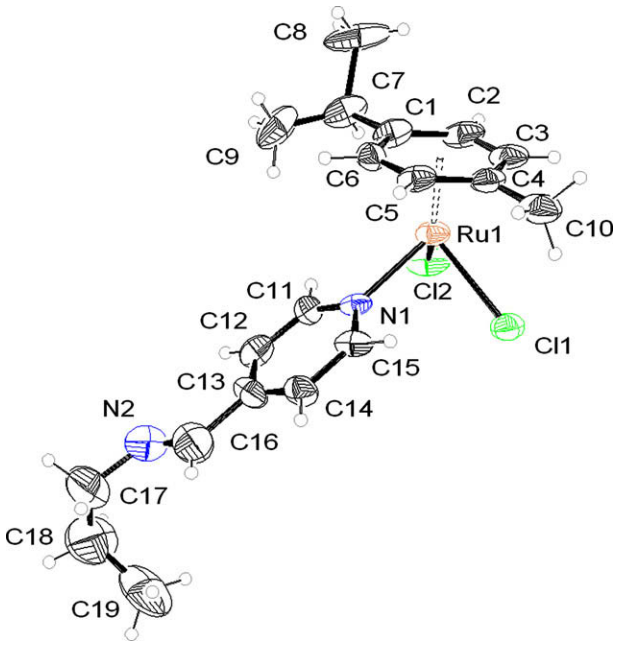


Fig. 3. Molecular structure of the mononuclear complex **5** showing ellipsoids at the 50% probability level. Selected bond lengths (Å) and angles (°): Ru(1)–N(1) 2.128(9), Ru(1)–Cl(1) 2.406(3), Ru(1)–Cl(2) 2.405(3), N(2)–C(16) 1.34(3), N(2)–C(17) 1.49(3); N(1)–Ru(1)–Cl(1) 84.8(2), N(1)–Ru(1)–Cl(2) 87.0(2), Cl(1)–Ru(1)–Cl(2) 87.65(12), C(13)–C(16)–N(2) 115(2), C(16)–N(2)–C(17) 125(2).

3. Experimental

3.1. General remarks

All reagents were purchased either from Aldrich or Fluka and used as received. [(*p*-cymene)$RuCl_2$]$_2$, [(hexamethylbenzene)$RuCl_2$]$_2$ [37] were prepared according to the literature methods. The NMR spectra were recorded on a Varian Unity 400 spectrometer (^{1}H: 400 MHz, ^{13}C: 100 MHz) or Varian Mercury 300 (^{1}H: 300 MHz, ^{13}C: 75 MHz) at ambient temperature unless stated otherwise. Infrared (IR) absorptions were measured on a Perkin-Elmer Spectrum One FT-IR spectrometer in dichloromethane using NaCl solution cells. Microanalyses were carried out using a Fisons EA 110 CHNS elemental analyser. For certain dendrimers, the analyses are outside acceptable limits, and are ascribed to the encapsulation of solvent molecules and other inorganic salts by dendritic compounds. Melting points were determined using a Kofler hot stage microscope (Riechart Thermover) and are corrected. Mass spectrometry was carried out at the University of Stellenbosch on a Waters API Quattro Micro triple quadrupole mass spectrometer. Data were recorded using Electrospray Ionisation (ESI) mass spectrometry in the positive-ion mode.

3.2. Synthesis of dendrimers **G^1** and **G^2**

A solution of 4-pyridinecarboxaldehyde (1.23 mL, 0.129 mmol) in dry toluene (5.00 mL) was added dropwise to an ice-cooled solution of DAB-$(NH_2)_4$ (1.006 g, 3.18 mmol) in dry toluene (50.0 mL). The reaction mixture was stirred at room temperature in the presence of anhydrous $MgSO_4$ (~10 g) for 24 h. The slurry was filtered and the solvent removed by rotary evaporation yielding an orange residue. The residue was dissolved in dichloromethane (20 mL), and washed copiously with H_2O (6 × 20 mL). The organic layer was collected and dried over anhydrous $MgSO_4$. After filtration by gravity, the solvent removed by rotary evaporation to yield the product as an oil, which was dried *in vacuo*.

*3.2.1. Data for dendritic ligand **G¹***

Pale yellow oil, yield 1.48 g (67.9%). ^{1}H NMR (400 MHz, $CDCl_3$): δ_{ppm} = 1.42 (m, 4H, CH_2), 1.83 (qn, 8H, CH_2), 2.38 (br t, 4H, CH_2), 2.52 (m, 8H, CH_2), 3.63 (t, 8H, ^3J = 7.53 Hz, CH_2), 7.52 (d, 8H, ^3J = 6.04 Hz, Ar_{pyr}), 8.23 (s, 4H, imine), 8.63 (d, 8H, ^3J = 6.02 Hz, Ar_{pyr}). ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$): δ_{ppm} = 25.3, 28.3, 51.7, 54.1, 59.8 (CH_2); 121.8, 150.4 (CH, Ar_{pyr}); 143.0 (C, Ar_{pyr}); 159.0 (CH, imine). IR (NaCl cells, CH_2Cl_2, cm^{-1}): $\nu_{(imine\ C=N)}$ 1648 (s), $\nu_{(aromatic\ C=N)}$ 1599 (s). Anal Calc. for $C_{40}H_{52}N_{10}$. $^1/_2CH_2Cl_2$: C, 68.00; H, 7.47; N, 19.58. Found: C, 68.42; H, 7.50; N, 20.02%.

*3.2.2. Data for dendritic ligand **G²***

Pale yellow oil, yield 1.47 g (75.4%). ^{1}H NMR (300 MHz, $CDCl_3$): δ_{ppm} = 1.39 (br m, 4H, CH_2), 1.47 (br m, 8H, CH_2), 1.72 (m, 16H, CH_2), 1.94-2.44 (overlapping m, 36H, CH_2), 3.56 (br t, 16H, ^3J = 6,49 Hz, CH_2), 7.48 (d, 16H, ^3J = 6.03 Hz, Ar_{pyr}), 8.17 (s, 8H, imine), 8.57 (d, 16H, ^3J = 5.99 Hz, Ar_{pyr}). ^{13}C {^{1}H} NMR (75 MHz, $CDCl_3$): δ_{ppm} = 24.8, 25.2, 28.3, 51.7, 52.2, 52.3, 54.3, 59.8 (CH_2); 121.8, 150.4 (CH, Ar_{pyr}); 142.9 (C, Ar_{pyr}); 159.0 (CH, imine). IR (NaCl cells, CH_2Cl_2, cm^{-1}): $\nu_{(imine\ C=N)}$ 1648 (s), $\nu_{(aromatic\ C=N)}$ 1599 (s). Anal. Calc. for $C_{88}H_{120}N_{22}$.$^1/_2CH_2Cl_2$: C, 69.54; H, 7.98; N, 20.16. Found: C, 69.24; H, 8.18; N, 20.49%.

*3.3. Synthesis of complexes **1–4***

The dimer $[(arene)RuCl_2]_2$ (0.35 mmol, 0.213 g for **1;** 0.081 mmol, 0.056 g for **2;** 0.307 mmol, 0.199 g for **3**; 0.248 mmol, 0.168 g for **4**) was dissolved in dry dichloromethane (30 mL). To this was added a solution of the dendritic ligand in dichloromethane (5 mL): ***G¹*** (0.17 mmol, 0.117 g for **1**; 0.041 mmol, 0.027 g for **2**), and ***G²*** (0.077 mmol, 0.114 g for **3**; 0.041 mmol, 0.062 g for **4**). The reaction mixture was allowed to stir at room temperature for 5 h. The solvent was reduced to 3 mL, and the product was precipitated with petroleum ether. The resulting yellow–orange precipitate was filtered, washed with petroleum ether and dried *in vacuo*.

*3.3.1. [{(p-cymene)$RuCl_2$}$_4$**G¹**)] (**1**)*

Yellow-orange solid, yield 0.26 g (79.1%). M.p.: 165 °C (decompose, without melting). ^{1}H NMR (300 MHz, $CDCl_3$): δ_{ppm} = 1.30 (d, 24H, ^3J = 6.87 Hz, CH*Me*$_2$), 1.47 (br m, 4H, CH_2), 1.83 (br m, 8H, CH_2), 2.09 (s, 12H, CH_3), 2.44–2.97 (overlapping m, 12H, H_2, H_3), 2.97 (m, 4H, C*H*Me$_2$), 3.67 (m, 8H, H_5), 5.28 (d, 8H, ^3J = 6.09 Hz, $Ar_{p\text{-}cy}$), 5.70 (d, 8H, ^3J = 5.70 Hz, $Ar_{p\text{-}cy}$), 7.49 (d, 8H, ^3J = 6.15 Hz, Ar_{pyr}), 8.20 (s, 4H, imine), 9.06 (d, 8H, ^3J = 5.42 Hz, Ar_{pyr}). ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$): δ_{ppm} = 18.3, 22.3 (CH_3, p-cy), 27.1, 31.5, 51.2, 53.7, 58.8 (CH_2), 122.5, 155.3 (CH, pyr); 139.8 (C, pyr), 30.7, 82.1, 83.3 (CH, p-cy); 97.5, 103.3 (C, p-cy); 158.4 (CH, imine). IR (NaCl cells, CH_2Cl_2, cm^{-1}): $\nu_{(imine,\ C=N)}$ 1646 (s), $\nu_{(pyr,\ C=N)}$ 1615 (s). Anal. Calc. for $C_{80}H_{108}Ru_4Cl_8N_{10}$.$1^1/_2CH_2Cl_2$: C, 48.34; H, 5.52; N, 6.92. Found: C, 48.22; H, 5.15; N, 6.74%. MS (ESI, *m/z*): 565.0 $[M + 4H + 4CH_2Cl_2 + H_2O]^{4+}$.

*3.3.2. [{(hexamethylbenzene)$RuCl_2$}$_4$**G¹**] (**2**)*

Yellow-orange solid, yield 0.050 g (86.5%). M.p.: 188 °C (decompose, without melting). ^{1}H NMR (300 MHz, $CDCl_3$, δ ppm): 1.44 (br m, 4H, CH_2), 1.85 (br m, 8H, CH_2), 1.97 (s, 72H, C*H*$_3$), 2.49–2.58 (overlapping m, 12H, CH_2), 3.67 (m, 8H, CH_2), 7.51 (d, 8H, ^3J = 6.4 Hz, Ar_{pyr}), 8.23 (s, 4H, imine), 8.78 (d, 8H, ^3J = 6.3 Hz, Ar_{pyr}). ^{13}C {^{1}H} NMR (75 MHz, $CDCl_3$, δ ppm): 15.4 (CH_3, HMB); 24.2, 26.2, 51.0, 53.4, 58.2 (CH_2); 91.4 (C, HMB); 122.5, 155.0 (CH, pyr); 143.9 (C, pyr); 158.9 (CH, imine). IR (NaCl cells, CH_2Cl_2, cm^{-1}): $\nu_{(imine,\ C=N)}$ 1646 (s), $\nu_{(pyr,\ C=N)}$ 1614 (s). Anal. Calc. for $C_{88}H_{124}Ru_4Cl_8N_{10}$. CH_2Cl_2: C, 51.03; H, 6.06; N, 6.69. Found: C, 51.01; H, 5.85; N, 6.39%. MS (ESI, *m/z*): 635.0 $[M-3Cl]^{3+}$.

*3.3.3. [{(p-cymene)$RuCl_2$}$_8$**G²**] (**3**)*

Yellow-orange solid, yield 0.30 g (98.1%). M.p.: 214 °C (decompose, without melting). ^{1}H NMR (300 MHz, $CDCl_3$): δ_{ppm} = 1.32 (d, 48H, ^3J = 6.92 Hz, CH*Me*$_2$), 1.35–1.48 (overlapping m, 12H, CH_2), 1.78 (m, 16H, CH_2), 2.09 (s, 24H, CH_3), 2.30–2.53 (overlapping m, 36H, CH_2), 2.97 (m, 8H, C*H*Me$_2$), 3.67 (m, 16H, CH_2), 5.28 (d, 16H, ^3J = 6.02 Hz, $Ar_{p\text{-}cy}$), 5.49 (d, 16H, ^3J = 6.01 Hz, $Ar_{p\text{-}cy}$), 7.49 (d, 16H, ^3J = 6.61 Hz, Ar_{pyr}), 8.19 (s, 8H, imine), 9.05 (d, 16H, J_3 = 6.53 Hz, Ar_{pyr}). ^{13}C {^{1}H} NMR (75 MHz, $CDCl_3$): δ_{ppm} = 18.2, 22.2 (CH_3, p-cy), 27.1, 38.8, 51.4, 55.2, 58.8 (CH_2), 122.5, 155.3 (CH, pyr), 144.2 (C, pyr), 30.6, 82.0, 83.0 (CH, p-cy), 97.4, 103.2 (C, p-cy), 158.3 (CH, imine). IR (NaCl cells, CH_2Cl_2, cm^{-1}): $\nu_{(imine,\ C=N)}$ 1646 (s), $\nu_{(pyr,\ C=N)}$ 1614 (s). Anal. Calc. for $C_{168}H_{232}Ru_8Cl_{16}N_{22}$.$4CH_2Cl_2$: C, 48.32; H, 5.66; N, 7.21. Found: C, 48.37; H, 6.03; N, 6.61%. MS (ESI, *m/z*): 569.0 $[M\text{-}7Cl + 3CH_2Cl_2 + CH_3CN]^{7+}$.

*3.3.4. [{(hexamethylbenzene)$RuCl_2$}$_8$**G²**] (**4**)*

Yellow-orange solid, yield 0.23 g (91.7%). M.p.: 194 °C (decompose, without melting). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 1.31 (br m, 4H, CH_2), 1.63 (br m, 8H, CH_2), 1.87 (m, 8H, CH_2), 1.99 (s, 144H, CH_3), 2.15–2.38 (overlapping m, 24H, CH_2), 2.50 (m, 4H, CH_2,), 2.60 (m, 16H, CH_2), 3.69 (m, 16H, CH_2), 7.54 (d, 16H, ^3J = 6.0 Hz, Ar_{pyr}), 8.23 (s, 8H, imine), 8.78 (d, 16H, ^3J = 5.6 Hz, Ar_{pyr}). ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$, δ ppm): 15.4 (CH_3, HMB); 25.3–58.9 (CH_2), 91.4 (C, HMB), 122.5, 155.0 (CH, pyr); 144.1 (C, pyr); 158.9 (C, imine). IR (NaCl cells, CH_2Cl_2, cm^{-1}): $\nu_{(imine,\ C=N)}$ 1646 (s), $\nu_{(pyr,\ C=N)}$ 1613 (s). Anal. Calc. for $C_{184}H_{264}Ru_8Cl_{16}N_{22}$.$2CH_2Cl_2$: C, 51.60; H, 6.24; N, 7.12. Found: C, 51.69; H, 6.43; N, 6.82%. MS (ESI, *m/z*): 631.0 $[M\text{-}7Cl + 5CH_2Cl_2 + 2CH_3CN]^{7+}$.

*3.4. Synthesis of the mononuclear complexes **5** and **6***

The *N*-(pyridin-4-ylmethylene)propan-1-amine (L), was prepared by the reaction of 4-pyridinecarboxaldehyde (0.107 g, 0.723 mmol for **5**; 0.030 g, 0.200 mmol for **6**) with *n*-propylamine in diethyl ether. [(*p*-cymene)$RuCl_2$]$_2$ (0.223 g, 0.362 mmol) or [(hexamethylbenzene)$RuCl_2$]$_2$ (0.068 g, 0.100 mmol) was dissolved in dry dichloromethane (30 mL). A solution of the *N*-(pyridin-4-ylmethylene)propan-1-amine (0.107 g, 0.723 mmol) in dry dichloromethane (5 mL) was added dropwise and the reaction mixture was allowed to stir for 5 hours. The solvent was reduced to approximately 3 mL, and the product was precipitated with petroleum ether. The orange-yellow precipitate was washed with petroleum ether and dried *in vacuo*.

*3.4.1. [(p-cymene)$RuCl_2$(L)] (**5**)*

Yellow–orange solid, yield 0.15 g (91.1%). M.p.: 163-166 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ_{ppm} = 0.97 (t, 3H, ^3J = 7.39 Hz, CH_3), 1.32 (d, 6H, ^3J = 6.93 Hz, CH*Me*$_2$), 1.75 (m, 2H, CH_2), 2.11 (s, 3H, C*H*$_3$), 3.00 (m, 1H, C*H*Me$_2$), 3.66 (t, 2H, ^3J = 6.47 Hz, CH_2), 5.23 (d, 2H, ^3J = 5.93 Hz, $Ar_{p\text{-}cy}$), 5.45 (d, 2H, ^3J = 5.92 Hz, $Ar_{p\text{-}cy}$), 7.60 (d, 2H, ^3J = 6.53 Hz, Ar_{pyr}), 8.27 (s, 1H, imine), 9.10 (d, 2H, ^3J = 6.43 Hz, Ar_{pyr}). ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$): δ_{ppm} = 11.8 (CH_3), 18.2, 22.3 (CH_3, p-cy), 23.8, 63.6 (CH_2), 30.7, 82.2, 83.1 (CH, p-cy), 97.3, 103.6 (C, p-cy), 122.5, 155.3 (CH, pyr), 144.6 (C, pyr), 157.3 (CH, imine). IR (NaCl cells, CH_2Cl_2, cm^{-1}): $\nu_{(imine,\ C=N)}$ 1647 (s), $\nu_{(pyr,\ C=N)}$ 1615 (s). Anal. Calc. for $C_{19}H_{26}RuCl_2N_2$: C, 50.22; H, 5.77; N, 6.16. Found: C, 49.96; H, 5.38; N, 5.99%. MS (ESI, *m/z*): 419.1 $[M-Cl]^+$.

*3.4.2. [(hexamethylbenzene)$RuCl_2$(L)] (**6**)*

Orange solid, yield 0.15 g (91.1%). M.p.: 139 °C (decompose, without melting). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 0.96 (t, 3H, ^3J = 7.4 Hz, CH_3), 1.74 (m, 2H, CH_2), 2.03 (s, 18H, C*H*$_3$), 3.66 (t, 2H, ^3J = 6.9 Hz, CH_2), 7.57 (d, 2H, ^3J = 6.4 Hz, Ar_{pyr}), 8.26 (s, 1H, imine), 8.86 (d, 2H, ^3J = 6.2 Hz, Ar_{pyr}). ^{13}C {^{1}H} NMR (100 MHz, $CDCl_3$, δ ppm): 11.8 (CH_3), 15.4 (CH_3, HMB), 23.8, 63.6 (CH_2), 91.4 (C,

HMB), 122.5, 155.1 (CH, pyr), 144.2 (C, pyr), 157.5 (CH, imine). IR (NaCl cells, CH_2Cl_2, cm^{-1}): $\nu_{(imine,\ C=N)}$ 1646 (s), $\nu_{(pyr,\ C=N)}$ 1614 (s). Anal. Calc. for $C_{21}H_{30}RuCl_2N_2$: C, 52.28; H, 6.27; N, 5.81. Found: C, 51.84; H, 5.94; N, 5.47%. MS (ESI, *m/z*): 447.1 $[M-Cl]^+$.

3.5. X-ray crystallography

X-ray data for **5**; $C_{19}H_{26}Cl_2N_2Ru$, $M = 454.39\ gmol^{-1}$, monoclinic, $P\ 2_1/c$ (no 14), $a = 18.246(3)$, $b = 15.057(2)$, $c = 7.3464(11)$ Å, $\beta = 101.360(14)^\circ$, $U = 1978.7(5)$ Å^3, $T = 173$ K, $Z = 4$, μ (Mo Kα) = 1.065 mm^{-1}, 3530 reflections measured, 1316 unique ($R_{int} = 0.2154$) which were used in all calculations. The final $wR(F^2)$ was 0.1830 (all data. The data were measured using a Stoe Image Plate Diffraction system equipped with a ϕ circle goniometer, using Mo Kα graphite monochromated radiation ($\lambda = 0.71073$ Å) with ϕ range 0–200°, increment of 1.0°, $D_{max}-D_{min} = 12.45–0.81$ Å. The structure was solved by direct methods using the program SHELXS-97 [38]. The refinement and all further calculations were carried out using SHELXL-97 [39]. The hydrogen atoms were included in calculated positions and treated as riding atoms using the SHELXL default parameters. All non-H atoms were refined anisotropically, using weighted full-matrix least-square on F^2. Fig. 3 was drawn with ORTEP [40].

3.6. Cytotoxicity study

While imine bonds are susceptible to hydrolysis, the presence of the aromatic substituent together with a possible dendrimer effect reduces the rate of hydrolysis, and for the compounds studied herein only a slow decomposition is observed. The human A2780 ovarian cancer cell line was obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were grown routinely in RPMI medium containing glucose, 5% foetal calf serum (FCS) and antibiotics at 37 °C and 5% CO_2. Cytotoxicity was determined using the MTT assay (MTT = 3(4,5dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). Cells were seeded in 96-well plates as monolayers with 100 μL of cell solution (approximately 20,000 cells) per well and pre-incubated for 24 hours in medium supplemented with 10% FCS. Compounds were prepared as DMSO solution then dissolved in the culture medium and serially diluted to the appropriate concentration, to give a final DMSO concentration of 0.5%. One hundred micro litre of drug solution was added to each well and the plates were incubated for another 72 h. Subsequently, MTT (5 mg/mL solution) was added to the cells and the plates were incubated for a further 2 h. The culture medium was aspirated, and the purple formazan crystals formed by the mitochondrial dehydrogenase activity of vital cells were dissolved in DMSO. The optical density, directly proportional to the number of surviving cells, was quantified at 540 nm using a multiwell plate reader and the fraction of surviving cells was calculated from the absorbance of untreated control cells. Evaluation is based on means from two independent experiments, each comprising 3 microcultures per concentration level.

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Appendix A. Supplementary material

CCDC 723508 (**5**) contains 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. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jorganchem.2009.06.028.

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