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Synthesis and anticancer activity of carbosilane metallodendrimers based on arene ruthenium (II) complexes

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Abstract

A series of new organometallic carbosilane dendrimers (first and second generation) and the corresponding non-dendritic mononuclear based on ruthenium arene fragments are described. The metallodendrimers were prepared by reaction of precursor $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ with carbosilane dendrimers functionalized with *N*- donor monodentate ligands such as NH_2 - and pyridine, or with *N,O*-, *N,N*- chelating imine ligands. While the dendrimer precursors are insoluble in DMSO or water, novel metallodendrimers are soluble in DMSO and some of them are even highly soluble in water. The molecular structure of the “Ru- NH_2 ” mononuclear compound (zero generation) was determined by single-crystal X-ray crystallography. The cytotoxicity activity of these dendritic structures was evaluated in several human cancer cell lines and compared with that of the corresponding mononuclear ruthenium complexes. Most compounds display significant cytotoxic activities in the low micromolar range with the first generation ruthenium dendrimers being the most active compounds. The cell death type for selected compounds has been studied along with their reactivity towards relevant biomolecules such as DNA, Human Serum Albumin (HSA) and Cathepsin-B. All the data points to a mode of action different from that of cisplatin for most complexes. First generation ruthenium dendrimers inhibit Cathepsin-B which may suggest potential antimetastatic properties of these compounds.

Graphical abstract

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Carbosilane-based ruthenium dendrimers have been tested as anticancer agents. These metallodendrimers are active against a number of cisplatin resistant cell lines in the low micromolar range while showing a dendritic effect.

Ruthenium (II) carbosilane dendrimers as anticancer agent

Keywords

ruthenium; carbosilane; dendrimer; nitrogen ligands; anticancer activity; Cathepsin-B inhibition

Introduction

Cancer is an uncontrolled cell proliferation that invades and destroys adjacent tissues and spreads to distant sites, with formation of new colonies or metastatic spread which causes deterioration of vital organs functions and may result in the death of the individual. Cancer causes 1 in 8 deaths worldwide and is rapidly becoming a global pandemic. In 2012, the WHO estimated there were 8.2 million deaths due to cancer on the previous year. If incidence rates do not change, the global cancer burden is expected to nearly double to 19.3 million cases by 2025.^{1, 2} The discovery of the antitumoral properties of cis-diamminedichloroplatinum (II) (cisplatin) by Rosenberg in 1965 paved the way to the development of metallodrugs-based cancer chemotherapy.³ Cisplatin has however, a number of drawbacks including its limited spectrum activity, acquired resistance and severe side effects. A number of follow-up platinum analogues (carboplatin, oxaliplatin or satraplatin) which have fewer side effects have been developed.^{4–6} In the search of more effective and selective potential anticancer metallodrugs^{7, 8} different approaches have been pursued, including the study of ruthenium compounds.^{9, 10} As platinum-based drugs, ruthenium compounds can exchange *N* and *O* donor molecules of interest in reactions with DNA. Ruthenium compounds can easily access three different oxidation states (II, III and possible IV) in biological fluids. Ruthenium (III) compounds could potentially behave as pro-drugs as they can be reduced to ruthenium (II) derivatives in solid tumor masses where the low content in oxygen may act as a reducing environment. In addition, ruthenium derivatives probably use transferrin^{11, 12} to accumulate into tumors due to the similarities with iron. To date, three coordination ruthenium (III) compounds have entered clinical trials for cancer: NAMI-A,^{9, 13, 14} KP1019^{9, 15} and NKP-1339^{16, 17} (Fig. 1).

Another family of ruthenium compounds that are becoming attractive candidates for further development as potential cancer chemotherapeutics are stable organoruthenium (II)

complexes.^{18–20} A number of pseudo-octahedral “piano-stool” organometallic ruthenium (II) arene complexes of the type $[(\eta^6\text{-arene})\text{Ru}(\text{chelate})\text{Cl}]^+$ where the chelating ligands are *N,N*- or *N,O*-, or with monodentate phosphine or amine ligands $[(\eta^6\text{-arene})\text{Ru}(\text{L})\text{Cl}_2]$ have been reported.^{21–35} Some relevant examples from the groups of Sadler (RM175),²⁴ Dyson (RAPTA-T²⁷ analogue of RAPTA-C),^{26, 30} and Contel (IM-Ru)³³ which have undergone advanced preclinical studies (*in vivo* and, in some cases^{26, 33} pharmacokinetic studies in mammals) are depicted in Fig. 2.

One of the main drawbacks of cancer chemotherapeutics is the lack of selectivity which induces severe adverse effects on healthy tissues and organs. It would be desirable to develop carrier platforms that could improve the efficacy and reduce toxicity of cancer treatment by specific delivery of the therapeutic agents to the tumor sites. Passive drug targeting and delivery (DTD) exploits the features of solid tumor vasculature which is disorganized, twisted and dilated with numerous pores resulting in a higher number of fenestrations by the enhanced permeability and retention effect (EPR effect).³⁶

Taking advantage of this effect, there have been reports on the use of nanoparticle-based platforms for the delivery of anticancer drugs. A number of such delivery systems have been approved for cancer therapy in the clinic, with many more currently undergoing clinical trials or preclinical evaluations.^{37–42}

In the field of nanoscopic delivery systems, dendrimers could potentially be advantageous for drug delivery applications.⁴³ Recently, the antitumoral properties of some metallodendrimers based on DAB or PPI scaffolds have been described,^{44–47} including η^6 -*p*-cymene-ruthenium (II) systems.^{48–50} Carbosilane dendrimers display relevant features, such as their chemical and thermal stability, their inert framework, biocompatibility and its hydrophobic nature that might enhance interactions with biological membranes. They have been studied in different biomedical applications such as antibacterial or antiviral agents, or as non-viral vehicles for the delivery of nucleic acids (oligonucleotides or siRNA).^{51–58} However, their application as delivery vehicles for metal-fragments in cancer therapy has not yet been described.

We report here on the synthesis, anticancer activity and reactivity with relevant biomolecules of new ruthenium η^6 -*p*-cymene-based carbosilane dendrimers and the corresponding non-dendritic mononuclear ruthenium derivatives.

Results and Discussion

1.- Synthesis and characterization of dendritic ligands

We are describing the synthesis of carbosilane dendrimers functionalized with η^6 -*p*-cymene-ruthenium (II) species containing four different ligands: two *N*- monodentate and *N,O*- and *N,N*-, chelating ligands. To clarify the discussion, the dendrimers will be named as $G_n\text{-}[X]_m$, where “*n*” indicates dendrimer generation (0, 1 or 2), “*X*” states the nature of the groups present on the dendrimer surface and “*m*” the number of terminal groups. With this nomenclature the non-dendritic mononuclear ruthenium complexes that will be used in subsequent biological studies, become G_0 or “zero generation” dendrimers.

The starting materials used to prepare the dendritic ligands are carbosilane dendrimers with terminal amine $-NH_2$ groups, $G_n[NH_2]_m$ ($n=0, m=1$ (**a**); $n=1, m=4$ (**b**); $n=2, m=8$ (**c**)).⁵⁹ These dendrimers were selected as they can be easily modified and new coordination sites can be introduced on the dendrimer surface.

The Schiff-base condensation reaction of dendrimers **a–c** with three different aldehydes, 4-pyridinecarboxaldehyde, 2-pyridinecarboxaldehyde and salicylaldehyde, leads to the formation of the corresponding dendrimers with terminal *N*-monodentate ligands $G_n[NCPH(p-N)]_m$ (**1–3**); *N,N*-chelating ligands $G_n[NCPH(o-N)]_m$ (**4–6**); and *N,O*-chelating ligands $G_n[NCPH(o-OH)]_m$ (**7–9**), respectively (Fig. 3). All reactions were carried out under nitrogen atmosphere and in the presence of $MgSO_4$ for water removal.

The addition of a slight excess of aldehyde (in order to ensure the complete reaction of all peripheral amine groups of the dendrimer) was required for dendrimers G_1 and G_2 . The dendrimer crude products were subsequently purified by size exclusion chromatography in THF. The resulting colorless (**1–3**), brown (**4–6**) or yellow (**7–9**) compounds, were isolated as oily products in high yields (see Experimental Section). All compounds are air stable and stable in THF or $CHCl_3$ solutions for months. The final products (**1–9**) are soluble in all common organic solvents but insoluble in water, methanol and DMSO. This lack of solubility prevented the biological evaluation of these complexes as controls.

Compounds **1–9** were characterized by elemental analysis, multinuclear NMR spectroscopy (1H , ^{13}C { 1H }, ^{15}N , ^{29}Si -NMR) and mass spectrometry. The analytical and spectroscopic data (see Experimental Section and Supplementary Information) are consistent with the proposed structures depicted in Fig. 3. The most relevant NMR resonances in all compounds are those corresponding to the imine bond and the new methylene $-CH_2N$ group. The aldimine proton was observed in the 1H -NMR spectra at δ ca. 8.20, whereas in the ^{13}C { 1H }-NMR spectra the resonance for the corresponding carbon was observed in the range 154.6–161.5 ppm. Furthermore, in the 1H -NMR spectra the resonance of the new methylene group $-CH_2N$ was shifted to a higher frequency (δ ca. 3.60) than that for the precursor amine dendrimers (2.63 ppm) (Fig. 4). The resonances for the carbosilane framework appear as broadened peaks in the 1H -NMR spectra and at similar chemical shifts to those of their precursor dendrimers.⁵⁹

In the 1H -NMR and ^{13}C -NMR spectra, the presence of aromatic rings in compounds **1–9** gave rise to several signals at the expected values. 1H - ^{29}Si -HMBC-NMR spectra also showed cross peaks similar to those observed in the parent $-NH_2$ ended dendrimers. The 1H - ^{15}N HMBC-NMR spectra show two signals for the pyridine derivatives corresponding to the pyridine and imine nitrogen's at ca. -64.0 and -32.0 ppm (compounds **1–3**) and at ca. -67.0 and -38.0 ppm (compounds **4–6**). The 1H - ^{15}N HMBC-NMR spectra for the phenolato-imine derivatives **7–9** show one signal at ca. -90.0 ppm which corresponds to the imine nitrogen. The higher value may be due to the formation of a hydrogen bond between the imine-nitrogen and the phenol group in compounds **7–9**.

2.- Synthesis and characterization of ruthenium complexes

The ligands **a–c** and **1–9** described above were used to coordinate organo-ruthenium complexes with the idea to evaluate their anticancer activity and to establish an initial structure *versus* biological activity relationship. In this respect, two monodentate ligands, one with primary terminal amine groups $-NH_2$ (**a–c**) and another one with terminal pyridine groups (**1–3**), and two chelating ligands, one of them containing two nitrogen donor atoms (possibility for *N,N*-coordination) **4–6**, and other one containing a nitrogen and an oxygen donor atom (possibility for *N,O*-coordination) **7–9**, were used.

The reaction of dendrimers **a–c** and **1–9** with the ruthenium precursor $[Ru(\eta^6\text{-}p\text{-cymene})Cl_2]_2$ was carried out in a ratio 1:1 branch:[Ru]. The general synthetic procedure for the synthesis of ruthenium dendrimers is shown in Scheme 1 and further details are provided in the Experimental Section. Neutral or cationic species are obtained depending on the nature of the ligand. For compounds **10–15**, where the dendritic ligand is acting as a monodentate ligand, the dimer is cleaved and two chlorine atoms remain coordinated to the ruthenium. For compounds **16–21**, the dendritic ligands are chelates and only one chlorine atom remains coordinated to the metal center (Scheme 1). Compounds **10–21** were characterized by elemental analysis and 1H , ^{13}C { 1H } and ^{29}Si -NMR spectroscopy (full details are given in the Experimental Section and Supplementary Information). These data along with the reaction stoichiometry support the presence of one Ru moiety per branch. For instance, if this procedure is carried out employing a ratio 2:1 branch:[Ru] a different compound is observed (see Supplementary Information, Scheme S1, Fig. S18–S19). Additionally, the reaction of excess 2-pyridinecarboxyaldehyde with $[Ru(\eta^6\text{-}p\text{-cymene})Cl_2]_2$ only led to the formation of a monosubstituted species (see Supplementary Information, Scheme S2–S3, Fig. S20–S22).

Compounds **16–18** are highly soluble in water (> 100 mg/ml), whereas compounds **10–15** are not soluble in water but are soluble in 1:99 DMSO:H₂O mixtures (relevant for subsequent biological evaluation). Metallodendrimers **19–21** are not soluble in water or DMSO:H₂O mixtures but they can be solubilized in DMSO or MeOH.

The coordination of ruthenium to dendritic ligands (**a–c** and **1–9**) could be confirmed by the shifting of the signals corresponding to the arene fragment in the 1H -NMR and in the ^{13}C -NMR spectra. 1H -NMR and ^{13}C -NMR spectra for the compounds **10–12** show the methylene group $-CH_2N$ shifted to low field (higher frequency) with respect to the chemical shift values observed in the corresponding ligands, **a–c**. This is due to the coordination of the electron-withdrawing metal center that causes the deshielding of the signals previously commented. The presence of a symmetry plane in the neutral compounds $G_n[NH_2Ru(\eta^6\text{-}p\text{-cymene})Cl_2]_m$ (**10–12**) makes the arene protons equivalent and only two doublets were observed at δ 5.30 and 5.40. Moreover, the 1H -NMR spectra showed a multiplet around δ 3.00 assigned to the arene protons and the methylene group $-CH_2N$ bonded to the amine ligand (See Supplementary Information, Fig. S11). The protons of the $-NH_2$ group appeared around δ 2.90.

The same behavior for the arene ligand was observed for the family of compounds $G_n[NPh(p\text{-N})Ru(\eta^6\text{-}p\text{-cymene})Cl]_m$ (**13–15**) in the 1H and ^{13}C -NMR spectra. In contrast,

the ^1H -NMR spectra (See Supplementary Information, Fig. S15) for the family G_n -[[NCP $\text{h}(\alpha\text{-N})\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}]_m$ (**16–18**) show that the symmetry of the arene ring is affected by the coordination of this chelating ligand, leading to a loss of symmetry that generates chirality around the ruthenium center. This chirality was clearly observed in the NMR spectra of these compounds. Three peaks at 5.82, 6.06 and 6.17 ppm corresponding to the arene and two doublets at δ 1.15 and 1.21 related to the two methyls of the isopropyl group were observed. This effect was confirmed by ^{13}C -NMR spectroscopy where four peaks around δ 87.00 were clearly observed. The diastereotopic effect around the methylene group adjacent to the imine nitrogen induced by the chiral ruthenium center is observed by the presence of two sets of signals around δ 4.00. Again, the coordination of ruthenium to two nitrogen atoms (imine-pyridine) shifts signals to a higher frequency with respect to the chemical shift values observed in the corresponding ligands. A similar effect (due to a chiral ruthenium) was also observed in the family G_n -[NCP $\text{h}(\alpha\text{-O})\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}]_m$ (**19–21**) for the arene and methylene groups. - In general, Si atoms are almost not affected by ruthenium coordination since no significant changes were observed with respect to the dendritic ligands (**a–c** and **1–9**) in the ^1H - ^{29}Si HMBC spectra.

For all these compounds, a pseudo-octahedral structure can be proposed in solution with the arene group occupying three of these six coordination sites, according to the X-ray structure obtained for compound **10** (Fig. 5). Complex G_0 -[$\text{NH}_2\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2$]**1** (**10**) crystallizes in the triclinic space group P-1 with one molecular formula in the asymmetric unit. Selected bond distances and angles are collected in Table S2 (see Supplementary Information).

In this complex, the arene ligand is planar and the distance between the ruthenium atom and the centroid of the aromatic ring is 1.668 Å, a normal value if compared with the data of similar structures found in the CSD (mean value 1.653 Å). All the distances and angles of this structure are in the expected range having values comparable to those in the related $\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2\text{NH}_2\text{R}$ species.⁶⁰

Taking into account that these metallodendrimers have been prepared for biological evaluation, we tested their stability in buffer and in DMSO, since mixtures DMSO:H₂O or DMSO:PBS (<1:99) were used in subsequent assays. Initially, we selected mononuclear compounds **10**, **13** and **16** for these tests and then we extended the study to the rest of dendritic compounds.

The stability of compound **16** was evaluated by ^1H -NMR spectroscopy in D₂O, PBS-D₂O or neat DMSO-*d*₆. We did not observe any appreciable changes in the ^1H -NMR spectra of this derivative - after 72 hours (see Supplementary Information Fig. S23–S27).

In the case of the first and second generation dendrimers with the same dendritic scaffold (compounds **17** and **18**), we also did not observe changes in the ^1H -NMR spectra for 72 hours, (see Supplementary Information Fig. S28–29).

The stability of monodentate derivatives (**10–15**) was evaluated by ^1H -NMR spectroscopy in neat DMSO-*d*₆ due to their low solubility in mixtures DMSO:H₂O or DMSO:PBS in concentrations higher than 100 μM . In these cases a displacement of the amine or nitrogen

heterocyclic based dendritic ligands (**a–c** and **1–3**, respectively) from the ruthenium coordination sphere was observed. It is not uncommon that to observed the discoordination of nitrogen ligands from the ruthenium center in the presence of coordinating solvents such as DMSO. The ^1H -NMR spectra of these derivatives (see for example figures S30–S34 for mononuclear compounds **10** and **13** and S35 for first generation dendrimer **14**) show the presence of $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2(\text{DMSO})]$, together with the signals corresponding to our compounds, indicating a competition between DMSO and the dendritic ligands for the coordination of ruthenium. We think that this process could be an equilibrium similar to others already described for ruthenium compounds bearing nitrogen ligands.^{61, 62} In order to get more information about the DMSO exchange process -, we treated CDCl_3 solutions of mononuclear compounds **13** and **16** with growing quantities of DMSO, at ratios $[\text{Ru}]/\text{DMSO}$ ranging from 1:0.25 to 1:4. In these conditions the ^1H NMR spectra of both monodentate and chelate complexes remain unchanged after 24 hours. (see Supplementary Information Fig. S26–S27). So, we believe that only at high concentration ratios of DMSO the exchange occurs.

3.- Biological Activity

3.1.- Cytotoxicity Studies—The cytotoxic activity of the new ruthenium complexes **10–21** was assayed by monitoring their ability to inhibit cell growth using the MTT or XTT assays (see Experimental Section). Cytotoxic activity of the compounds was determined as described in the Experimental Section in several human cancer cell lines: cervical HeLa, breast MCF-7, colon HT-29 and triple negative breast MDA-MB-231. In order to assess the compounds selectivity for cancerous cells with respect to normal cell lines, the compounds were also screened for their cytotoxic effects on the non-tumorigenic human embryonic kidney cells HEK-293T. The results are summarized in Table 1. All the compounds studied are cytotoxic in these cell lines but the non-dendritic G_0 mononuclear complexes (**10**, **13** and **16**) display lower cytotoxicity (especially compounds **10** and **13**). Compound **16** with the N,N' -chelating ligand $G_0[\text{NCPH}(o\text{-N})]_1$ (**4**) displays a higher cytotoxicity for cervical, breast and colon cancer cell lines (*ca.* 6–7 μM) but lower (10 fold) cytotoxicity for triple-negative breast cancer. These non-dendritic species have a different selectivity and while compound **10** results more selective (3-fold) towards colon cancer cell lines (when compared to non-tumorigenic HEK-293T), **13** is more selective (2-fold) to the cervical cancer cell lines. Compound **16** is selective (6–7-fold) to cervical, breast and colon cancer cell lines but as mentioned before is far less active on triple negative breast MDA-MB-231 cancer cell lines. There seems to be a general trend that indicates that the ruthenium dendrimers of first and second generation are in general more cytotoxic than their analogous mononuclear zero generation counterparts. For these G_1 and G_2 dendrimers the cytotoxicity is comparable for the cancerous and HEK-293T cells. However, HEK-293T cell lines are immortalized cells that may display a higher sensitivity to chemicals. Some of us have described recently an IM ruthenium compound $[(\eta^6\text{-}p\text{-cymene})\text{Ru}\{(\text{Ph}_3\text{P}=\text{N}-\text{CO}-2\text{-N}-\text{C}_5\text{H}_4)-\kappa\text{-}N,O\}\text{Cl}]\text{Cl}$ (Ru-IM, Fig. 2) which displayed similar IC_{50} values *in vitro* for human cancer cell versus HEK-293T but which was very effective *in vivo* on MDA-MB-231 xenografts in NOD.CB17-Prkdc SCID/J mice while having low systemic toxicity.³³ The cytotoxicity of the most active dendrimers is in the range of the cytotoxicity obtained for two arene-ruthenium octanuclear dendrimers on cervical cancer cell lines (A2780 and A2780cisR).⁴⁹ In the case of these

previously reported compounds (containing chelating *N,O*- and *N,N*-ruthenium (II) arene functionalized poly(propyleneimine) dendrimer scaffolds a correlation between size dependency of the dendrimers and cytotoxicity was found.⁴⁹ With the systems described here, this correlation is not that clear. While the cytotoxicity of the G_1 and G_2 dendrimers is higher than that of the mononuclear derivatives in most cases, there are not big differences between G_1 and G_2 . The activities found for the tetranuclear G_1 derivatives are higher than those found for other tetranuclear arene-ruthenium compounds described in the literature.⁶³ All these data suggest that first generation dendrimers may be sufficient in order to get compounds with a high biological activity.

The three ruthenium compounds (**19–21**) with the *N,O*- chelating ligands G_n -[NCPh(*o*-OH)]_{*m*} (**7–9**) were also evaluated although their lack of solubility in mixtures 1:99 DMSO-media precluded the acquisition of reliable data. Consistent IC₅₀ values were obtained only for HeLa cell lines (IC₅₀(μ M) = 33.3 ± 3.2 (**19**); 52.6 ± 3.2 (**20**) and 20.6 ± 4.4 (**21**)) and thus results for these compounds have not been included in Table 1.

First generation dendrimers (**11**, **14** and **17**) were in general more soluble than their respective G_2 dendrimers (in 1:99 DMSO:media or 1:99 DMSO:H₂O mixtures) while having a similar cytotoxicity. For that, we selected first generation dendrimers **11** and **14** and all water-soluble dendrimers **16–18** (with ligands G_n -[NCPh(*o*-N)]_{*m*} (**4–6**)) for further evaluation based on their cytotoxicity and solubility properties.

3.2.- Mechanism of cell death—We selected highly cytotoxic water-soluble first generation dendrimer (G_1 -[NCPh(*o*-N)Ru(η^6 -*p*-cymene)Cl₂]₄) (**17**) as a model to evaluate the mechanism of cell death of the most active metallodendrimers in triple negative MDA-MB-231 cancer cells (Fig. 6). MDA-MB-231 viability, necrosis, and apoptotic activity were analysed by the ApoTox-Glo Triplex (see Experimental Section) which combines three chemical assays to assess viability, cytotoxicity, and caspase activation events within a single well. These three critical parameters characterize the effects of the treatment with compound **17** on triple negative breast cancer cells. Viability and cytotoxicity are inversely proportional, such that if the viability of cells is reduced, cytotoxicity is usually stimulated. The cytotoxicity may be programmed cell death (apoptosis) or primary necrosis. Our studies showed compound **17** decreases cell viability. The cytotoxicity results show that **17** does stimulate necrosis as 27% of the cells are necrotic, although it also induces apoptosis to a lesser degree as 16% of the cells are apoptotic. About 15% of non-viable cells we undetected by neither the apoptotic nor the necrotic indicator suggesting that those cells might die via different modalities such as necroptosis, autophagy, or maybe pyroptosis that is driven by the activation of caspase proteases Caspase-1 which our assay does not detect.

The induction of apoptotic cell death has been considered the main pathway by which cytotoxic metal complexes exert their effect. However, a number of cancers are able to develop resistance to cisplatin and follow-on derivatives due to defects on the cellular apoptotic machinery. A recent report has reviewed the metal complexes that trigger alternative cell pathways to apoptosis and has identified some candidates to be explored as potential chemotherapeutics.⁶⁴

3.3.- Reactivity with biomolecules

a) Interactions with DNA: Since DNA replication is a key event for cell division, it is among critically important targets in cancer chemotherapy. Most cytotoxic platinum drugs form strong covalent bonds with DNA bases.⁶⁵ However, a variety of platinum compounds act as DNA intercalators upon coordination to the appropriate ancillary ligands.⁶⁶ The more thoroughly studied ruthenium antitumor agents have displayed differences with respect to their interactions with DNA depending on their structure.⁹ Thus, while NAMI-A is known to have fewer and weaker interactions with DNA than cisplatin,⁹ indazoliumbisindazoletrichlororuthenate (KP1019) undergoes interactions similar to cisplatin but with a lower intensity in terms of DNA-DNA and DNA-protein crosslinks.¹⁵ Organometallic piano-stool ruthenium (II) compounds based on biphenyl rings RM175 interact strongly with DNA binding to guanines and by intercalation.^{67, 68} Organometallic ruthenium (II) RAPTA derivatives, characterized by the presence of water soluble TPA phosphine, exhibit pH-dependent DNA damage: at the pH typical of hypoxic tumor cells DNA was damaged, whereas at the pH characteristic of healthy cells little or no damage was detected. A number of organometallic piano-stool ruthenium (II) compounds with *N,N*-, *N,O*- or *C,N*-chelating ligands have shown no or weak interaction with DNA,^{25, 68–70} while two selected compounds containing chelating *N,O*- and *N,N*-ruthenium (II) arene functionalized poly(propyleneimine) dendrimer scaffolds showed interaction with DNA and their cytotoxicity was based on the extent of these interactions.⁴⁹ Cycloruthenated compounds based on pincer *C,N*-ligands (RDC family) displayed a much weaker interaction with *plasmid* (*pBR322*) DNA when compared to cisplatin.⁷¹ Complexes of the type $[\text{Ru}(\text{Cp})(2,2\text{-bipy})(\text{PR}_3)][\text{CF}_3\text{SO}_3]$ have shown no observable interaction with DNA.²⁰

In this context, we evaluated the effect of DNA interactions that could, to some extent, contribute to the observed cytotoxicity of the new metallodendrimers. We followed the interaction of selected compounds (**10**, **11**, **13–18**) by electrophoresis in agarose gel with *plasmid* (*pBR322*) DNA. For compounds (**16–18**) we also studied their interaction with *Calf Thymus* DNA (CT DNA) by circular dichroism (CD). The CD spectral technique is very sensitive to diagnose alterations on the secondary structure of DNA that result from DNA-drug interactions.

A typical CD spectrum of CT DNA shows a positive band with a maximum at 275 nm due to base stacking, and a negative band with a minimum at 248 nm due to helicity, characteristic of the B conformation.⁷² Therefore, changes in the CD signals can be assigned to corresponding changes in DNA secondary structure. In addition, it is known that simple groove binding or electrostatic interaction of small molecules cause little or no alteration in any of the CD bands when compared to major perturbations induced by covalent binding or intercalation.

CD spectra of CT DNA incubated with compounds **16–18** (see Fig. 7) at 37°C and pH = 7.30 in Tris/HCl buffer up to molar ratio drug/DNA = 1.0 show no modification of the DNA bands with respect to untreated CT DNA, indicating that drug-DNA interactions, if existing, do not induce any observable perturbation on the DNA secondary structure under our experimental conditions. Higher ratios were also tested, although loss of CD signal was

observed due to precipitation of the DNA induced by **17–18**, most likely because of phosphate charge neutralization by the cationic compounds, which suggests the existence of an electrostatic attraction. DNA condensation or precipitation by neutralization of backbone charges has been previously described for other ionic ruthenium drugs.^{33, 73}

In order to gain further insight on the nature of the compound DNA interactions, gel electrophoresis studies were also performed with the ruthenium (II) complexes **10**, **11**, **13–18** on *plasmid* (*pBR322*) DNA (Fig. 8). For these experiments, cisplatin and the starting dimeric organometallic ruthenium (II) complex $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ were also measured as controls. *Plasmid* (*pBR322*) DNA presents two main forms, OC (open circular or relaxed) and CCC (covalently closed or supercoiled), which display different electrophoretic mobility. Changes in the electrophoretic mobility of any of the forms upon incubation of the plasmid with a compound are usually interpreted as evidence of interaction.⁷⁴ Generally, a drug that induces unwinding of the CCC form will produce a retardation of the electrophoretic mobility, while coiling of the OC form will result in increased mobility. Fig. 8 shows the effect of cisplatin, $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ and ruthenium (II) dendrimers **10**, **11**, **13–18** on *plasmid* (*pBR322*) DNA after incubation at 37°C for 20 h in Tris/HCl buffer up to drug/DNA ratio 2.0. As previously reported, cisplatin is able to both increase and decrease the mobility of the OC and the CCC forms, respectively.⁷⁵ All the new ruthenium dendrimers tested and the ruthenium starting dimer do not seem to induce any alteration on the mobility of the plasmid like other organometallic piano-stool ruthenium (II) compounds with *N,N*-, *N,O*- or *C,N*-chelating ligands.^{33, 35}

Since it has been shown that for some ruthenium (II) organometallic complexes (RAPTA series) at pH typical of hypoxic tumor cells, DNA is damaged while for the pH characteristic of healthy cells little or no damage was detected,²⁵ we evaluated the interaction of first generation dendrimers (**11**, **14** and water-soluble **17**) with *plasmid* (*pBR322*) DNA at a more acidic or basic pH (Fig. 9). We could confirm that there is no significant change with respect to the effects at neutral pH and therefore all these first generation dendrimers do not interact with *plasmid* (*pBR322*) DNA.

The results of CD, and gel electrophoresis taken together suggest that the new ruthenium dendrimers undergo no or weakly electrostatic interactions with DNA. This conclusion is supported by three main facts: 1) results obtained by CD spectroscopy do not show evidence of CT DNA modifications of secondary structure, suggesting that drug-DNA interactions, if any, are of weak nature, but neither covalent nor intercalation; 2) precipitation of CT DNA is observed in CD experiments (**16–18**) at high ratios drug/DNA, suggesting backbone charge neutralization for these ionic dendrimers, and 3) no retardation of the plasmid DNA electrophoretic mobility is observed. Thus, we hypothesize that the anti-tumor properties observed for these metallodendrimers are due to non-DNA related mechanisms/factors, as previously observed for other $\eta^6\text{-}p\text{-cymene}$ ruthenium compounds^{9, 10, 21, 33} but different to that found for previously described ruthenium dendrimers with *N,N*- and *N,O*-chelating ligands.⁴⁹

b) Interactions with HSA: Human serum albumin (HSA) is the most abundant carrier protein in plasma and is able to bind a variety of substrates including metal cations,

hormones and most therapeutic drugs. It has been demonstrated that the distribution, the free concentration and the metabolism of various drugs can be significantly altered as a result of their binding to the protein.⁷⁶ HSA possesses three fluorophores, namely tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues, with Trp214 being the major contributor to the intrinsic fluorescence of HSA. This Trp fluorescence is sensitive to the environment and binding of substrates, as well as changes in conformation that can result in quenching (either dynamic or static).

Thus, the fluorescence spectra of HSA in the presence of increasing amounts of the compounds **10**, **11**, **13–18**, and cisplatin were recorded in the 300–450 nm range upon excitation of the tryptophan residue at 295 nm (Fig. 10A and Fig. S36, S37, S39–41, S43 and S44 in Supplementary Information). The compounds caused a concentration dependent quenching of fluorescence without changing the emission maximum or the shape of the peak. All these data indicate an interaction of the compounds with HSA. In order to get more information about the mode of interaction between HSA and ruthenium complexes, the fluorescence data were analysed using the Stern-Volmer equation. While a linear Stern-Volmer plot is indicative of a single quenching mechanism, either dynamic or static, the positive deviation observed in the plots of F_0/F versus $[Q]$ of compounds **10**, **11**, **13–15**, **17** and **18** (Fig. S38, S42 and S45 in Supplementary Information) suggests the presence of different binding sites in the protein with different binding affinities.⁷⁷ On the other hand, the Stern-Volmer plot for complex **16** (Fig. 10B) shows a linear relationship, suggesting the existence of a single quenching mechanism, most likely dynamic, and a single binding affinity. The Stern-Volmer constants for complex **16** is $3.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$.

In general, higher quenching by the ruthenium dendrimers was observed compared to that of cisplatin under the chosen conditions, most likely due to the faster reactivity of these compounds with HSA, as compared to cisplatin. A similar behaviour has been observed for other piano-stool ruthenium (II) compounds with *N,N*-, *N,O*- or *C,N*-chelating ligands^{33, 35} indicating that the dendritic scaffolds do not affect much the interaction of the peripheral ruthenium centers with HSA.

3.4.- Inhibition of Cathepsin-B—Cathepsin-B (Cat-B) is an abundant and ubiquitously expressed cysteine peptidase of the papain family, which has turned out to be a prognostic marker for several types of cancers.⁷⁸ Cathepsin-B seems to be involved (along with other Cathepsins) in metastasis, angiogenesis and tumor progression.⁷⁹ It has been proposed that Cat-B may be a possible therapeutic target for the control of tumor progression.⁸⁰ RAPTA-Ru compounds which inhibit Cat-B with IC_{50} in the low micromolar range can reduce the mass and number of metastases *in vivo*.⁸¹ Therefore, we studied the inhibition of Cathepsin-B by highly cytotoxic first generation dendrimers **11**, **14** and **17** (see Experimental Section for further details and IC_{50} values in Table 2).

All these compounds were able to inhibit Cathepsin-B. Dendrimer **11** and especially water-soluble dendrimer **17** inhibited Cathepsin-B in the low micromolar range. Similar IC_{50} values were obtained with bovine Capthesin B for some cycloruthenated compounds of the RAPTA series, especially RAPTA-T and RAPTA-C⁸¹ ($\text{IC}_{50} = 1.5 \text{ } \mu\text{M}$ and $2.5 \text{ } \mu\text{M}$, respectively) which have been shown to have antimestatatic activity *in vivo*.

Conclusions

To conclude, we have demonstrated the potential of new carbosilane-based ruthenium dendrimers as anticancer agents. These metallodendrimers are active against a number of cisplatin resistant cell lines in the low micromolar range while showing a dendritic effect (enhancement of the activity when compared to their mononuclear counterparts). The ruthenium dendrimers can be easily synthesized and obtained in high yields, including highly active first and second generation water-soluble dendrimers based on $G_n[\text{NCP}(\text{o-N})]_m$ scaffolds. Initial mechanistic studies indicate that the cell death type for selected water-soluble first generation **17** is through both apoptotic and necrotic pathways. The interaction of selected metallodendrimers described here with DNA is weak and electrostatic in nature. In general, higher quenching of the HSA fluorescence by the ruthenium dendrimers was observed compared to that of cisplatin which indicate the existence of an interaction of the compounds with this protein. These facts point to a mode of action of these ruthenium derivatives different from that of cisplatin. First generation dendrimers show relevant inhibitory properties on Cathepsin-B (a protease that has been proposed as possible therapeutic target for the control of tumor progression.) with **17** having an IC_{50} in the range of the antimetastatic agents RAPTA-C and RAPTA-T.

Overall this study supports the idea that the cytotoxic activities displayed by a number of *p*-cymene based ruthenium mononuclear complexes with chelating ligands, does not decrease while they get incorporated into carbosilane dendritic scaffolds. Moreover, the biocompatibility and the hydrophobic nature of the ruthenium metallodendrimers reported here, together with their nanoscopic size may enhance interactions with biological membranes and facilitate their delivery.

Experimental Section

General considerations

All the reactions were carried out under inert atmosphere and solvents were purified from appropriate drying agents when necessary. NMR spectra were recorded on a Varian Unity VXR-300 (300.13 (^1H), 75.47 (^{13}C) MHz) or on a Bruker AV400 (40.56 (^{15}N), 79.49 (^{29}Si) MHz). Chemical shifts (δ) are given in ppm. ^1H and ^{13}C resonances were measured relative to internal deuterated solvents peaks considering TMS = 0 ppm, meanwhile ^{15}N and ^{29}Si resonances were measured relative to external CH_3NO_2 and TMS, respectively. When necessary, assignment of resonances was done from HMBC and HSQC NMR experiments. Elemental analyses were performed on a LECO CHNS-932. Mass Spectra were obtained from an Agilent 6210 spectrometer. Compounds salicylaldehyde, 2-pyridinecarboxaldehyde, 4-pyridinecarboxaldehyde, triethylsilane, allylamine and $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ (Aldrich) were obtained from commercial sources. Compounds $G_n[\text{NH}_2]_m$, where $n = 0, 1$ or 2 and $m = 1, 4$ or 8 , were synthesized as published.⁵⁹

Electrophoresis experiments were carried out in a Bio-Rad Mini sub-cell GT horizontal electrophoresis system connected to a Bio-Rad Power Pac 300 power supply. Photographs of the gels were taken with an Alpha InnotechFluorChem 8900 camera. Fluorescence intensity measurements were carried out on a PTI QM-4/206 SE Spectrofluorometer (PTI,

Birmingham, NJ) with right angle detection of fluorescence using a 1 cm path length quartz cuvette. Circular dichroism spectra were recorded using a Chirascan CD Spectrometer equipped with a thermostated cuvette holder. The inhibition of Cathepsin-B experiments were performed by Reaction Biology Corporation.

1.- Synthesis of selected compounds

The synthesis of all compounds is described in the Supplementary Information section. We have included here a selection of the most relevant derivatives.

Synthesis of zero generation dendritic ligands— $G_0[\text{NCPH}(p\text{-N})]_1$ (**1**), $G_0[\text{NCPH}(o\text{-N})]_1$ (**4**) and $G_0[\text{NCPH}(o\text{-OH})]_1$ (**7**).

In a typical procedure, to a solution of $G_0[\text{NH}_2]_1$ (500 mg, 2.88 mmol) in dry THF, the corresponding aldehyde, 4-pyridinecarboxaldehyde (308.9 mg, 2.88 mmol) for (**1**), 2-pyridinecarboxaldehyde (308.9 mg, 2.88 mmol) for (**4**), and salicylaldehyde (281.7 mg, 2.30 mmol) for (**7**) was added. The reaction mixture was stirred at room temperature in the presence of anhydrous MgSO_4 for 24 hours. The resulting solution was evaporated to dryness and the residue extracted in $\text{CH}_2\text{Cl}_2/\text{H}_2\text{O}$. The excess of the aldehyde was removed in the aqueous phase.

$G_0[\text{NCPH}(p\text{-N})]_1$ (1**)**—Orange oil, yield 598 mg (79%). $^1\text{H-NMR}$ (CDCl_3): δ (ppm) = 0.53 (overlapping m, 8H, $-\text{Si}(\text{CH}_2\text{CH}_3)_3$ and $-\text{SiCH}_2\text{CH}_2$); 0.92 (m, 9H, $-\text{Si}(\text{CH}_2\text{CH}_3)_3$); 1.69 (m, 2H, $-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 3.63 (t, $^3J_{(H-H)} = 7.1$ Hz, 2H, $-\text{CH}_2\text{CH}_2\text{N}$); 7.57 (m, 2H, Ar); 8.67 (m, 2H, Ar); 8.24 (s, 1H, $-\text{CH}_{\text{imine}}$). ^{13}C $\{^1\text{H}\}$ NMR (CDCl_3): δ (ppm) = 3.4 ($-\text{Si}(\text{CH}_2\text{CH}_3)_3$); 7.6 ($-\text{Si}(\text{CH}_2\text{CH}_3)_3$); 9.1 ($-\text{SiCH}_2\text{CH}_2$); 25.4 ($-\text{CH}_2\text{CH}_2\text{CH}_2$); 65.6 ($-\text{CH}_2\text{CH}_2\text{N}$); 122.0, 143.2, 150.5 (CAr); 158.9 ($-\text{CH}_{\text{imine}}$). $^{29}\text{Si-NMR}$ (CDCl_3): δ (ppm) = 6.98 ($-\text{Si}(\text{CH}_2\text{CH}_3)_3$). $^{15}\text{N-NMR}$ (CDCl_3): δ (ppm) = -64.1 (N_{pyr}); -32.2 (N_{imine}). Elemental Analysis (%): Calc. For $\text{C}_{15}\text{H}_{26}\text{N}_2\text{Si}$ (262.47): C, 68.64; H, 9.98; N, 10.67; Found: C, 68.49; H, 9.59; N, 10.61. Mass ($\text{M} + \text{H}^+$) = 263.19.

$G_0[\text{NCPH}(o\text{-N})]_1$ (4**)**—Brown oil, yield 628 mg (83%). $^1\text{H-NMR}$ (CDCl_3): δ (ppm) = 0.48 (overlapping m, 8H, $-\text{Si}(\text{CH}_2\text{CH}_3)_3$ and $-\text{SiCH}_2\text{CH}_2$); 0.89 (m, 9H, $-\text{Si}(\text{CH}_2\text{CH}_3)_3$); 1.68 (m, 2H, $-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 3.62 (t, $^3J_{(H-H)} = 6.6$ Hz, 2H, $-\text{CH}_2\text{CH}_2\text{N}$); 7.26 (m, 1H, Ar); 7.69 (m, 1H, Ar); 7.95 (m, 1H, Ar); 8.61 (m, 1H, Ar); 8.34 (s, 1H, $-\text{CH}_{\text{imine}}$). ^{13}C $\{^1\text{H}\}$ NMR (CDCl_3): δ (ppm) = 3.4 ($-\text{Si}(\text{CH}_2\text{CH}_3)_3$); 7.6 ($-\text{Si}(\text{CH}_2\text{CH}_3)_3$); 9.1 ($-\text{SiCH}_2\text{CH}_2$); 25.4 ($-\text{CH}_2\text{CH}_2\text{CH}_2$); 65.3 ($-\text{CH}_2\text{CH}_2\text{N}$); 121.3, 124.7, 136.6, 149.5, 161.8 (CAr); 154.8 ($-\text{CH}_{\text{imine}}$). $^{29}\text{Si-NMR}$ (CDCl_3): δ (ppm) = 6.88 ($-\text{Si}(\text{CH}_2\text{CH}_3)_3$). $^{15}\text{N-NMR}$ (CDCl_3): δ (ppm) = -67.0 (N_{pyr}); -37.2 (N_{imine}). Elemental Analysis (%): Calc. For $\text{C}_{15}\text{H}_{26}\text{N}_2\text{Si}$ (262.47): C, 68.64; H, 9.98; N, 10.67; Found: C, 68.52; H, 10.17; N, 10.75. Mass ($\text{M} + \text{H}^+$) = 263.19.

$G_0[\text{NCPH}(o\text{-OH})]_1$ (7**)**—Yellow oil, yield 595 mg (93%). $^1\text{H-NMR}$ (CDCl_3): δ (ppm) = 0.54 (overlapping m, 8H, $-\text{Si}(\text{CH}_2\text{CH}_3)_3$ and $-\text{SiCH}_2\text{CH}_2$); 0.96 (m, 9H, $-\text{Si}(\text{CH}_2\text{CH}_3)_3$); 1.69 (m, 2H, $-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 3.57 (t, $^3J_{(H-H)} = 6.7$ Hz, 2H, $-\text{CH}_2\text{CH}_2\text{N}$); 6.84 (m, 1H, Ar); 6.97 (m, 1H, Ar); 7.26 (m, 2H, Ar); 8.33 (s, 1H, $-\text{CH}_{\text{imine}}$); $-\text{OH}$ is not observed. ^{13}C

{¹H} NMR (CDCl₃): δ (ppm) = 3.2 (-Si(CH₂CH₃)₃); 7.4 (-Si(CH₂CH₃)₃); 8.8 (-SiCH₂CH₂); 25.5 (-SiCH₂CH₂CH₂N); 62.9 (-CH₂CH₂N); 117.0, 118.3, 118.7, 131.0, 131.9, 164.4 (C_{Ar}); 161.4 (-CH_{imine}). ²⁹Si-NMR (CDCl₃): δ (ppm) = 7.12 (-Si(CH₂CH₃)₃). ¹⁵N-NMR (CDCl₃): δ (ppm) = -82.2 (N_{imine}). Elemental Analysis (%): Calc. For C₁₆H₂₇NOSi (277.48): C, 69.26; H, 9.81; N, 5.05; Found: C, 69.53; H, 9.86; N, 5.41. Mass (M + H⁺) = 278.19.

First generation dendrimers

G₁[NCPH(*p*-N)]₄ (**2**), G₁[NCPH(*o*-N)]₄ (**5**) and G₁[NCPH(*o*-OH)]₄ (**8**).

To a solution of G₁[NH₂]₄ (180 mg, 0.27 mmol) in THF, the corresponding aldehyde, 4-pyridinecarboxaldehyde (119.5 mg, 1.12 mmol) for (**2**), 2-pyridinecarboxaldehyde (119.5 mg, 1.12 mmol) for (**5**), and salicylaldehyde (136.3 mg, 1.12 mmol) for (**8**) was added. The mixture was stirred under inert atmosphere at room temperature in the presence of anhydrous MgSO₄ for 24 hour. Subsequently, the solvent was evaporated to give an oil that was purified by size exclusion chromatography.

G₁[NCPH(*p*-N)]₄ (2**)**—Yellow pale oil, yield 183 mg (69%). ¹H-NMR (CDCl₃): δ (ppm) = -0.04 (s, 24H, -(CH₃)₂SiCH₂CH₂CH₂N); 0.53 (overlapping m, 24H, -SiCH₂CH₂CH₂Si and -SiCH₂CH₂CH₂N); 1.28 (m, 8H, -SiCH₂CH₂CH₂Si); 1.66 (m, 8H, -SiCH₂CH₂CH₂N); 3.62 (t, ³J(*H*-*H*) = 6.9 Hz, 8H, -(CH₃)₂SiCH₂CH₂CH₂N); 7.57 (m, 8H, Ar); 8.67 (m, 8H, Ar); 8.24 (s, 4H, -CH_{imine}). ¹³C {¹H} NMR (CDCl₃): δ (ppm) = -3.3 (-SiCH₂CH₂CH₂N); 13.0 (-SiCH₂CH₂CH₂N); 17.5, 18.5, 20.1 (-SiCH₂CH₂CH₂Si); 25.3 (-SiCH₂CH₂CH₂N); 65.3 (-SiCH₂CH₂CH₂N); 121.9, 143.0, 150.3 (C_{Ar}); 158.7 (-CH_{imine}). ²⁹Si-NMR (CDCl₃): δ (ppm) = 0.55 (-SiCH₂CH₂CH₂Si); 2.01 (-SiCH₂CH₂CH₂N). ¹⁵N-NMR (CDCl₃): δ (ppm) = -64.3 (N_{pyr}); -32.2 (N_{imine}). Elemental Analysis (%): Calc. For C₅₆H₉₂N₈Si₅ (1017.81): C, 66.08; H, 9.11; N, 11.01; Found: C, 66.11; H, 8.72; N, 10.79. Mass (M + H⁺) = 1017.63.

G₁[NCPH(*o*-N)]₄ (5**)**—Brown oil, yield 202 mg (73%). ¹H-NMR (CDCl₃): δ (ppm) = -0.01 (s, 24H, -(CH₃)₂SiCH₂CH₂CH₂N); 0.53 (overlapping m, 24H, -SiCH₂CH₂CH₂Si and -SiCH₂CH₂CH₂N); 1.30 (m, 8H, -SiCH₂CH₂CH₂Si); 1.70 (m, 8H, -SiCH₂CH₂CH₂N); 3.64 (t, ³J(*H*-*H*) = 7.0 Hz, 8H, -(CH₃)₂SiCH₂CH₂CH₂N); 7.29 (m, 4H, Ar); 7.72 (m, 4H, Ar); 7.98 (m, 4H, Ar); 8.63 (m, 4H, Ar); 8.35 (s, 4H, -CH_{imine}). ¹³C {¹H} NMR (CDCl₃): δ (ppm) = -3.4 (-SiCH₂CH₂CH₂N); 13.2 (-SiCH₂CH₂CH₂N); 17.6, 18.7, 20.3 (-SiCH₂CH₂CH₂Si); 25.5 (-SiCH₂CH₂CH₂N); 65.1 (-SiCH₂CH₂CH₂N); 121.3, 124.7, 136.6, 149.5, 161.8 (C_{Ar}); 154.8 (-CH_{imine}). ²⁹Si-NMR (CDCl₃): δ (ppm) = 0.59 (-SiCH₂CH₂CH₂Si); 1.93 (-SiCH₂CH₂CH₂N). ¹⁵N-NMR (CDCl₃): δ (ppm) = -68.2 (N_{pyr}); -38.2 (N_{imine}). Elemental Analysis (%): Calc. For C₅₆H₉₂N₈Si₅ (1017.81): C, 66.08; H, 9.11; N, 11.01; Found: C, 66.21; H, 9.22; N, 10.59. Mass (M + H⁺) = 1017.63.

G₁[NCPH(*o*-OH)]₄ (8**)**—Yellow oil, yield 232 mg (79%). ¹H-NMR (CDCl₃): δ (ppm) = -0.03 (s, 24H, -(CH₃)₂SiCH₂CH₂CH₂N); 0.55 (overlapping m, 24H, -SiCH₂CH₂CH₂Si and -SiCH₂CH₂CH₂N); 1.30 (m, 8H, -SiCH₂CH₂CH₂Si); 1.67 (m, 8H, -SiCH₂CH₂CH₂N); 3.55 (t, ³J(*H*-*H*) = 6.8 Hz, 8H, -(CH₃)₂SiCH₂CH₂CH₂N); 6.85 (m, 4H, Ar); 6.95 (m, 4H, Ar);

7.25 (m, 8H, Ar); 8.30 (s, 4H, $-\text{CH}_{\text{imine}}$); $-\text{OH}$ is not observed. ^{13}C $\{^1\text{H}\}$ NMR (CDCl_3): δ (ppm) = -3.2 ($-(\text{CH}_3)_2\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 13.1 ($-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 17.6 , 18.7 , 20.2 ($-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{Si}$); 25.7 ($-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 62.9 ($-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 117.2 , 118.4 , 118.9 , 131.2 , 132.4 , 164.5 (CAr); 161.6 ($-\text{CH}_{\text{imine}}$). ^{29}Si -NMR (CDCl_3): δ (ppm) = 0.53 ($-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{Si}$); 1.95 ($-(\text{CH}_3)_2\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$). ^{15}N -NMR (CDCl_3): δ (ppm) = -81.2 (N_{imine}). Elemental Analysis (%): Calc. For $\text{C}_{60}\text{H}_{96}\text{N}_4\text{O}_4\text{Si}_5$ (1077.86): C, 66.86; H, 8.98; N, 5.20; Found: C, 66.59; H, 9.15; N, 5.23. Mass ($\text{M} + \text{H}^+$) = 1077.63.

Second generation dendrimers

$\text{G}_2[\text{NCPH}(p\text{-N})]_8$ (**3**), $\text{G}_2[\text{NCPH}(o\text{-N})]_8$ (**6**) and $\text{G}_2[\text{NCPH}(o\text{-OH})]_8$ (**9**).

Second generation dendrimers are prepared in a similar way to that described for first generation derivatives. Full details for these complexes are collected in the Supplementary Information section.

General procedure for the preparation of monodentate $-\text{NH}_2$ (**10–12**) and chelating N,N-cationic metallodendrimers (**16–18**)

Neutral N- metallodendrimers— $\text{G}_0[\text{NH}_2\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_1$ (**10**), $\text{G}_1[\text{NH}_2\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_4$ (**11**) and $\text{G}_2[\text{NH}_2\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_8$ (**12**).

To a solution of the dendritic ligand with general formula $\text{G}_n[\text{NH}_2]_m$ (75.0 mg, 0.43 mmol of (**a**); 53.0 mg, 0.08 mmol of (**b**); and 59 mg, 0.03 mmol of (**c**)) in dichloromethane, the dimer $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ (132.5 mg, 0.21 mmol) was slowly added to obtain $\text{G}_0[\text{NH}_2\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_1$ (**10**). In order to obtain compounds $\text{G}_1[\text{NH}_2\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_4$ (**11**) and $\text{G}_2[\text{NH}_2\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_8$ (**12**), we added 98.1 mg, 0.16 mmol and 88.4 mg, 0.14 mmol of $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ respectively. The reaction was stirred at room temperature for 5h. The mixture solution was concentrated and the product, a yellow-orange solid, was precipitated with diethyl ether and dried *in vacuo*.

$\text{G}_0[\text{NH}_2\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_1$ (10**):** Yellow-orange solid, yield 189 mg (91%). ^1H -NMR (CDCl_3): δ (ppm) = 0.49 (overlapping m, 8H, $-\text{Si}(\text{CH}_2\text{CH}_3)_3$ and $-\text{SiCH}_2\text{CH}_2$); 0.91 (m, 9H, $-\text{Si}(\text{CH}_2\text{CH}_3)_3$); 1.32 (d, $^3J_{(\text{H-H})} = 6.9$ Hz, 6H, $-(\text{CH}_3)_2\text{CH}_{\text{cye}}$); 1.53 (m, 2H, $-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 2.26 (s, 3H, $-\text{CH}_{3\text{cye}}$); 2.76 (m, 2H, $-\text{NH}_2$); 3.01 (overlapping m, 3H, $-(\text{CH}_3)_2\text{CH}_{\text{cye}}$ and $-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 5.26 (d, $^3J_{(\text{H-H})} = 6.0$ Hz, 2H, Ar_{cye}); 5.40 (d, $^3J_{(\text{H-H})} = 6.0$ Hz, 2H, Ar_{cye}). ^{13}C $\{^1\text{H}\}$ NMR (CDCl_3): δ (ppm) = 3.3 ($-\text{Si}(\text{CH}_2\text{CH}_3)_3$); 7.6 ($-\text{Si}(\text{CH}_2\text{CH}_3)_3$); 8.6 ($-\text{SiCH}_2\text{CH}_2$); 19.0 ($-\text{CH}_{3\text{cye}}$); 22.3 ($-(\text{CH}_3)_2\text{CH}_{\text{cye}}$); 28.0 ($-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 31.0 ($-(\text{CH}_3)_2\text{CH}_{\text{cye}}$); 53.1 ($-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 80.6, 80.7 ($-\text{CH}_{\text{cye}}$); 96.3, 102.5 (C_{cye}). ^{29}Si -NMR (CDCl_3): δ (ppm) = 7.13 ($-\text{Si}(\text{CH}_2\text{CH}_3)_3$). Elemental Analysis (%): Calc. For $\text{C}_{19}\text{H}_{37}\text{Cl}_2\text{NRuSi}$ (479.57): C, 47.59; H, 7.78; N, 2.92; Found: C, 47.45; H, 7.35; N, 3.22.

$\text{G}_1[\text{NH}_2\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_4$ (11**):** Yellow-orange solid, yield 142 mg (94%). ^1H -NMR (CDCl_3): δ (ppm) = -0.04 (br s, 24H, $-(\text{CH}_3)_2\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 0.49 (overlapping m, 24H, $-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{Si}$ and $-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 1.31 (overlapping m, 32H, $-(\text{CH}_3)_2\text{CH}_{\text{cye}}$ and $-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{Si}$); 1.53 (br m, 8H, $-\text{SiCH}_2\text{CH}_2\text{CH}_2\text{N}$); 2.26 (s, 12H, $-\text{CH}_{3\text{cye}}$); 2.85 (m,

8H, -NH₂); 2.97 (overlapping m, 12H, -(CH₃)₂CH_{cyc} and -SiCH₂CH₂CH₂N); 5.33 (m, 8H, Ar_{cyc}); 5.43 (m, 8H, Ar_{cyc}). ¹³C {¹H} NMR (CDCl₃): δ (ppm) = -3.2 (- (CH₃)₂SiCH₂CH₂CH₂N); 12.4 (-SiCH₂CH₂CH₂N); 17.6, 18.6, 18.9 (-SiCH₂CH₂CH₂Si); 20.0 (-CH₃_{cyc}); 22.4 (- (CH₃)₂CH_{cyc}); 27.8 (-SiCH₂CH₂CH₂N); 31.0 (- (CH₃)₂CH_{cyc}); 52.6 (-SiCH₂CH₂CH₂N); 80.3, 81.0 (-CH_{cyc}); 95.9, 102.6 (C_{cyc}). ²⁹Si-NMR (CDCl₃): δ (ppm) = 0.56 (-SiCH₂CH₂CH₂Si); 1.94 (- (CH₃)₂SiCH₂CH₂CH₂N). Elemental Analysis (%): Calc. For C₇₂H₁₃₆Cl₈N₄Ru₄Si₅ (1886.21): C, 45.85; H, 7.27; N, 2.97; Found: C, 45.95; H, 7.14; N, 3.29.

G₂-[NH₂Ru(η⁶-*p*-cymene)Cl]₂]₈ (12): Yellow-orange solid, yield 142 mg (94%). ¹H-NMR (CD₃OD): δ (ppm) = -0.08 (br s, 12H, -CH₃SiCH₂CH₂CH₂Si); -0.04 (br s, 48H, - (CH₃)₂SiCH₂CH₂CH₂N); 0.54 (overlapping br m, 64H, -SiCH₂CH₂CH₂Si, -CH₃SiCH₂CH₂CH₂Si and -(CH₃)₂SiCH₂CH₂CH₂N); 1.31 (overlapping br m, 72H, -SiCH₂CH₂CH₂Si, -CH₃SiCH₂CH₂CH₂Si and -(CH₃)₂CH_{cyc}); 1.55 (br m, 16H, - (CH₃)₂SiCH₂CH₂CH₂N); 2.26 (s, 24H, -CH₃_{cyc}); 2.88 (m, 16H, -NH₂); 3.02 (overlapping br m, 24H, -(CH₃)₂CH_{cyc} and -(CH₃)₂SiCH₂CH₂CH₂N); 5.35 (m, 16H, Ar_{cyc}); 5.43 (m, 16H, Ar_{cyc}). ¹³C {¹H} NMR (CD₃OD): δ (ppm) = -4.3 (- (CH₃)₂SiCH₂CH₂CH₂N); -3.3 (-CH₃SiCH₂CH₂CH₂Si); 13.1 (- (CH₃)₂SiCH₂CH₂CH₂N); 18.6, 18.8, 19.8, 20.9, 22.5, 22.7 (-SiCH₂CH₂CH₂Si, -CH₃SiCH₂CH₂CH₂Si and -CH₃_{cyc}); 23.3 (- (CH₃)₂CH_{cyc}); 28.3 (- (CH₃)₂SiCH₂CH₂CH₂N); 32.5 (- (CH₃)₂CH_{cyc}); 43.7 (- (CH₃)₂SiCH₂CH₂CH₂N); 77.6, 79.8 (-CH_{cyc}); 94.7, 98.4 (C_{cyc}). ²⁹Si-NMR (CD₃OD): δ (ppm) = -SiCH₂CH₂CH₂Si is not observed; 0.93 (-CH₃SiCH₂CH₂CH₂Si); 2.01 (- (CH₃)₂SiCH₂CH₂CH₂N). Elemental Analysis (%): Calc. For C₁₆₀H₃₀₈Cl₁₆N₈Ru₈Si₁₃ (4085.13): C, 47.04; H, 7.60; N, 2.74; Found: C, 47.61; H, 8.24; N, 3.28.

Chelating N,N- cationic metallodendrimers—G₀-[NCPH(*o*-N)Ru(η⁶-*p*-cymene)Cl]₂]₁ (16), G₁-[NCPH(*o*-N)Ru(η⁶-*p*-cymene)Cl]₂]₄ (17) and G₂-[NCPH(*o*-N)Ru(η⁶-*p*-cymene)Cl]₂]₈ (18).

The corresponding chelating *N,N*-ligand (86.5 mg, 0.33 mmol of G₀-[NCPH(*o*-N)]₁; 63.0 mg, 0.06 mmol of G₁-[NCPH(*o*-N)]₄; 100 mg, 0.04 mmol of G₂-[NCPH(*o*-N)]₈) was dissolved in dry ethanol and then, the dimer [Ru(η⁶-*p*-cymene)Cl]₂ (100 mg, 0.16 mmol for (4); 75.8 mg, 0.12 mmol for (5) and 104 mg, 0.17 mmol for (6)) was added slowly to that solution. The solution was stirred overnight at room temperature. The solvent was evaporated under reduced pressure, affording compounds **16–18** in moderate yields.

G₀-[NCPH(*o*-N)Ru(η⁶-*p*-cymene)Cl]₂]₁ (16): Brown solid, 121 mg (65%). ¹H-NMR (CDCl₃): δ (ppm) = 0.50 (overlapping m, 8H, -Si(CH₂CH₃)₃ and -SiCH₂CH₂); 0.90 (m, 9H, -Si(CH₂CH₃)₃); 1.01 (d, ³J(*H-H*) = 6.8 Hz, 3H, -(CH₃)₂CH_{cyc}); 1.10 (d, ³J(*H-H*) = 6.8 Hz, 3H, -(CH₃)₂CH_{cyc}); 1.84 (br m, 1H, -SiCH₂CH₂CH₂N); 1.99 (br m, 1H, -SiCH₂CH₂CH₂N); 2.17 (s, 3H, -CH₃_{cyc}); 2.69 (m, 1H, -(CH₃)₂CH_{cyc}); 4.31 (br m, 1H, -SiCH₂CH₂CH₂N); 4.48 (br m, 1H, -SiCH₂CH₂CH₂N); 5.77 (m, 1H, Ar_{cyc}); 5.93 (m, 2H, Ar_{cyc}); 6.21 (m, 1H, Ar_{cyc}); 7.69 (m, 1H, Ar); 7.97 (m, 1H, Ar); 8.25 (m, 1H, Ar); 9.78 (m, 1H, Ar); 8.85 (s, 1H, -CH_{imine}). ¹³C {¹H} NMR (CDCl₃): δ (ppm) = 3.1 (-Si(CH₂CH₃)₃); 7.5 (-Si(CH₂CH₃)₃); 9.1 (-SiCH₂CH₂); 18.8 (-CH₃_{cyc}); 21.9, 22.5 (- (CH₃)₂CH_{cyc}); 24.7 (-

SiCH₂CH₂CH₂N); 31.1 (-(CH₃)₂CH_{cye}); 70.9 (-SiCH₂CH₂CH₂N); 84.6, 85.2, 85.8, 87.3 (-CH_{cye}); 102.4, 106.3 (C_{cye}); 128.7, 129.1, 139.2, 154.4, 156.7 (CAr); 166.5 (-CH_{imine}). ²⁹Si-NMR (CDCl₃): δ (ppm) = 7.05 (-Si(CH₂CH₃)₃). Elemental Analysis (%): Calc. For C₂₅H₄₀Cl₂N₂RuSi (568.66): C, 57.68; H, 7.71; N, 2.49; Found: C, 57.87; H, 7.58; N, 2.61. Mass m/z ([M-Cl]⁺) = 533.16.

G₁-[NCP(*o*-N)Ru(η⁶-*p*-cymene)Cl]Cl₄ (17): Brown solid, yield 75.8 mg (55%). ¹H-NMR (CD₃OD): δ (ppm) = 0.00 (br s, 24H, -(CH₃)₂SiCH₂CH₂CH₂N); 0.60 (overlapping m, 24H, -SiCH₂CH₂CH₂Si and -SiCH₂CH₂CH₂N); 1.02 (d, ³J(*H*-*H*) = 6.6 Hz, 12H, -(CH₃)₂CH_{cye}); 1.15 (d, ³J(*H*-*H*) = 6.6 Hz, 12H, -(CH₃)₂CH_{cye}); 1.32 (br m, 8H, -SiCH₂CH₂CH₂Si); 1.99 (br m, 8H, -SiCH₂CH₂CH₂N); 2.25 (s, 12H, -CH_{3cye}); 2.67 (br s, 4H, -(CH₃)₂CH_{cye}); 4.25 (br m, 4H, -SiCH₂CH₂CH₂N); 4.67 (br m, 4H, -SiCH₂CH₂CH₂N); 5.81 (m, 8H, Ar_{cye}); 6.05 (m, 4H, Ar_{cye}); 6.17 (m, 4H, Ar_{cye}); 7.77 (br s, 4H, Ar); 8.17 (overlapping m, 8H, Ar); 9.48 (br s, 4H, Ar); 8.67 (s, 4H, -CH_{imine}). ¹³C {¹H} NMR (CD₃OD): δ (ppm) = -2.9 (-(CH₃)₂SiCH₂CH₂CH₂N); 13.7 (-SiCH₂CH₂CH₂N); 18.6, 19.2, 19.8 (-SiCH₂CH₂CH₂Si); 21.1 (-CH_{3cye}); 21.9, 23.0 (-(CH₃)₂CH_{cye}); 25.8 (-SiCH₂CH₂CH₂N); 32.4 (-(CH₃)₂CH_{cye}); 71.6 (-SiCH₂CH₂CH₂N); 85.8, 86.4, 87.1, 90.0 (-CH_{cye}); 104.4, 106.9 (C_{cye}); 129.7, 129.9, 141.0, 156.2, 157.1 (CAr); 168.5 (-CH_{imine}). ²⁹Si-NMR (CD₃OD): δ (ppm) = 0.49 (-SiCH₂CH₂CH₂Si); 1.90 (-(CH₃)₂SiCH₂CH₂CH₂N). Elemental Analysis (%): Calc. For C₉₆H₁₄₈Cl₈N₈Ru₄Si₅ (2242.59): C, 51.42; H, 6.65; N, 5.00; Found: C, 51.63; H, 6.71; N, 5.23.

G₂-[NCP(*o*-N)Ru(η⁶-*p*-cymene)Cl]Cl₈ (18): Brown solid, yield 97 mg (48%). ¹H-NMR (CD₃OD): δ (ppm) = -0.06 (overlapping br m, 60H, -CH₃SiCH₂CH₂CH₂Si and -(CH₃)₂SiCH₂CH₂CH₂N); 0.61 (overlapping br m, 64H, -SiCH₂CH₂CH₂Si, -CH₃SiCH₂CH₂CH₂Si and -(CH₃)₂SiCH₂CH₂CH₂N); 1.00 (br m, 24H, -(CH₃)₂CH_{cye}); 1.15 (br m, 24H, -(CH₃)₂CH_{cye}); 1.38 (overlapping br m, 24H, -SiCH₂CH₂CH₂Si and -CH₃SiCH₂CH₂CH₂Si); 1.98 (br m, 16H, -(CH₃)₂SiCH₂CH₂CH₂N); 2.25 (s, 24H, -CH_{3cye}); 2.66 (br s, 8H, -(CH₃)₂CH_{cye}); 4.23 (br m, 8H, -SiCH₂CH₂CH₂N); 4.66 (br m, 8H, -SiCH₂CH₂CH₂N); 5.81 (m, 16H, Ar_{cye}); 6.03 (m, 8H, Ar_{cye}); 6.16 (m, 8H, Ar_{cye}); 7.77 (br s, 8H, Ar); 8.17 (overlapping m, 16H, Ar); 9.47 (br s, 8H, Ar); 8.66 (s, 4H, -CH_{imine}). ¹³C {¹H} NMR (CD₃OD): δ (ppm) = -4.2 (-(CH₃)₂SiCH₂CH₂CH₂N); -2.8 (-CH₃SiCH₂CH₂CH₂Si); 13.7 (-(CH₃)₂SiCH₂CH₂CH₂N); 19.1, 19.8, 20.0, 21.1, 21.9 (-SiCH₂CH₂CH₂Si, -CH₃SiCH₂CH₂CH₂Si and -CH_{3cye}); 23.3 (-(CH₃)₂CH_{cye}); 25.8 (-(CH₃)₂SiCH₂CH₂CH₂N); 32.4 (-(CH₃)₂CH_{cye}); 71.6 (-(CH₃)₂SiCH₂CH₂CH₂N); 85.7, 86.4, 87.1, 88.8 (-CH_{cye}); 104.3, 107.0 (C_{cye}); 129.7, 129.8, 141.0, 156.2, 157.0 (CAr); 163.4 (-CH_{imine}). ²⁹Si-NMR (CD₃OD): δ (ppm) = -SiCH₂CH₂CH₂Si is not observed; 0.89 (-CH₃SiCH₂CH₂CH₂Si); 2.14 (-(CH₃)₂SiCH₂CH₂CH₂N). Elemental Analysis (%): Calc. For C₂₀₈H₃₃₂Cl₁₆N₁₆Ru₈Si₁₃ (4797.89): C, 52.07; H, 6.97; N, 4.67; Found: C, 51.58; H, 7.20; N, 4.67.

Full details of the synthetic procedure and analytical and structural data for monodentate pyridine metallodendrimers (**13–15**) and salicylaldehyde metallodendrimers (**19–21**) are provided in the Supplementary Information section.

2.- X-Ray crystallography

Details of the X-ray experiment, data reduction, and final structure refinement calculations are summarized in Table S2. A suitable single (orange) crystal of compound **10** was selected. Data collection was performed on a crystal stuck to a glass fibre using an inert per-fluorinated ether oil and mounted in a low temperature N₂ stream at 200(2) K, in a Bruker-Nonius Kappa CCD single crystal diffractometer equipped with a graphite-monochromated Mo-K α radiation (λ = 0.71073 Å), and an Oxford Cryostream 700 unit. Data was collected with an exposure time of 20 s per frame (four sets; 179 frames), ω/ψ scans, 2.0° scan-width. Raw data were corrected for Lorenz and polarization effects. The structure was solved, using the WINGX package,⁸² by direct methods (SHELXS-97),⁸³ completed by the subsequent difference Fourier techniques and refined by using full-matrix least-squares against F² (SHELXL-97). All non-hydrogen atoms were anisotropically refined. Hydrogen atoms were geometrically placed and left riding on their parent atoms. Crystals were obtained from a solution of **10** in CH₃OH by slow diffusion of Et₂O at RT.

3.- Cell culture, inhibition of cell growth and cell death analysis

Cell culture and MTT assays for HeLa, HT-29 and MCF7 cells

The human cervical carcinoma cell line HeLa, human colon adenocarcinoma cell line HT-29 and human breast adenocarcinoma cell line MCF7 utilized in this assay were kindly donated by Unidad de Cultivos at the University of Alcalá. Cells were grown routinely in DMEM (Dulbecco's modified Eagle Medium) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin B (all from Sigma-Aldrich) at 37°C and 5% CO₂. Cells were seeded in 96-well plates (Nunclon Delta Surface, Thermo Fischer Scientific) as monolayers (approximately 5×10⁴) and grown for 72 h in complete medium (90 µL). Solutions of compounds were prepared by diluting a freshly prepared stock solution (in DMSO) of the corresponding compound in aqueous medium (DMEM). Afterward, the intermediate dilutions of the compounds were serially diluted to the appropriate concentration (ranging from 0 to 100 µM) and the cells were incubated for another 24 h (0.6% (v/v) is the maximal final content of DMSO). DMSO at comparable concentrations did not show any effects on cell cytotoxicity. Cytotoxicity was determined using the MTT assay (MTT 3-(4,5dimethyl 2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). Subsequently, MTT (5.0 mg/mL solution) was added to the cells and the plates were incubated for a further 3.5 h. Then the culture medium was removed and the purple formazan crystals formed by the mitochondrial dehydrogenase and reductase activity of vital cells were dissolved in DMSO. The optical density, directly proportional to the number of surviving cells, was quantified at 570 nm (background correction at 690 nm) using a multiwell plate reader and the fraction of surviving cells was calculated from the absorbance of untreated control cells. The IC₅₀ value indicates the concentration needed to inhibit a biological function of the cells by half and is presented as a mean (±SE) from three independent experiments, each comprising three microcultures per concentration level.

Cell culture and XTT assays for MDA-MB 231 and HEK-293T cells

The human breast adenocarcinoma MDA-MB 231 cell line in comparison with healthy human embryonic kidney (HEK-293T) cells, were used to study the ruthenium dendrimers' cytotoxic activity. Human breast adenocarcinoma cell lines MDA-MB 231 as well as HEK-293T cells were grown adherently and maintained in Dulbecco's Modified Eagle's Medium supplemented with 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 10% fetal bovine serum (FBS). All cell lines were cultured in a humidified chamber at 37°C and under 5% CO₂ and 95% O₂.

For evaluation of cell viability, cells were seeded at a concentration of 5×10^3 cells/well in 90 μL of DMEM without phenol red and without antibiotics, supplemented with 10% FBS and 2 mM L-glutamine into tissue culture grade 96-well flat bottom microplates (BioLiteMicrowell Plate, Fisher Scientific, Waltham, Massachusetts, USA) and grown for 24h at 37°C under 5% CO₂ and 95% O₂ in a humidified incubator. Afterwards, the intermediate dilutions of the compounds were added to the wells (10 μL) to obtain a final concentration ranging from 0.1 to 50 μM , and the cells were incubated for 24 h or 48 h. Following 24 h or 48 h drug exposure, 50 μL per well of 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2Htetrazolium-5-carboxanilide (XTT) (Roche Diagnostics Indianapolis, Indiana, USA) labeling mixture was added to the cells at a final concentration of 0.3 mg.mL^{-1} and incubated for 4 h at 37°C under 5% CO₂ and 95% O₂ in a humidified incubator. The optical absorbance of each well in a 96-well plate was quantified using BioTek ELx808 absorbance microplate reader (BioTek Winooski, VT) set at 450 nm wavelength. The percentage of surviving cells was calculated from the ratio of absorbance of treated to untreated cells. The IC₅₀ value indicates the concentration needed to inhibit a biological function of the cells by half and is presented as a mean ($\pm\text{SE}$) from three independent experiments, each comprising three microcultures per concentration level.

4.- Cell viability for Compound 17. ApoTox-Glo Triplex Assay

For apoptosis, viability, and cytotoxicity assays, the MDA-MB-231 cells were seeded in 96-well opaque-walled tissue culture plates with clear bottoms (Thermo Scientific Nunc; Somerset, NJ, USA) at an initial density of 5×10^4 cells/mL in DMEM without phenol red and without antibiotics, supplemented with 10% FBS and 2 mM L-glutamine. Following 24h incubation, cells were treated with 10.7 μM of compound **17** for 24 h. The cells were then assayed using the ApoTox-Glo Triplex Assay (Promega GmbH, High-Tech-Park, Mannheim, Germany). 20 μL of viability/cytotoxicity reagent containing both glycyphenylalanyl-aminofluorocoumarin (GF-AFC) and bis-alanylalanyl-phenylalanyl-rhodamine 110 (bis-AAF-R110) substrates was added to each well, and they were briefly mixed by orbital shaking at 200 rpm for 30 seconds and then incubated at 37 °C for 2 hours. Fluorescence was measured at a 400 nm for excitation /505 nm for emission (viability) and 485 nm for excitation /520 nm for emission (cytotoxicity/necrosis) using a BioTek Fluorescence Microplate Reader (BioTek U.S., Winooski, VT)). Next, 100 μL of Caspase-Glo 3/7 reagent was added to each well, and the samples were briefly mixed by orbital shaking at 200 rpm for 30 seconds and then incubated at room temperature for 1 hour. Luminescence was measured for 1 second and is proportional to the amount of caspase

activity present (BioTek U.S., Winooski, VT). The results for each treatment were expressed as fold change between non-treated (0.1% DMSO) and treated samples. ApoTox-Glo Triplex assays were repeated twice ($n = 2$), and each repetition was run in quadruplicate. The average of the four values was used for statistical calculations. The data are presented as the mean value.

5.-Interaction of compounds 10, 11, 13–18, $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ and cisplatin with *plasmid* (*pBR322*) DNA by Electrophoresis (Mobility Shift Assay)

10 μL aliquots of *plasmid* (*pBR322*) DNA (20 $\mu\text{g/mL}$) in buffer (5 mM Tris/HCl, 50 mM NaClO_4 , pH = 7.39) were incubated with different concentrations of the compounds (10, 11, 13–18, $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ in the range 0.25 and 4.0 metal complex:DNAbp) at 37 °C for 20 h in the dark. Samples of free DNA and cisplatin-DNA were prepared as controls. After the incubation period, the samples were loaded onto the 1 % agarose gel. The samples were separated by electrophoresis for 1.5 h at 80 V in Tris-acetate/EDTA buffer (TAE). Afterwards, the gel was stained for 30 min. with a solution of GelRed Nucleic Acid stain.

6.-Interaction of compounds 16–18 with *Calf Thymus* DNA by Circular Dichroism

Stock solutions (5 mM) of each complex were freshly prepared in water prior to use. The right volume of those solutions was added to 3 ml samples of an also freshly prepared solution of CT DNA (48 μM) in Tris/HCl buffer (5 mM Tris/HCl, 50 mM NaClO_4 , pH=7.39) to achieve molar ratios of 0.1, 0.25, 0.5, 1.0 and 2.0 drug/DNA. The samples were incubated at 37°C for a period of 20 h. All CD spectra of DNA and of the DNA-drug adducts were recorded at 25°C over a range 220–420 nm and finally corrected with a blank and noise reduction. The final data is expressed in molar ellipticity (millidegrees).

7.-Interaction of compounds 10, 11, 13–18 and cisplatin, with HSA by Fluorescence Spectroscopy

A solution of each compound (8 mM) in DMSO was prepared and ten aliquots of 2.5 μL were added successively to a solution of HSA (10 μM) in phosphate buffer (pH = 7.4) to achieve final metal complex concentrations in the range 10–100 μM . The excitation wavelength was set to 295 nm, and the emission spectra of HSA samples were recorded at room temperature in the range of 300 to 450 nm. The fluorescence intensities of all the metal compounds, the buffer and the DMSO are negligible under these conditions. The fluorescence was measured 240 s. after each addition of compound solution. The data were analysed using the classical Stern-Volmer equation $F_0/F = 1 + K_{SV}[Q]$.

8.- Inhibition of Cathepsin-B

Cathepsin-B, purified from human liver (Accession # P07858) and substrate Peptide sequence: Z-FR-AMC [AMC=7-amino-4-methylcoumarin] were dissolved on a buffer: 25

mM MES pH = 6, 50 mMNaCl, 0.005% Brij35, 5 mM DTT and 1% DMSO with a final concentration of 10 μ M. The enzyme solution was delivered into the reaction well. Compounds **11**, **14** and **17** (1% DMSO solution) were delivered into the enzyme mixture by Acoustic technology (Echo550; nanoliter range), incubate for 10 min. at room temp. The substrate solution was delivered into the reaction well to initiate the reaction. The enzyme activity was monitored (Ex/Em = 355/460 nm) as a time-course measurement of the increase in fluorescence signal from fluorescently-labeled peptide substrate for 120 min. at room temperature. The data was analyzed data by taking slope (signal/time) of linear portion of measurement. The slope is calculated by using Excel, and curve fits are performed using Prism software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Coordination ruthenium (III) complexes that have entered clinical trials for cancer treatment.^{9, 13–17}

Fig. 2.

Ruthenium (II) arene complexes which have undergone advanced preclinical studies.^{24, 26, 27, 33}

Fig. 3.

Abbreviate nomenclature of carbosilane dendrimers: a) zero generation: $G_0[X]_1$; b) first generation: $G_1[X]_4$; c) second generation: $G_2[X]_8$.

b)

c)

Fig. 4.
¹H-NMR spectra: a) G₀[NCPh(*p*-N)]₁ (**1**); b) G₁[NCPh(*o*-N)]₄ (**5**); and c) G₂[NCPh(*o*-OH)]₈ (**9**).

Fig. 5.
ORTEP drawing of the mononuclear complex $G_O[\text{NH}_2\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_I$ structure with the atoms labelling scheme, selected bond distances and angles are presented in Table S2 (see Supplementary Information).

Fig. 6.

Compound **17** impaired the viability of MDA-MB-231 by inducing necrosis and apoptosis. 10.7 μ M of **17** reduced MDA-MB-231 viability by ca. 58% (Apoptosis: 16%, Necrosis: 27%, Double Stain: 15%) while 42% of the treated cells remain viable. The effect **17** on viability, necrosis and apoptosis was assessed by measuring protease activity using the non-cell-permeable substrate, cells-permeable substrate and by measuring the total caspase-3 and -7 activities with the ApoTox-Glo Triplex Assay. The effect of each treatment was determined by comparing treated and untreated cells.

Fig. 7. CD spectra of CT DNA (97.5 μ M) and CT DNA incubated with 0.1, 0.25, 0.5 and 1.0 equivalents of compounds **16–18** for 20 h at 37 $^{\circ}$ C.

Fig. 8.

Electrophoresis mobility shift assays for cisplatin, $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ and dendrimers **10**, **11**, **13–18** (see Experimental Section for further details). DNA refers to untreated *p*lasmid (*pBR322*) DNA. Lanes *a*, *b*, *c* and *d* correspond to metal/DNAbp ratios of 0.25, 0.5, 1.0 and 2.0, respectively.

Fig. 9.

Electrophoresis mobility shift assays first generation dendrimers **11**, **14**, and **17** at metal/DNAbp ratio of 2.0 (see Experimental Section for further details) at three different pH values (6, 7 and 8). DNA refers to untreated *plasmid* (*pBR322*) DNA.

Fig. 10.

(a) Fluorescence titration curve of HSA with compound **16**. Arrow indicates the increase of quencher concentration (10–100 mM). (b) Stern-Volmer plot for HSA fluorescence quenching observed with compound **16**, the ruthenium dimer $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ and cisplatin.

Scheme 1.

Synthesis of new ruthenium non-dendritic zero generation $G_0[X]_1$ species (10, 13, 16, 19) and first $G_1[X]_4$ (11, 14, 17, 20) and second generation $G_2[X]_8$ (12, 15, 18, 21) dendrimers.

Table 1

IC₅₀ (μM) of Metal Complexes **10–18** in Human Cell Lines.

Cell line	Ru atoms per compounds	HeLa	MCF-7	HT-29	MDA-MB-231	HEK-293T
Compound	Ru	IC ₅₀ (μM)	IC ₅₀ (μM)	IC ₅₀ (μM)	IC ₅₀ (μM)	IC ₅₀ (μM)
10	1	89.7±4.9	22.0±3.7	14.6±2.0	63.6±4.7	44.8±0.1
11	4	6.3±0.3	3.6±0.3	6.6±0.4	4.5±0.4	5.0±0.5
12	8	68.8±3.2	19.9±0.1	14.4±0.1	–	–
13	1	26.4±6.3	56.0±5.1	41.7±2.8	53.3±0.9	51.9±6.6
14	4	4.4±0.1	2.5±0.1	3.3±0.2	4.9±0.2	5.2±0.3
15	8	9.1±0.4	3.4±0.1	7.0±0.2	6.7±0.0	6.3±0.5
16	1	7.2±0.5	6.3±0.4	6.9±0.2	74.1±3.1	46.7±3.2
17	4	8.1±0.4	6.9±0.5	5.6±0.1	6.1±1.0	10.7±0.0
18	8	6.9±0.3	8.5±0.5	13.4±1.7	10.1±0.8	10.5±1.5

* Freshly prepared stock solutions of complex in DMSO were used. All compounds were dissolved in 1% of DMSO and diluted with cell culture medium for a 24h incubation period.

Table 2Human Cathepsin B inhibition^a by first generation ruthenium metallodendrimers **11**, **14** and **17**

Compound	IC ₅₀ (μM)
11	14.4
14	47.1
17	4.4

^aCapthesin B purified from human liver.