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Ruthenium(III) Chloride Complex with a Tridentate Bis(arylimino)pyridine Ligand: Synthesis, Spectra, X-ray Structure, 9-Ethylguanine Binding Pattern, and In Vitro Cytotoxicity

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The synthetic, spectroscopic, structural, and biological studies of a bis(arylimino)pyridine Ru(III) chloride compound containing the ligand, 2,6-bis(2,4,6-trimethylphenyliminomethyl)pyridine are reported. The bis(arylimino)pyridine ligand, with three donor nitrogen atoms, was synthesized by condensation of 2,6-pyridinedicarboxaldehyde with 2,4,6-trimethylaniline. The Ru(III) complex, with formula $[RuCl_3(L1)](H_2O)$ (RuL1), where L1 = 2,6-bis(2,4,6-trimethylphenyliminomethyl)pyridine, was structurally determined on the basis of analytical and spectroscopic (IR, UV−vis, ESI-MS) studies. A straightforward strategy to fully characterize the paramagnetic compound using advanced ^{1}H NMR is reported. This new complex is a prototype for a series of new anticancer Ru(III) and Ru(II) compounds with improved cytostatic properties; likely to be modified in a desirable manner due to the relatively facile ligand modification of the bis(imino)pyridines and their molecular architecture. The present Ru(III) complex is the first example of this family of Ru(III)/Ru(II) anticancer compounds with the aimed physicochemical characteristics. Although the ligand itself is moderately active in selected cell lines (EVSA-T and MCF-7), the activity of the $[Ru(L1)Cl_3]$ complex has increased significantly for a broad range of cancer cell lines tested in vitro (IC_{50} values = 11∼17 μM). Reaction of the RuL1 species with the DNA model base 9-ethylguanine (9EtGua) was found to produce in a redox reaction the species *trans*-$[Ru(II)(L1)(9EtGua)_2(H_2O)](ClO_4)_2$ (abbreviated as RuL1−9EtGua), which was studied in solution and also in the solid state, by X-ray crystallography. The structure comprises the as yet unknown *trans*-bis(purine)Ru(II) unit.

Introduction

The application of metal ions in medicine is not a new trend. Despite the historical method of drug discovery, based on trial-and-error testing of chemical substances, the ongoing trend is the purposeful design of metal-based therapeutics. The physical and chemical design of a drug is a complex challenge. When talking about a metal-based drug and once the metal is selected, the first and simple choice could be a variation in the ligands coordinated to the metal; this will allow the tuning of the chemical and physical properties that finally will control the endogenous distribution of the drug in the body, or even specific tissue targeting.

In particular, after the serendipitous discovery of cisplatin,[1,2] the most successful platinum-based anticancer compound, attention to other anticancer metal-based compounds has been directed[3–8] in a search for less toxic and more effective drugs.

Among all of the metals used in the synthesis of potential anticancer drugs, a wide range of ruthenium compounds have been described in the literature, some of them with outstand-

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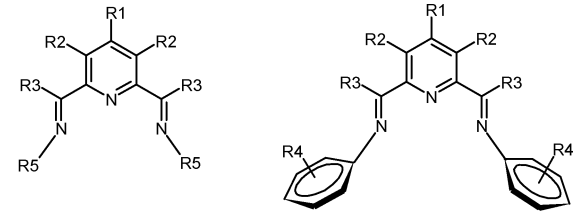
ing anticancer activity[9–16] and two of them, for instance NAMI-A and KP1019, are currently involved in clinical trials.[17–19] It is known that ruthenium compounds are well suited for medical applications due to the fact of having convenient rates of ligand exchange,[20] a range of accessible oxidation states, and the ability of the ruthenium to mimic iron in binding to certain biological molecules.[9,10,17] Under aqueous conditions, three predominant oxidation states are known for Ru, for instance, Ru(II), Ru(III), and Ru(IV), all of them mostly presenting an octahedral configuration. This octahedral geometry appears to be partially responsible for the differences observed in the mechanism of action compared with cisplatin. The hypoxic environment of many tumors may favor the reduction of Ru(III) compounds (which are relatively slow to bind to most biological substrates) to Ru(II) species, which bind more rapidly.[10] Among ruthenium complexes studied for anticancer application, the group of ruthenium complexes with pyridyl-based ligands is of special interest, due to a combination of easily constructed rigid chiral structures and useful photophysical properties. They mostly have been studied because, when presenting chirality, they are capable of enantioselective recognition of DNA and cleavage properties as well.[21–38] As the majority of these

Scheme 1. Schematic Representation of Tridentate Bis(imino)pyridine and Bis(arylimino)pyridine Derivatives



R1,R2,R3=H, Me, alkoxy
R4=H, hydrocarbyl, fluorohydrocarbyl, halo, alkoxy, hydroxyl; possibility of asymmetry
R5=NH2, N-alkyl, N-pyrrolyl, N-benzimidazolyl

complexes contain bidentate ligands with functional auxiliary ligands, research on Ru(III)/Ru(II) complexes with more rigid, tridentate ligands, and additional chloride ligands is a new challenge.

In fact, considerable cytotoxic activity of complexes with structural formulas: [Ru(bpy)(terpy)Cl]Cl and *mer*-[Ru(terpy)Cl_3] (bpy = 2,2-bipyridyl, terpy= 2,2′:6′,2″-terpyridine) has been demonstrated in murine and human tumor cell lines.[39,40] *mer*-[Ru(III)Cl_3(terpy)] exhibits a remarkably higher cytotoxicity than the other complexes and even displays the highest[36] and remarkable DNA interstrand cross-linking properties. Unfortunately, solubility problems and − even more importantly − difficulties in preparation of terpyridine derivatives have reduced the attention for this system.

During the past decade, bis(imino)pyridine ligands (Scheme 1) have attracted significant attention[41–47] because of their easy synthesis, possibility of steric and electronic tuning, and well-documented ability to support a range of catalytically active metal centers (especially for iron and cobalt) and other interesting structural types. In particular their redox activity has been studied intensely, and in general the variety of chemistry displayed for this ligand system is remarkable.[48]

The tridentate ligand 2,6-bis(2,4,6-trimethylphenylimino-methyl)pyridine (abbreviated L1, schematically represented in Scheme 2) presents three in-plane bonding positions, in which only the three meridional positions of an octahedron

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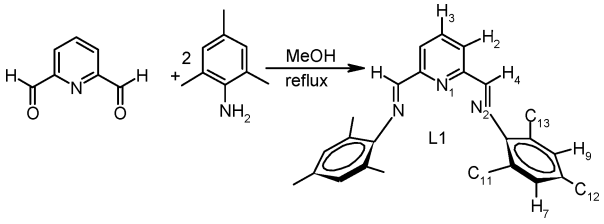
Scheme 2. Schematic Representation of the Synthesis of L1[a]



[a] The hydrogen atoms belonging to the methyl groups have been omitted for clarity.

can be occupied by the donor nitrogen atoms. In this respect L1 behaves like 2,2′:6′,2″-terpyridine.

In a search for new anticancer-active systems based on ruthenium, we have prepared and characterize a prototype of a new class of ruthenium compounds, using a versatile tridentate bis(imino)pyridine-type of molecule as chelating ligand. The resulting octahedral complex keeps three coordination sites occupied by labile chloride ligands. The bis(imino)pyridine ligand can be chemically modified to tune its solubility, its cytotoxicity, and also the pharmacokinetics and pharmacodynamics in the human body. We have studied its interaction with the DNA model base, 9-ethylguanine (9EtGua), in solution, but also by X-ray diffraction of the isolated crystals of this adduct.

Experimental Section

Materials. All of the chemicals and analytical grade solvents were purchased from various commercial sources and were used without further purification treatments unless otherwise stated. Ruthenium trichloride was a generous gift from Johnson Matthey, U.K. All synthesized compounds are reasonably thermally stable and air-stable, both in the solid state and in solution. For the sake of caution, however, their preparation and manipulation in solution were carried out under an inert atmosphere (argon).

Preparation of the Compounds. Synthesis of 2,6-Pyridinedicarboxaldehyde. The synthetic procedure has been reported previously by Papadopolous[49] and was later modified by Vance.[50] Activated Mn(IV) dioxide (Across) was prepared by heating overnight at 110 °C. An excess of MnO_2 (100 g) and 10.0 g (71.9 mmol) of 2,6-bis(hydroxymethyl)pyridine (Aldrich) were refluxed with stirring for 5 h in 500 mL of chloroform (Biosolve, spectrophotometric grade). The oxide residue was separated from the solution by vacuum filtration, and the black residue was rinsed four times with 100 mL of chloroform. Solvent was removed from the solution by rotary evaporation, and then the crude product was dissolved in the minimal amount of chloroform and passed through a silica gel column (ca. 15 cm long, ca. 4 cm diameter). The pure dialdehyde elutes easily and can be seen as an opaque white band in the clear silica gel, while impurities remain at the top of the column. Removal of the solvent by rotary evaporation gives the product in 59% yield; mp = 114−118 °C. ^{1}H NMR spectrum (400 MHz, chloroform, 21 °C, *s* = singlet, *d* = doublet, *t* = triplet, and *m* = multiplet): 10.1782 (s, CH, 2H), 8.1975 (d, pyH, 2H), 8.0912 (t, pyH, 1H) ppm.

Synthesis of 2,6-Bis(2,4,6-trimethylphenyliminomethyl)pyridine, L1. The procedure followed was previously reported by Balamurugan.[46] To a solution of 2,6-pyridinedicarboxaldehyde (0.68 g, 5.0 mmol) in absolute methanol (25 mL) (Biosolve) were successively added 2,4,6-trimethylaniline (Aldrich) (1.35 g, 10.0 mmol), and the resulting mixture was refluxed for 2 h over molecular sieves (4A). The reaction mixture was filtered while hot. Upon cooling, a yellow crystalline solid (L1), was obtained in high yield (1.7736 g, 96%). Diffraction-quality crystals were grown from dmf. Elemental analysis for $C_{25}H_{27}N_3$: Calcd (%): C, 81.26; N, 11.37; H, 7.36. Found (%): C, 81.20; N, 11.47; H, 7.64. ESI-MS: *m*/*z* = 465.23, $[(C_{25}H_{28}N_3)(CH_3CN)(H_2O)_3]^{1+}$. IR: 3100−2800, 1640−1565, 1481, 1451−1430, 1205, 1139, 852, 815, 733, 642, 588−573 and 384 cm^{-1}. UV−vis in dmf (λ_{max} (log ε_M)): 300.1(3.67) and 356(3.69). ^{1}H NMR (300 MHz, dmf, 21 °C, *s* = singlet, *d* = doublet, *t* = triplet and *m* = multiplet): δ = 8.43(d, 2H, H_2 and H_{2a}), 8.41(s, 2H, H_4 and H_{4a}), 8.23(t, 1H, H_3), 6.93(s, 4H, H_7, H_{7a}, H_9 and H_{9a}), 2.26(s, 6H, $3H_{12}$ and $3H_{12a}$), and 2.12 ppm (s, 12H, $3H_{11}$, $3H_{11a}$, $3H_{13}$ and $3H_{13a}$).

Synthesis of Trichlorido(2,6-bis(2,4,6-trimethylphenyliminomethyl)pyridine)ruthenium(III) hydrate, RuL1. $RuCl_3 \cdot 3H_2O$ (0.1 g, 0.382 mmol; Johnson Matthey Chemicals) was dissolved in an ethanolic solution (ethanol/water, 3:2) (Riedel-deHaen) and was gently refluxed at 109 °C with continuous purging of argon for 4.5 h. After that, the hot reaction mixture was cooled to room temperature. The resulting solution was filtered through a glass filter and placed in a new round-bottom flask. Then, 0.6 mL of concentrated HCl (Riedel−deHaen) and 0.1483 g (1.05 eq, 0.4014 mmol) of L1 was added. The reaction mixture was further refluxed for 2 h and cooled down and again stirred for further 12 h at room temperature. The dark-brown solid formed after this time was collected by filtration, washed with plenty of cold dichloromethane, cold ethanol, cold water, and finally dried with dry diethyl ether. Yield: 92% (0.3514 mmol, 0.2090 g). Elemental analysis for $RuC_{25}H_{27}N_3Cl_3 \cdot (H_2O)$: Calcd (%): C, 50.47; N, 7.06; H, 4.91. Found (%): C, 50.37; N, 7.05; H, 5.03. ESI-MS: *m*/*z* = 582.07, $[Ru(C_{25}H_{27}N_3)Cl_2CH_3CN]^{1+}$. IR: 3050−2860, 1595.5, 1476−1440, 1377, 1334, 858.6, 606.8, 452.1, 374.3, and 326 cm^{-1}. UV−vis in dmf (λ_{max} (log ε_M)): 317(3.74), 390(3.80), 482(3.40), and 594(3.1). ^{1}H NMR (300 MHz, dmf, 21 °C, *s* = singlet, *d* = doublet, *t* = triplet, and *m* = multiplet): δ = 4.636(s, 4H, H_7, H_{7a}, H_9 and H_{9a}), 1.5983(s, 6H, $3H_{12}$ and $3H_{12a}$), −1.850 (broad s, 2H, H_2 and H_{2a}), −2.417 (broad s, 12H, $3H_{11}$, $3H_{11a}$, $3H_{13}$ and $3H_{13a}$), −4.291(broad s, 1H, H_3), and −27.850 ppm (broad, 2H, H_4 and H_{4a}).

Synthesis of Aquabis(9-ethylguanine)(2,6-bis(2,4, 6-trimethylphenyliminomethyl)pyridine)ruthenium(II) Perchlorate, RuL1−9EtGua. This compound was synthesized by the procedure described by van Vliet[39] for Ru(terpy)(9EtGua)$_2$Cl$_3$ synthesis, with minor modifications: 30 mg (0.0504 mmol) of RuL1 and 27.11 mg(3 Eq, 0.1513 mmol) of 9-ethylguanine were dissolved in 6 mL ethanol/water (70:30). The reaction mixture was kept under reflux for 24 h. After reflux, the volume of the solution was reduced by a half-by rotary evaporation and 1.5 mL of aqueous saturated $NaClO_4$ solution was added. After two days, the formed solid was collected by filtration, washed with plenty of cold water, cold chloroform, and dried with dry diethyl ether. Yield: 70.85% (0.03571 mmol, 37.36 mg). X-ray quality crystals were obtained by slow evaporation of a concentrated solution of RuL1−9EtGua in methanol. Elemental analysis for $RuC_{32}H_{38}N_8Cl_2O_9$: Calcd (%): C, 44.79; N, 17.41 and H, 4.53. Found (%): C, 44.82; N, 17.28 and H, 4.78. ESI-MS: *m*/*z* = 946.75, [**RuL1−9EtGua** − ClO_4]$^+$; *m*/*z* = 927.74, [**RuL1−9EtGua** − H_2O − ClO_4]$^+$; *m*/*z*=434.73,

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[**RuL1−9EtGua** + H_2O − $2ClO_4$]$^{2+}$ *m*/*z* = 413.80, [**RuL1−9EtGua** − H_2O − $2ClO_4$]$^{2+}$, 100%. IR: 3340, 3200−2900, 1661, 1634.4, 1603.5, 1568−1423, 1081.5, 622, and 374 cm^{-1}. UV−vis in methanol (λ_{max} (log ε_M)): 317(4.01), 363(3.93), 477(3.76), and 552(3.2). ^{1}H NMR (300 MHz, methanol, 21 °C, *s* = singlet, *d* = doublet, *t* = triplet, and *m* = multiplet): δ = 8.44(s, 2H, H_4 and H_{4a}), 8.40(d, 2H, H_2 and H_{2a}), 8.06(t, 1H, H_3), 6.77(s, 4H, H_7, H_{7a}, H_9 and H_{9a}), 6.68(s, 2H, H_{18} and H_{18a}), 4.61(broad s, 4H, N_5−H), 3.94(m, 4H, $2H_{19}$ and $2H_{19a}$) 2.22(s, 6H, $3H_{12}$ and $3H_{12a}$), 1.32(s, 12H, $3H_{11}$, $3H_{11a}$, $3H_{13}$ and $3H_{13a}$), and 1.17 ppm (t, 6H, H_{20} and H_{20a}).

Caution! *Perchlorate salts of metal complexes with organic ligands are potentially explosive. Only small amounts of the compound should be prepared and handled with great care.*

Methods and Instrumentation. (a) X-ray Crystallography. All reflections intensities were measured at 150(2) K using a Nonius KappaCCD diffractometer (rotating anode for L1 and fine-focus sealed tube for RuL1−9EtGua) equipped with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å) under the program *COLLECT*.[51] The program *PEAKREF*[52] was used to determine the cell dimensions. The two sets of data were integrated using the program *EVALCCD*.[53] The structure of L1 was solved with the program *SHELXS86*[54] and that of RuL1−9EtGua with the program *DIRDIF99*.[55] The two structures were refined on F^2 with *SHELXL97*.[56] Multiscan semiempirical absorption corrections were applied to the two sets of data using *SADABS*.[57] For L1, 2026 reflections were unique (R_{int} = 0.037), of which 1637 were observed (θ_{max} = 26°) with the criterion of $I > 2\sigma(I)$; for RuL1−9EtGua, 5448 reflections were unique (R_{int} = 0.015), of which 5276 were observed (θ_{max} = 27.5°) with criterion of $I > 2\sigma(I)$. The *PLATON* software[58] was used for molecular graphics, structure checking, and calculations. The H-atoms were placed at calculated positions (except as specified) with isotropic displacement parameters having values 1.2 or 1.5 times U_{eq} of the attached atom. For L1, the H-atoms of the two methyl groups C11 (ortho position) and C12 (para position) were found to be disordered by a rotation of 60° and were treated using the *AFIX 123* instruction. The occupation factors for the two major components of the disorder refined to 0.73(2) and 0.77(3). For RuL1−9EtGua, the H-atoms for the atoms O1 and N5 of the ruthenium complex were located from the difference Fourier map and the O−H and N−H bond distances were restrained to be 0.84 and 0.88 Å (using the *DFIX* instruction). All crystallographic data are listed in Table 1.

(b) NMR Spectroscopy. ^{1}H NMR experiments were recorded on a Bruker 300 DPX spectrometer using 5 mm NMR tubes. All spectra were recorded at 21 °C, unless otherwise indicated. Temperature was kept constant using a variable temperature unit. The software XWIN-NMR and XWIN-PLOT were used for edition of the NMR spectra. Tetramethylsilane (TMS) or the deuterated solvent residual peaks were used for calibration. In addition 2D ^{1}H COSY spectra were recorded to confirm the proton assignments from 1D measurements.

Table 1. Crystallographic Data for L1 and RuL1−9EtGua

abbreviation:	**L1**	**RuL1−9EtGua**
empirical formula	$C_{25}H_{27}N_3$	$C_{39}H_{47}N_{13}O_3Ru\cdot 2(MeOH)\cdot 2(ClO_4)$
fw	369.50	1109.95
cryst symmetry	monoclinic	orthorhombic
space group	*C*2/*c* (No. 15)	*Fdd*2 (No. 43)
a, Å	12.5220(7)	25.2350(1)
b, Å	9.9495(8)	30.8420(2)
c, Å	16.9423(13)	12.2446(1)
β (°)	102.382(4)	90
V, Å^3	2061.7(3)	9529.95 (11)
Z	4	8
T, K	150(2)	150(2)
ρ_{calcd}, g/cm^3	1.190	1.547
μ, mm^{-1}	0.07	0.52
R1[a]	0.041	0.019
wR2[b]	0.103	0.048
GOF	1.04	1.06
$\Delta\rho_{max}$, e Å^{-3}	0.17	0.30
$\Delta\rho_{min}$, e Å^{-3}	−0.16	−0.30
Flack parameter		−0.025 (14)

[a] $R1 = \Sigma||F_o| - |F||/\Sigma|F_o|$. [b] $wR2 = \{\Sigma[w(F_o^2 - F_c^2)^2]/\Sigma[w(F_o^2)^2]\}^{1/2}$.

(c) Carbon, Hydrogen, and Nitrogen Analysis. Elemental analyses were performed with a PerkinElmer series II CHNS/O 2400 Analyzer.

(d) Mass Spectroscopy. Electrospray mass spectra were recorded on a Finnigan TSQ-quantum instrument using an electrospray ionization technique (ESI-MS). The eluent used was the mixture acetonitrile/water 80:20.

(e) Other Methods. The UV−vis (UV−vis) spectra were recorded using a Varian CARY 50 UV−vis spectrophotometer operating at room temperature. The electronic spectra were recorded in freshly prepared solutions of each compound. The IR spectra obtained for the products mentioned in this work, in the 4000−300 cm^{-1} range, were recorded as solid with a PerkinElmer FTIR Paragon 1000 spectrophotometer with a single-reflection diamond ATR P/N 10500. X-band powder EPR spectra were obtained on a Bruker-EMXplus electron spin resonance spectrometer (Field calibrated with DPPH (g = 2.0036))

(f) Cytotoxicity and IC_{50} Determination. The in vitro cytotoxicity test of compounds L1 and RuL1 were performed using the SRB test[59] for estimation of cell viability. The human cell lines MC7(breast cancer), EVSA(breast cancer), WIDR(colon cancer), IGROV(ovarian cancer), M19-MEL(melanoma cancer), A498(renal cancer), and H226(nonsmall cell lung cancer) were used. Cell lines WIDR, M19 MEL, A498, IGROV, and H226 belong to the currently used anticancer screening panel of the National Cancer Institute, USA.[60] The MCF7 cell line is an estrogen receptor $(ER)^+$/progesterone receptor $(PgR)^+$, and the cell line EVSA-T is $(ER)^-$/$(PgR)^-$. Prior to the experiments a mycoplasma test was carried out on all cell lines and found to be negative. All the cell lines were maintained in a continuous logarithmic culture in RPMI 1640 (Invitrogen, Paisley Scotland) medium with Hepes and phenol red. The medium was supplemented with 10% fetal calf serum (Invitrogen, Paisley Scotland), penicillin 100 IU/mL (Sigma, USA), and streptomycin 100 μg/mL (Sigma, USA). The cells were mildly trypsinized for passage and for use in the experiments. For the cell growth assay, cells (1500−2000 cells/150 μL of complete medium/well) were precultured in 96 multiwell plates (falcon 3072, BD) for 48 h at 37 °C in a 5% CO_2 containing incubator and

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subsequently treated with the tested compounds for 5 days. The stock solutions of the compounds were prepared in the corresponding medium. A 3-fold dilution sequence of 10 steps was made in full medium, starting with the 250 000 ng/mL stock solution. Every dilution was used in quadruplicate by adding 50 μL to a column of wells. The result in the highest concentration of 62 500 ng/mL is present in column 12. Column 2 was used for the blank, and column 1 was completed with medium to diminish interfering evaporation. After a 120 h incubation time, the surviving cells in cultures, treated with the compounds were detected using the sulforhodamine B (SRB, Sigma,USA) test.[59] After the incubation time cells were fixed with 10% of trichloroacetic acid (Sigma, USA) in PBS (Emmer-Compascuum, NL). After three washing cycles with tap water, the cells were stained for at least 15 min with 0.4% SRB dissolved in 1% of acetic acid (Baker BV, NL). After staining, the cells were washed with 1% acetic acid to remove the unbound stain. The plates were air-dried, and the bound stain was dissolved in 150 μL of 10 mM Tris-base. The absorbance was read at 540 nm using an automated microplate reader (Labsystems Multiskan MS). Data were used for construction of concentration−response curves and determination of the ID_{50} values was graphically done by use of *Deltasoft 3* software.

The variability of the in vitro cytotoxicity test depends on the cell line used and the serum applied. With the same batch of cell lines and the same batch of serum the interexperimental CV (coefficient of variation) is 1−11% depending on the cell line, and the intraexperimental CV is 2−4%. These values may be higher when using other batches of cell lines and/or serum.

Results and discussion

Synthesis and Characterization. Condensation of 1 equiv of 2,6-bis(aldehyde)pyridine with 2 equiv of the required aniline[42] to produce 2,6-bis(imino)pyridine ligands is the most commonly synthetic procedure used. A few earlier results have been reported[48,61,62] related to the rich chemistry developed by these bis(imino)pyridine ligands, which is a result of the many favorable reactive sites (Scheme 1), including the nitrogen and carbon centers of the imine moiety as well as the pyridine ring. Little attention has been given to changes of the substituents at the imine carbon, although most of the earlier research has been directed to bis(imino)pyridine frame modifications in the groups attached to the imine nitrogen.[48] Some synthetic strategies for the preparation of bis(imino)pyridine derivatives with different symmetry are known; for instance, the method of reacting 2,6-bis(acetyl)pyridine, first, with 1 equiv of a substituted aniline and subsequently with 1 equiv of either a primary amine or a different aniline has been successfully applied in the synthesis of (2-arylimino-6-alkylimino)pyridines or 2,6-bis(arylimino)pyridines.[42,63–65] Also, variable substitution

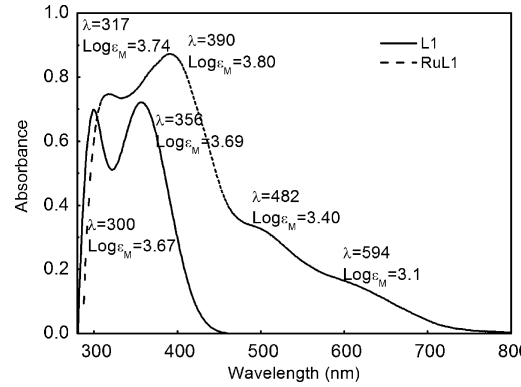

Figure 1. Absorption spectra of L1(solid line) and RuL1(dashed line) in dmf.

patterns on the aryl rings bound to the imine nitrogen atoms can easily be obtained as well as different substituents located in the pyridine moiety. For instance, the introduction of a bulky alkyl group at the 4 position in the pyridine ring that can impair a better hydrophobic nature could be easily obtained through a radical attack,[66] or to double the 2,6-bis(imino)pyridyl moiety to give polydentate ligands (6N) capable of coordinating two metal centers.[67] All of these possibilities clearly underline the facile tuneability of the chemical and physical properties of the ligands by themselves but also of the coordination complexes formed with them, which finally will be reflected in the cytotoxicity.

The 2,6-bis(2,4,6-trimethylphenyliminomethyl)pyridine ligand, used in the synthesis of the entitled ruthenium compounds, was prepared in one single step with high yields from the condensation of 2 equivs of 2,4,6-trimethylaniline with 1 equiv of 2,6-pyridinedicarboxaldehyde (Scheme 2).

L1 was fully characterized by elemental analysis, ^{1}H NMR, mass spectroscopy, and IR and UV−vis studies as well, and the results agree with data previously reported.[46] In addition, the free ligand was studied by single-crystal X-ray diffraction studies. The molecule lies about a mirror plane, which passes through the N1 and C3 atoms (Scheme 2).

The RuL1 compound was synthesized in good yields by treating $RuCl_3 \cdot 3H_2O$ with L1 in a refluxing mixture of ethanol/water. Despite promising catalytic properties and increased attention to study such metal−ligand systems,[41] attempts to synthesize ruthenium complexes with such bis(imino)pyridine ligands and different starting ruthenium compounds have remained largely unsuccessful;[68] in fact only one related ruthenium compound has been described in literature.[68,69]

The compound RuL1−9EtGua was prepared by treatment of RuL1 with 3 equivs of 9EtGua in ethanol/water (Experimental Section) as shown in Scheme 3. The complexes were characterized by a variety of techniques including elemental analysis, ESI-MS spectrometry and UV−vis, IR, EPR, and ^{1}H NMR spectroscopy. In addition, RuL1−9EtGua was

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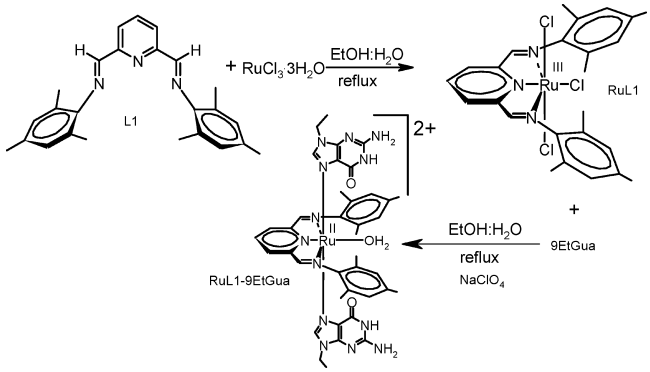
Scheme 3. Schematic Representation of the Synthesis of RuL1 and RuL1−9EtGua



studied by single-crystal X-ray diffraction.

From IR studies, several changes were observed in the spectrum of RuL1 when comparing with the spectrum obtained from the free ligand. Table S1 in the Supporting Information summarizes the most important IR peaks, the corresponding assignment, and frequencies in the mid-IR region, confirming the presence of the ligand and coordinating to ruthenium. A sharp vibration peak assigned to the ν(Ru−Cl) stretching mode was observed in RuL1 at 325 cm^{-1}, a value which is in accordance with the proposed structure. The absorption spectra for the ligand and its complex, in the UV−vis region, were recorded using a Varian CARY 50 UV−vis spectrophotometer operating at room temperature, using freshly prepared dmf solutions (0.148 mM and 0.136 mM), due to the poor solubility in water. The spectrum of RuL1 is characterized by intense peaks in the region that comprises 200−700 nm. The spectrum in the visible region is dominated by the expected d $\rightarrow \pi^*$ MLCT bands and in the UV region by ligand-centered $\pi \rightarrow \pi^*$ transitions. The bands appearing at 317 nm (log $\varepsilon_M = 3.74$) and 390 nm (log $\varepsilon_M = 3.80$) are considered mainly as intraligand charge-transfer transitions, as they have high molar absorption coefficients and could be observed in the free ligand[70] as well (Figure 1). The energy of the $\pi \rightarrow \pi^*$ transition in free L1 (at 300 nm and 356) is lower for RuL1 (at 317 nm and 390), which is consistent with the coordination of L1. The transitions observed in the visible region in this compound, are comparable to other Ru(III) complexes involving nitrogen donor molecules.[71,72]

The ESI-MS spectrum of RuL1 exhibits a positive peak at $m/z = 582.07$, which corresponds to the cationic structure, $[Ru(C_{25}H_{27}N_3)Cl_2CH_3CN]^{1+}$. A mixture of CH_3CN/H_2O, 80:20 was used as eluent. The MS peak exhibited the correct isotopomer distribution, as expected from the number of chlorine atoms and the ruthenium isotope distribution.

Although RuL1 is paramagnetic, ^{1}H NMR spectroscopy can provide important structural information for such compounds.[73,74] Figure 2 shows the ^{1}H NMR spectrum of RuL1 and the corresponding assignments. Because of this paramagnetic nature, the spectrum of RuL1 shows six paramagnetically shifted and broadened peaks that were assigned, on the basis of integration and proximity to the paramagnetic ruthenium center, and which are distributed in a wide frequency range. Because of the symmetry in the complex, the protons forming part of the structure are magnetically equivalent in pairs, so only six resonances are observed in the spectrum. Striking similarities have been observed for paramagnetic complexes of cobalt and iron with similar bis(imino)pyridine ligands.[41,42,61] The lost of multiplicity is also attributed to the proximity of the paramagnetic center. The integration values are in agreement with the proposed structure (assignment data: $\delta = 4.636$(s, 4H, H_7, H_{7a}, H_9 and H_{9a}), 1.5983(s, 6H, $3H_{12}$ and $3H_{12a}$), −1.850 (broad s, 2H, H_2 and H_{2a}), −2.417(broad s, 12H, $3H_{11}$, $3H_{11a}$, $3H_{13}$ and $3H_{13a}$), −4.291(broad s, 1H, H_3), and −27.850 ppm (broad, 2H, H_4 and H_{4a})). The strong coordination of the aryl-substituted imine arm to the paramagnetic ruthenium center is confirmed by the large downfield shift ($\delta = -27.850$ ppm) of the *N*═CH resonance as well as its broad line width. Particular attention should be directed to the resonance of the hydrogen atoms, H_{11} and H_{13}, belonging to the methyl moieties in the aryl group, as they are shifted to high field and presents a very broad line width, which suggests that this aromatic ring is spatially very close to the paramagnetic ruthenium center on the NMR time scale. The presence of just one signal for the methyl groups located at the ortho position in the aryl ring suggests a free rotation about the N−C axis as they are magnetically equivalent. The powder EPR of the solid RuL1 just shows a single very broad, uninformative line centered around $g = 2.10$.

The very clean spectrum indicates a high purity of the sample. No relevant change in the spectrum was found after several hours at 298 K. Only, after 9 days a partial reduction and probably coordination of solvent is observed.

Although the mechanism of action of the cytotoxic ruthenium compounds is not yet elucidated in detail, a direct interaction with DNA is a likely possibility, among other mechanisms.[17,40,75–78] To shed some light on the coordination interactions of RuL1 and DNA, the reaction with the model base 9-ethylguanine was studied in detail. Even though this model reaction does not mimic the real interaction with DNA in the cells, it provides useful information on the reactivity of the complex, leaving-group liability, and structural characteristics of the formed adduct. Furthermore, the ruthenium−nucleobase model complex formed could be a useful reference compound for the identification of analogous

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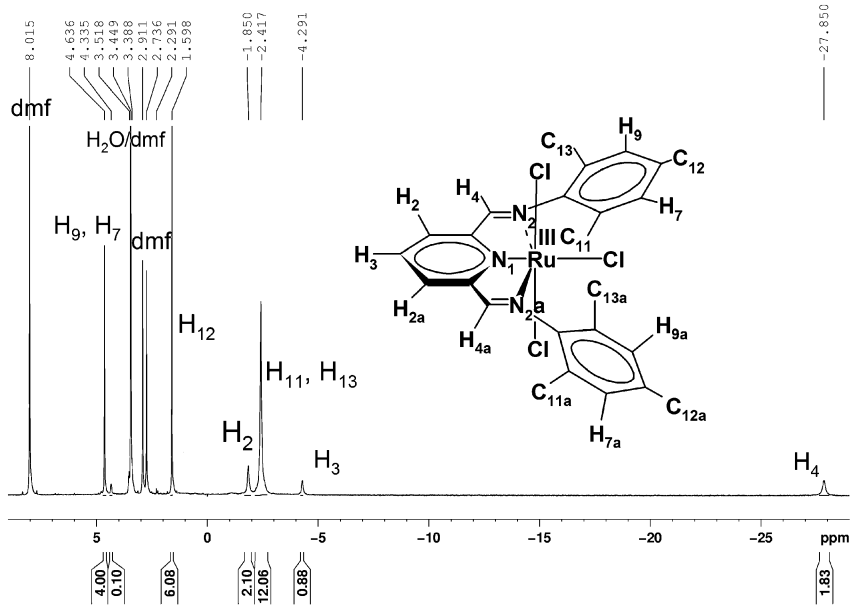

Figure 2. ^{1}H NMR spectrum of the paramagnetic compound, RuL1, and corresponding assignment recorded in deuterated dmf at 21 °C. In the schematic representation, the hydrogen atoms belonging to the methyl groups have been omitted for clarity. A trace of water is visible at 3.38 ppm.

guanine adducts in the cells. In the case of RuL1, two model 9-ethylguanine (9Etgua) bases were found coordinated to the structure in trans configuration, through N7, as was later confirmed by X-ray diffractions studies.

The experimental procedure followed for the synthesis of RuL1−9EtGua was similar to the procedure describe for the synthesis of a closely related ruthenium compound synthesized by van Vliet.[39] The reduction of Ru(III) takes part without any familiar reducing agent, as has been observed in other cases.[39,71] It appears that ethanol may serve as the reductant and the coordination of the nitrogen donor model base must favor even more the reduction process, as the donor properties tend to stabilize the Ru(II) stage. The elemental analysis corresponds with reduction of ruthenium and coordination of two molecules of 9EtGua and one molecule of water. The IR spectrum further confirms the presence of water coordinated to the structure (also Table S1 in the Supporting Information). The ESI-MS spectrum of RuL1−9EtGua exhibits many positive peaks, all them confirming the presence of the proposed complex. The MS peaks exhibited the correct isotopomer distribution mainly derived from the ruthenium atom. The electronic spectrum of RuL1−9EtGua shows broad and intense visible bands between 300 and 600 nm due to the metal to ligand charge transfer transition (Figure 3) and the intense peaks by ligand-centered $\pi \rightarrow \pi^*$ transitions, previously assigned.

The synthesis, stability, and isolation of RuL1−9EtGua as a monoaqua complex are favored by the intramolecular hydrogen-bonding properties of the 9EtGua molecules. The complex and ligands display resolved ^{1}H NMR spectra in deuterated methanol, thereby providing important structural evidence. Figure 4 shows the ^{1}H NMR spectrum of RuL1−9EtGua, along with the free 9EtGua spectrum and the corresponding assignments, which were confirmed by 2D NMR studies. The presence of just a few peaks suggests a high symmetry in the system. The frequency range where the resonance peaks are placed demonstrate the diamagnetic

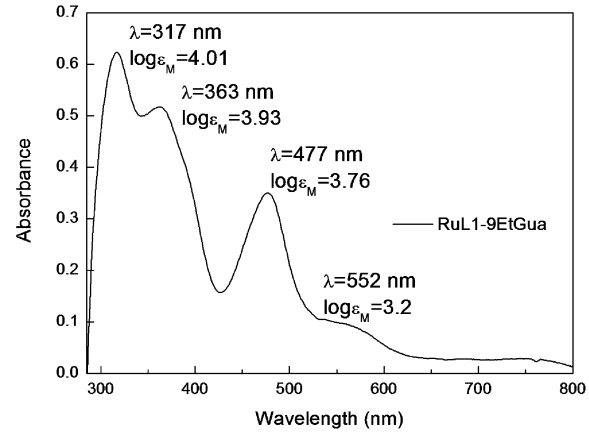

Figure 3. Absorption spectrum of RuL1−9EtGua in methanol.

nature of the compound formed. The integration values are in agreement with the proposed structure (assignment data: δ = 8.44(s, 2H, H_4 and H_{4a}), 8.40(d, 2H, H_2 and H_{2a}), 8.06(t, 1H, H_3), 6.77(s, 4H, H_7, H_{7a}, H_9 and H_{9a}), 6.68(s, 2H, H_{18} and H_{18a}), 4.61(broad s, 4H, N_5−H), 3.94(m, 4H, $2H_{19}$ and $2H_{19a}$) 2.22(s, 6H, $3H_{12}$ and $3H_{12a}$), 1.32(s, 12H, $3H_{11}$, $3H_{11a}$, $3H_{13}$ and $3H_{13a}$) and 1.17(t, 6H, H_{20} and H_{20a})). The very clean spectrum indicates a high purity of the sample. No relevant change in the spectrum was found after several days.

Overall, the ^{1}H NMR spectrum of this diamagnetic RuL1−9EtGua compound presents the same pattern of resonances that are present in L1 by itself (Experimental Section). The presence of one signal for the methyl groups, H_{11}, and H_{13} is consistent with its high symmetry and the presence of a hindered rotation of the aryl ring about the N−C axis and was also observed in related compounds of cobalt and iron.[64]

The influence of the temperature was recorded in a methanolic solution of RuL1−9EtGua (Figure S1 in the Supporting Information). At room temperature (298 K) and at low temperature (218 K), the ^{1}H NMR spectra of

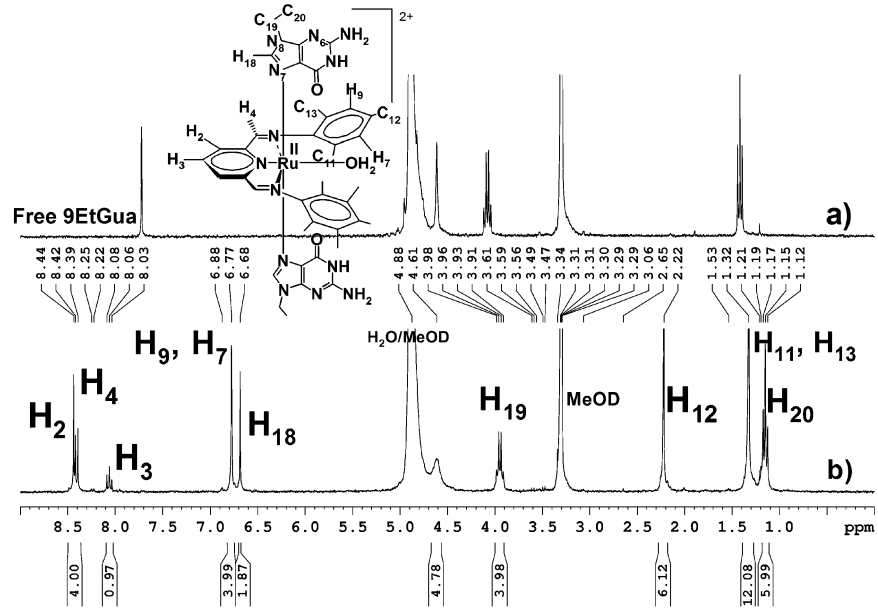

Figure 4. [1]H NMR spectrum of free 9EtGua (a) and RuL1−9EtGua (b) recorded in deuterated methanol at 21 °C.

RuL1−9EtGua are very similar, showing just a slight shift of the resonance peaks corresponding to protons H_9 or H_7 and H_{18}, and the originally broad peak assigned to the amino group in 9EtGua (4.61 ppm) shows a well-resolved resonance at low temperature.

Crystallography. Crystals of L1 suitable for X-ray diffraction studies were grown from a concentrated dimethylformamide solution. One pale-yellow block crystal was mounted on a glass fiber. In the structure of L1 (*C*2/*c*, $Z = 4$), the molecules lie at sites of 2-fold symmetry. A molecular plot of the structure is presented in the supplementary Figure S2 in the Supporting Information.

Like that observed in crystal structures of related systems,[79–82] L1 shows, in the solid state, that the imino nitrogen atoms prefers the trans conformation with respect to the central pyridine nitrogen. This spatial organization provides the least steric hindrance within the phenyl radicals located at C5. Also to be mentioned is the fact that the phenyl substituted radicals are slightly twisted to reduce the methyl hindrance between both aromatic rings. The bond lengths within the ligand are as expected. The double bond nature of C4−N2 is shown by the bond length of 1.2494(17) Å and the C1−N1 length, slightly longer, with 1.3419(15) Å, typical value for an aromatic double bond. The sp^2 nature of the C4 atom is confirmed by the planarity of the C1−C4−N2−H4 moiety. Table 2 includes selected bond distances and angles for L1.

Crystals of RuL1−9EtGua suitable for X-ray diffraction studies were grown from a concentrated methanol solution. One dark-brown block crystal was mounted on a glass fiber.

Table 2. Selected Geometric Parameters (Angstroms, Degrees) for L1

	Distances (Angstroms)		
C1−N1	1.3419(15)	C1−C4	1.4733(18)
C4−N2	1.2494(17)	C1−C2	1.384(2)
C5−N2	1.4225(17)	C5−C10	1.4019(19)
	Angles (Degrees)		
C1−N1−C1a	117.37(16)	N2−C4−C1	122.54(13)
C4−N2−C5	119.25(12)	C6−C5- N2	123.30(12)
N1−C1−C2	123.22(13)	C5−C6−C11	121.75(12)
N1−C1−C4	115.26(12)		

In the structure of RuL1−9EtGua (*Fdd*2, $Z = 8$), the asymmetric unit contains one-half of the ruthenium complex because it is located at sites of 2-fold symmetry, one counteranion ClO_4^-, and one lattice methanol molecule. The structure of RuL1−9EtGua is ordered. The molecular structure of the ruthenium complex is shown in Figure 5.

In RuL1−9EtGua, the immediate ruthenium coordination sphere is a distorted octahedron, with the major distortion arising via the N2−Ru1−N2a angle, at 156.06 (7)°. This angle is considerably smaller than the ideal angle of 180°,

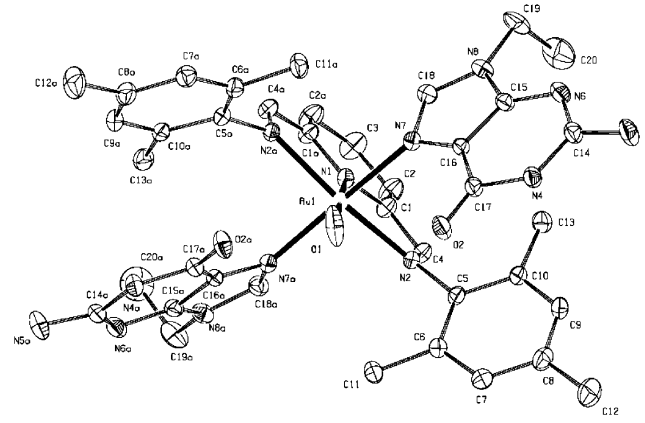

Figure 5. Displacement ellipsoid plot (50% probability level) of the asymmetric unit at 150 K. Half of the complex is symmetry generated via 2-fold symmetry (the 2-fold axis runs through the N1 and C3 atoms). The ClO_4^- counteranion, the lattice methanol molecule, and the H-atoms have been omitted for clarity.

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Table 3. Selected Geometric Parameters (Angstroms, Degrees) for RuL1−9EtGua

	Distances (Angstroms)		
Ru1−N1	1.928(2)	C5−N2	1.441(2)
Ru1−N2	2.1221(12)	C1−C4	1.440(2)
Ru1−N7	2.0990(12)	C1−C2	1.392(2)
Ru1−O1	2.084(2)	C5−C10	1.410(2)
C1−N1	1.3612(19)	N7−C18	1.319(2)
C4−N2	1.299(2)	N8−C18	1.356(2)
	Angles (Degrees)		
N1−Ru1−O1	180.00(2)	C4−N2−C5	116.09(13)
O1−Ru1−N2	101.97(4)	N1−C1−C2	120.74(16)
O1−Ru1−N7	87.92(4)	N1−C1−C4	111.62(15)
N1−Ru1−N2	78.03(4)	O2−C17−N4	118.00(15)
N1−Ru1−N7	92.08(4)	N2−C4−C1	118.04(14)
N2−Ru1−N7	85.13(5)	N2−C5−C6	119.75(13)
C1−N1−C1a	119.87(19)	C5−C6−C11	121.10(14)

and the same effect in related Ru(II) complexes is already reported in literature.[68,69] The Ru1−N1(pyridyl) bond [1.928(2) Å] is shorter than the Ru1−N2 (imino) bond [2.1221(13) Å]. The double-bond character of the imino linkage C4−N2 is retained [1.299(2) Å, for L1 is 1.2494(17) Å], although the difference with respect to the aromatic double bond distance [C1−N1, 1.3612(19) Å] is smaller than the difference observed in the free ligand (Table 2). Another important change is the reduction in the C1−C4 bond distance. It is noticeable that the planes of the substituted phenyl rings are oriented essentially orthogonal to the plane of the backbone (76.41°) as observed in other iron-, cobalt-, and ruthenium-related systems.[61,69] The ortho-methyl substituents in the phenyl rings are not bulky enough to protect the metal center as observed in other bulkier substituents as isopropyl.[61] The angle N1−Ru1−O1 is normal for an octahedral conformation [180.00(2)°].

The Ru1−N7 bond distance, 2.0990(12)Å, is slightly shorter when comparable with related structures[83] where the reported Ru−N7 bond distances are found between 2.122 and 2.131 Å. Worth mentioning is that the Ru1−N7 bond distance is an intermediate value between the Ru−pyridyl bond distance [Ru1−N1, 1.928(2) Å] and the Ru−imino distance [Ru1−N2, 2.122(12) Å]. The keto group belonging to the 9EtGua moiety is oriented to the center of the phenyl rings and slightly bent out of the plane with the O2−C17−C16−C15 torsion angle of 175.71(16)°. It is also important to stress the fact that the 9EtGua moieties are twisted by 38.53° from the plane describe by Ru1−O1 [torsion angle, O1−Ru1−N7−C16, 38.52(13)°]. The relative orientation of the two 9EtGua molecules is classified as head-to-tail. This energetically less-favored orientation could be due to the extra stabilization generated by hydrogen bonds D−H···A between the protons belonging to the water molecule coordinated and the oxygen from keto groups in 9EtGua [O1−H1···O2, O1···O2 = 2.5436(14) Å]. Table 3 includes selected bond distances and angles for RuL1−9EtGua.

Cytotoxic Activity Studies. The cytotoxicity of L1 and RuL1 and using cisplatin and doxorubicin as reference compounds was studied in the following tumor cell lines: A498, EVSA-T, H226, IGROV, M19, MCF-7, and WIDR.

(83) Zobi, F.; Hohl, M.; Zimmermann, W.; Alberto, R. *Inorg. Chem.* **2004**, *43*, 2771–2772.

Table 4. In Vitro Cytotoxicity Assay of Compounds Synthesized Incubated during 120 h

	cell line, IC_{50} (μM)						
compound	A498	EVSA-T	H226	IGROV	M19	MCF-7	WIDR
L1	93.7	23.9	36.3	82.8	57.9	15.0	59.5
RuL1	15.1	11.2	15.2	12.2	12.2	17.1	14.5
cisplatin	7.51	1.41	10.9	0.56	1.86	2.33	3.22
DOX	0.16	0.015	0.37	0.11	0.03	0.02	0.02

The most important data have been summarized in Table 4. The IC_{50} value represents the minimal amount of drug needed to inhibit 50% of the cancer cell growth. On the basis of these results, RuL1 and L1 show lower cytotoxic effects than cisplatin, but all of the compounds present IC_{50} values within the micromolar range, which is generally considered as a moderate cytotoxic activity.[59,60] RuL1 shows increased activity when compared with free L1, which stresses the influence of the metal center in the cytotoxic activity, with IC_{50} values ranging from 11.20−17.10 μM. The range of IC_{50} values for L1 is wider, presenting remarkable values for the breast-cancer cell lines EVSA-T and MCF-7. This high-moderate cytotoxic effect is synergistically increased when the metal is coordinated, for the EVSA-T (breast cancer) cell line ((ER)−/(Pgr)−), although the coordination of L1 has a negative impact in the IC_{50} in the MCF-7 (breast cancer) cell line ((ER)+/(Pgr)+). The presence of cytotoxic activity by itself probes that RuL1 and 1 are able to travel inside the cells.

These results clearly indicate that more studies with different cell lines and in vitro studies with biologically relevant structures, like proteins, DNA, and reducing agents, should be developed. Also structural modifications that improve the dissolution properties will be directed.

Conclusions. The search of ruthenium complexes with anticancer properties was started in the late 1970s. Because of their low toxicity and good selectivity for metastatic cancer, ruthenium complexes have now became the second option in the design of new metal anticancer drugs.

In this study a completely new ruthenium compound using a versatile tridentate bis(imino)pyridine-type of molecule as chelating ligand, was synthesized by a new method in high yield; the compounds has been extensively characterized. The resulting octahedral complex keeps three coordination sites occupied by labile chloride ligands. The interaction of the ruthenium complex with the DNA-model base 9-ethylguanine (9EtGua) has been demonstrated, both in the solid state and in solution with the coordination of two model guanine bases.

Remarkable inhibition properties for RuL1 were found, as the complex shows moderate cytotoxic activity and up to six times higher activity than the parental ligand. Even more encouraging results may be expected when structural modifications would improve the dissolution properties.

This research has led the development of a promising new generation of potential antineoplastic Ru(III) and Ru(II) complexes with a bis(arylimino)pyridine ligands. The potential interest lies mainly in the facility of modifications of the ligand moiety, which could help in the tuning of the biological properties but also represent a plausible active

catalytic compound in the field of metal−bis(imino)pyridine systems that have attracted significant attention in last years.

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Supporting Information Available: Table and discussion of the IR spectra of L1, RuL1 and RuL1−9EtGua; 1D,^{1}H NMR temperature dependence study of RuL1−9EtGua, and the X-ray diffraction plot of L1 is also attached. CIF file containing the crystallographic data for both compounds. Ordering information is available on any current masthead page. This material is available free of charge via the Internet at http://pubs.acs.org.

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