New Cytotoxic and Water-Soluble Bis(2-phenylazopyridine)ruthenium(II) Complexes

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New water-soluble bis(2-phenylazopyridine)ruthenium(II) complexes, all derivatives of the highly cytotoxic α-[Ru(azpy)$_2$Cl$_2$] (α denoting the coordinating pairs Cl, N(py), and N(azo) as cis, trans, cis, respectively) have been developed. The compounds 1,1-cyclobutanedicarboxylatobis(2-phenylazopyridine)ruthenium(II), α-[Ru(azpy)$_2$(cbdca-*O*,*O*′)] (**1**), oxalatobis(2-phenylazopyridine)ruthenium(II), α-[Ru(azpy)$_2$(ox)] (**2**), and malonatobis(2-phenylazopyridine)ruthenium(II), α-[Ru(azpy)$_2$(mal)] (**3**), have been synthesized and fully characterized. X-ray analyses of **1** and **2** are reported, and compound **1** is the first example in which the cbdca ligand is coordinated to a ruthenium center. The cytotoxicity of this series of water-soluble bis(2-phenylazopyridine) complexes has been determined in A2780 human ovarian carcinoma and A2780cisR, the corresponding cisplatin-resistant cell line. For comparison reasons, the cytotoxicity of the complexes α-[Ru(azpy)$_2$Cl$_2$], α-[Ru(azpy)$_2$(NO$_3$)$_2$], β-[Ru(azpy)$_2$Cl$_2$] (β indicating the coordinating pairs Cl, N(py), and N(azo) as cis, cis, cis, respectively), and β-[Ru(azpy)$_2$(NO$_3$)$_2$] have been determined in this cell line. All the bis(2-phenylazopyridine)ruthenium(II) compounds display a promising cytotoxicity in the A2780 cell line (IC$_{50}$ = 0.9−10 μM), with an activity comparable to that of cisplatin and even higher than the activity of carboplatin. Interestingly, the IC$_{50}$ values of this series of ruthenium compounds (except the β isomeric compounds) are similar in the cisplatin-resistant A2780cisR cell line compared to the normal cell line A2780, suggesting that the activity of these compounds might not be influenced by the multifactorial resistance mechanism that affect platinum anticancer agents.

Introduction

Cisplatin is one of the most widely used antitumor drugs, especially for the treatment of testicular and ovarian cancer.[1,2] However, cisplatin has some major drawbacks, like severe toxic side effects, a limited applicability to a relatively small range of tumors, and often occurring resistance. This resistance is either developed or intrinsic.[2] The second-generation platinum drug diammine(1,1-cyclobutanedicarboxylato)platinum-(II), carboplatin, is also in wide clinical use. It has less toxic side effects and can be administered in a significant higher dose than cisplatin.[2] In the search for other antitumor-active metal complexes, several ruthenium complexes have been reported to be promising as anticancer drugs.[3] For example, complexes such as *trans*-Hind[Ru^{III}Cl$_4$(ind)$_2$] (Hind = indazole), *mer*-[Ru-(terpy)Cl$_3$] (terpy = 2,2′:6′,6″-terpyridine), and [Ru^{IV}-(chd-H$_2$)Cl$_2$] (chd = 1,2-cyclohexanediamine tetraacetate) have been reported to be highly antitumor-active.[4−6] Recently, some Ru(II) arene complexes have also been reported, which show inhibition of cancer cell growth.[7,8] Currently, the most promising antimetastatic agent appears to be *trans*-(H$_2$im)[RuCl$_4$(dmso)(Him)] (nicknamed NAMI-A) (Him = imidazole), which has successfully undergone the preclinical stage of testing[9,10] and has recently finished phase I clinical trials as an antimetastatic drug.[11] So at present, a number of antitumor-active ruthenium complexes are known, but no structure−activity relationships (SARs) have been established as yet.

The isomeric dichlorobis(2-phenylazopyridine)ruthenium(II) complexes exhibit quite different cytotoxicities against a series of tumor cell lines.[12] The α-isomer α-[Ru(azpy)$_2$Cl$_2$] (α-Cl), in which α indicates the coordinating pyridines (trans), azo nitrogens (cis), and chlorides (cis) (Figure 1), has been reported to show a remarkably high cytotoxicity, even more pronounced than cisplatin in most of the tested cell lines (i.e., MCF-7, EVSA-T, WIDR, IGROV, M19, A498, and H266).[12] Noteworthy is the difference in cytotoxicity between the two cis isomers (Figure 1) α and β (β indicating the coordinating pairs Cl, N(py), and N(azo) as cis, cis, cis, respectively) of which the latter shows a cytotoxicity of a factor 10 lower than the α isomer in the panel of used cell lines.[12] Because the dichlorobis(2-phenylazopyridine)ruthenium(II) complexes are not water-soluble, the water-soluble compounds α-[Ru(azpy)$_2$(NO$_3$)$_2$] (α-NO$_3$) and β-[Ru(azpy)$_2$(NO$_3$)$_2$] (β-NO$_3$) (Figure 1) have been developed in our group.[13,14]

The present paper reports the structural characterization of a new series of water-soluble compounds: 1,1-cyclobutanedicarboxylatobis(2-phenylazopyridine)ru-

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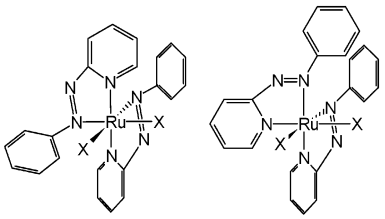

Figure 1. Schematic structures of α-[Ru(azpy)$_2$X$_2$] (left) and β-[Ru(azpy)$_2$X$_2$] (right) (X = Cl or NO_3).

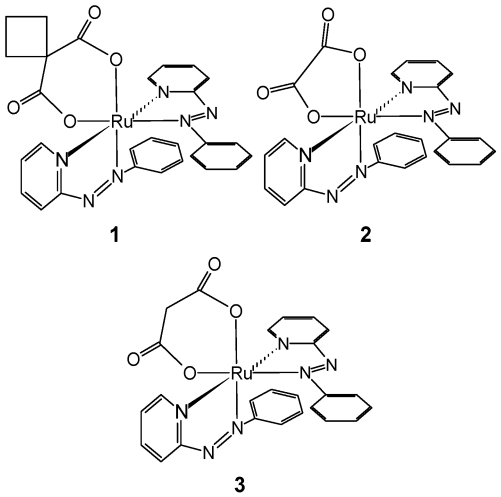

Figure 2. Schematic structures of α-[Ru(azpy)$_2$(cbdca-*O,O′*)] (**1**), α-[Ru(azpy)$_2$(ox)] (**2**), and α-[Ru(azpy)$_2$(mal)] (**3**).

thenium(II), α-[Ru(azpy)$_2$(cbdca-*O,O′*) (**1**), oxalatobis(2-phenylazopyridine)ruthenium(II), α-[Ru(azpy)$_2$(ox)] (**2**), and malonatobis(2-phenylazopyridine)ruthenium(II), α-[Ru(azpy)$_2$(mal)] (**3**) (Figure 2). The single-crystal X-ray diffraction studies of both **1** and **2** are reported. In fact, the X-ray structure of **1** is the first example of this carboxylato ligand coordinated to ruthenium. The choice of the carboxylato ligands was inspired by the compound carboplatin, which is known to be less toxic than cisplatin while being equally active as cisplatin.[15] In the present paper, the cytotoxic activity of these new water-soluble bis(2-phenylazopyridine)ruthenium(II) carboxylato complexes and of the water-soluble α-NO_3 and β-NO_3 is described and compared to the cytotoxicity of the parent compounds α-Cl and β-Cl. The cytotoxicity of the compounds has been tested in A2780 (human ovarian carcinoma) and A2780cisR, the corresponding cisplatin-resistant cell line. A2780cisR is endowed with multifactorial resistance mechanisms such as decreased uptake, increased glutathion levels, and increased DNA repair[15] and thus represents a good model for the screening of new anticancer metal-based agents.

Results and Discussion

X-ray Analyses. The bis(2-phenylazopyridine)ruthenium(II) complexes with carboxylato ligands have been synthesized using the water-soluble starting compound α-[Ru(azpy)$_2$(NO$_3$)$_2$] and the particular carboxylato acid based on the synthesis of carboplatin.[16]

A projection of the X-ray structures of α-[Ru(azpy)$_2$-(cbdca-*O,O′*)], **1**, and α-[Ru(azpy)$_2$(ox)], **2**, is shown in Figures 3 and 4, respectively. If the coordination pairs O, N(py), and N(azo) are considered in that order, the configuration of **1** and **2** is cis, trans, cis (ctc), so the

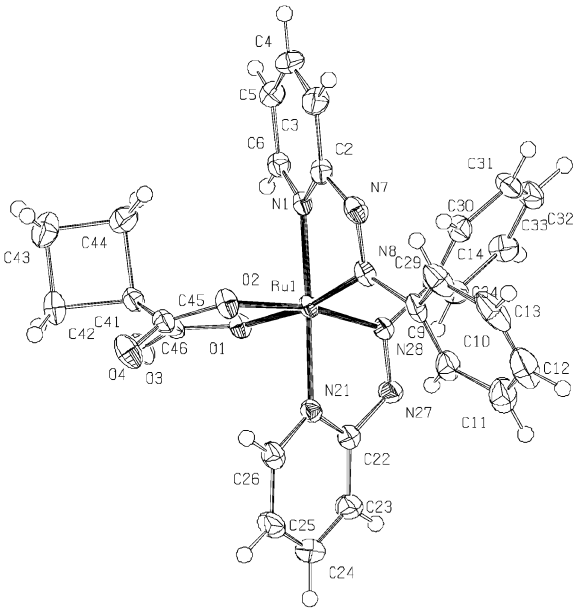

Figure 3. Atomic displacement ellipsoid plot[35] of **1** drawn at the 50% probability level. Hydrogen atoms are drawn as spheres of arbitrary radius.

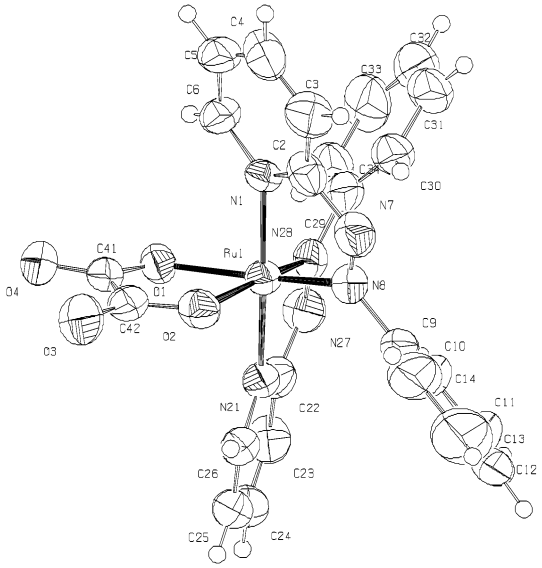

Figure 4. Atomic displacement ellipsoid plot[35] of **2** drawn at the 50% probability level. Hydrogen atoms are drawn as spheres of arbitrary radius.

original α configuration is retained during the reaction. The molecular structures clearly show the didentate *O,O′*-coordinated dicarboxylato ligands. The Ru−N(py) and Ru−N(azo) distances are comparable to those in similar structures.[17] The Ru−O distances of 2.044(2) and 2.051(2) Å in **1** and 2.056(4) and 2.054(5) Å in **2** are consistent with common single bonding, although slightly shorter than, for example, the average Ru−O_{ox} distance[18] in [(tpy)(C_2O_4)Ru^{III}ORu^{III}(tpy)(C_2O_4)]·8H_2O. The distance C_{46}−O_4 of 1.223(3) Å (and C_{45}−O_3 = 1.224-(4) Å) in **1** indicates the double-bonded keto functionality, whereas the distances C_{46}−O_1 and C_{45}−O_2 of 1.296(3) and 1.303(3) Å, respectively, indicate single bonding. These distances are similar to those found in compound **2**. The O−Ru−O angle in **1** is 88.43(8)° and 80.47(19)° in **2** and are comparable to the Cl−Ru−Cl angle[17] in α-[Ru(azpy)$_2$Cl$_2$] (89.4°) and the O−Ru−O

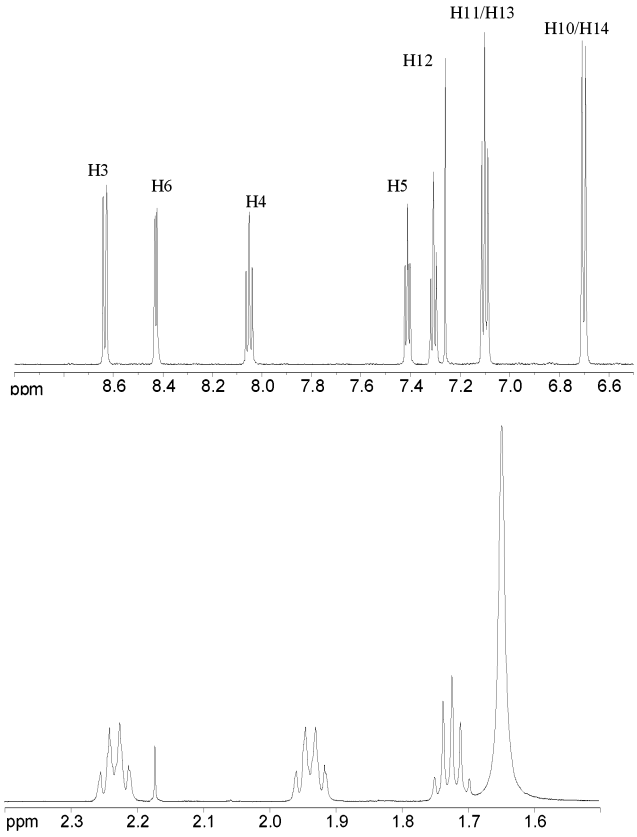

Figure 5. ^{1}H NMR spectrum of **1** in $CDCl_3$ showing the aromatic region (top) and high-field region (bottom). For the proton assignment, the numbering scheme of the X-ray structure is used.

angle[14] in α-[Ru(azpy)$_2$(NO$_3$)$_2$] (77.9°). The X-ray structure of **1** shows the C_{42} and C_{44} atoms to be inequivalent with Ru−C distances of 4.946(3) and 3.990(3) Å. This inequivalence of the cyclobutane ring carbon atoms in the solid-state structure was also observed in the case of [Pt(en)(cbdca-*O*,*O*′)].[19] In solution (vide infra) this inequivalence is apparently removed because the two azpy ligands are identical in NMR. Also, carboplatin shows inequivalence of the analogous C_α and C_β atoms in the solid state.[20] The malonate-like ring from the cbdca ligand coordinated to a metal ion may adopt three conformations: boat, chair, and planar.[21] In **1**, the conformation was found to be close to half-boat which is different from the boat conformation found for carboplatin.[20,22] In the complex [Pt(en)(cbdca-*O*,*O*′)] the Pt-cbdca chelate ring adapts a flattened boat conformation,[19] which seems comparable to the half-boat conformation found in **1**. In the X-ray structure of **1,** as well as in solution (vide infra), it appears that the H_{42A} and H_{42B} protons (and the H_{44A}/H_{44B} and H_{43A}/H_{43B} protons) are not equivalent. This is in contrast to the (in solid state and in solution) observed equivalence of these protons in carboplatin.[20,23]

Characterization and Stability Properties Studied by NMR Spectroscopy. The ^{1}H NMR spectra of the complexes **1**, **2**, and **3** in $CDCl_3$ (for ^{1}H NMR of **1**, see Figure 5) show one set of azpy signals, indicating two equivalent azpy ligands, so the complexes still possess a C_2 symmetry. The assignment of the aromatic signals has been made by the use of 2D correlation NMR spectroscopy, and H6 and H3 have been assigned on the basis of the ^3J coupling constants, which are known to be larger for H3. The same order of signals accounts for **1**, **2**, and **3**, which proves that the carboxylato ligands are chelating and that the α-configuration for the azpy ligands is retained, in agreement with X-ray data (vide supra). In the 600 MHz ^{1}H NMR spectrum of **1** (Figure 5) in $CDCl_3$, the cyclobutane signals are observed at high field. Three multiplet signals are observed at 2.22, 1.93, and 1.70 ppm all having an intensity of 2 and assigned to the cyclobutane ring. Because three signals appear, one would expect that C_{42} and C_{44} are inequivalent. However, on the other hand, the two azpy ligands are identical from NMR spectra. This might suggest some dynamic motion of the six-membered ring part of the cbdca ligand, which gives the C_2 axis of the compound. To solve this contradiction, a comparison with the NMR data of carboplatin is drawn. The NMR spectrum of carboplatin shows only two signals for the cyclobutane ring (a triplet and quartet), and it has been stated that the simplicity of the spectrum of carboplatin may arise from a rapid inversion at the oxygen atoms.[20,24] The ^{13}C{^{1}H} NMR spectrum of an analogue of carboplatin, i.e., [Pt(en)(cbdca-*O*,*O*′)], shows only three resonances for the four-membered ring of cbdca, so there is also rapid ring inversion in solution.[19] Ring flexibility seems to be confirmed by crystal structure studies of other malonato metal complexes. Both boat and chair conformations have been observed.[21] Moreover, because the cyclobutane ring is planar in carboplatin, protons are similar on either side of the cyclobutane ring.[20,24] Combined with the X-ray data of **1** (vide supra), it seems likely that rapid (on the NMR time scale) chelate ring flipping of the six-membered ring occurs in solution to account for the C_2 symmetry, which makes the azpy ligands equivalent. This flipping in a sense would also make the carbon atoms C_{42} and C_{44} equivalent. However, as mentioned before, three signals appear for the cbdca part of the compound. Combined with the complicated multiplet pattern, this may indicate the inequivalence of H_{42A} and H_{42B} (and also H_{44A}/H_{44B} and H_{43A}/H_{43B}). The three facts, i.e., the azpy ligands being equivalent, the assumed rapid ring motion, and the inequivalence of the hydrogens on either side of the cyclobutane ring, would result in one multiplet signal assignable as H_{44B}/H_{42B}, the other as H_{44A}/H_{42A}, and the final mulitplet at 1.70 ppm as H_{43A}/H_{43B} (which appears most upfield corresponding to hydrogens that are the most far away from the ruthenium metal ion). The exact assignment of the cyclobutane signals has not been attempted.

Stability of Complexes 1, 2, and 3 in Aqueous Solution. The stability of complexes **1**, **2**, and **3** in solution has been followed in time in D_2O by ^{1}H NMR spectroscopy. The aromatic resonances of the compounds **2** and **3** were not found to change over 4 weeks at room temperature, and no new signals appeared, indicating that these compounds are inert to hydrolysis in D_2O under these conditions. Compound **1** is less inert to hydrolysis in comparison to **2** and **3**. Following the resonances of **1** in D_2O in time shows the appearance of one new set of aromatic signals, probably from α-[Ru(azpy)$_2$(D$_2$O)$_2$]$^{2+}$ and free cbdca^{2-}, and after approximately 8 days the ratio is 1:1 between intact **1** and the new signals in the aromatic region. The ratio of **1** relative to the new aromatic signals and the occurring

free cbdca remained unchanged for the rest of the observation period. This reactivity deviates from carboplatin in water, where no hydrolysis has been observed ($k < 5 \times 10^{-9}$ s^{-1}).[23−25]

To investigate whether the complexes are stable under physiological conditions (pH 7.4 and 0.05 M phosphate, 0.1 M NaCl), samples of **1**, **2**, and **3** (1 mg/mL) have been followed by ^{1}H NMR spectroscopy for 1 month at room temperature. The spectra of **2** and **3** did not show any changes, proving that the compounds also remain intact for 1 month under physiological conditions. Compound **1**, however, appeared to be less stable under physiological conditions, and hydrolysis of the cbdca ligand does occur. After 5 days, two hydrolysis species are visible, but unfortunately, it was not possible to assign unambiguously the appearing resonances because of overlapping signals. Likely candidates are α-[Ru(azpy)$_2$(cbdca-O)(X)] (with X = D_2O, Cl, or HPO_4) and α-[Ru(azpy)$_2$(X)$_2$]$^{2+}$ (with X = D_2O or HPO_4). The approximate rate constant for the decrease in the concentration of **1** was determined to be $k = 1.3 \times 10^{-6}$ s^{-1} (assuming first-order decay). The rate of hydrolysis of **1** under physiological conditions is comparable to the observed hydrolysis rates of carboplatin. Different hydrolysis studies of carboplatin have been performed, and apparently, added nucleophiles or acids offer some assistance to the lability of the coordinated cbdca. For example, in phosphate buffer (310 K, 1 mM carboplatin, 0.1 M Na_2HPO_4), a rate constant of 7.2×10^{-7} s^{-1} has been reported.[25] A rate constant of 1.2×10^{-6} s^{-1} has been observed for an aqueous solution containing phosphate and chloride (20 mM carboplatin, 140 mM Cl, 0.1 M Na_2HPO_4).[23] In correlation to the cytotoxicity tests, the stability of the carboxylato complexes was also tested by dissolving compounds **1**, **2**, and **3** in DMSO-d_6 to which D_2O was added in the same ratio as that used in the cytotoxity tests. The NMR data show that DMSO does not coordinate to the ruthenium azpy moiety.

Cytotoxicity Tests. The IC_{50} values represent the concentration of a drug that is required for 50% reduction of cellular growth. Because α-Cl and β-Cl are poorly water-soluble, a DMSO stock solution was used for all compounds to perform a proper comparison between the compounds. NMR data, mass spectrometry, and conductivity measurements of α-Cl and α-NO_3 in aqueous solutions (0.1 mg/mL and 1% DMSO) show that DMSO does not coordinate, just like the carboxylate complexes (vide supra).[26] In the case of α-Cl, the chloride ligands remain coordinated, whereas upon dissolution of α-NO_3, the nitrate ligands are replaced by water ligands. The IC_{50} values of α-Cl and β-Cl, α-NO_3 and β-NO_3, **1**−**3**, carboplatin, and cisplatin are listed in Table 1. The order of cytotoxicities in A2780 is α-Cl > β-Cl > **2** ∼ **1** ∼ **3** ∼ α-NO_3 > β-NO_3. The cytotoxic activity of the complexes α-NO_3 (and to a lesser extent β-NO_3) and the complexes **1**, **2**, and **3**, which all show the improved water solubility, is very interesting because they show an appreciable cytotoxicity of only a factor 10 less than the extremely cytotoxic but water-insoluble parent complex α-Cl. Remarkable is the influence of the cis-coordinated ligands (i.e., Cl, NO_3, ox, mal, and cbdca) on the cytotoxicity and solubility. The cytotoxicity data of α-NO_3 , **1**, **2**, and **3** compared to the parent compound α-Cl show that changing the Cl ligands with the other ligands results in a decrease of activity but a parallel increase in water solubility.

Table 1. IC_{50} Values in A2780 and A2780cisR Cell Lines of α- and β-[Ru(azpy)$_2$Cl$_2$], α- and β-[Ru(azpy)$_2$(NO$_3$)$_2$], α-[Ru(azpy)$_2$(cbdca-*O*,*O*′)], **1**, α-[Ru(azpy)$_2$(ox)], **2**, and α-[Ru(azpy)$_2$(mal)], **3**, Cisplatin, and Carboplatin

	IC_{50} in A2780 (μM)	IC_{50} in A2780cisR (μM)
α-[Ru(azpy)$_2$Cl$_2$]	0.85	0.98
α-[Ru(azpy)$_2$(NO$_3$)$_2$]	8.5	6.3
β-[Ru(azpy)$_2$Cl$_2$]·CHCl$_3$	1.6	7.3
β-[Ru(azpy)$_2$(NO$_3$)$_2$]·CHCl$_3$	9.7	12.1
α-[Ru(azpy)$_2$(ox)], **2**	6.3	6.3
α-[Ru(azpy)$_2$(mal)], **3**	7.9	6.2
α-[Ru(azpy)$_2$(cbdca-*O*,*O*′)], **1**	7.2	4.9
cisplatin	2.3	7.8
carboplatin	8.2	41.6

For more insight into the structure−activity relationships for the azpy compounds in this study, the other cis isomer, i.e., the β isomer, has been taken into account also. Regarding the cytotoxicity data of the β-Cl and β-NO_3 complexes, it is concluded that the β isomer shows lower activity in A2780 and A2780cisR than the analogous α isomeric complexes but still shows considerable activity. Also, of these β isomeric compounds, the β-NO_3 shows less activity than the β-Cl complex but increased water solubility.

Most interestingly, for compounds **1**−**3** and α-NO_3, the IC_{50} values for the resistant cell line are slightly lower than or the same as for the normal cell line, in contrast to the platinum compounds. In A2780cisR, the multifactorial resistance mechanisms such as decreased uptake, increased levels of GSH (γ-glutamate-cysteine-glycine), and increased DNA repair cause a smaller activity of the platinum compounds compared to that for the normal cell line.

It is known that ruthenium complexes can bind to DNA,[3] and in particular, the bis(2-phenylazopyridine)-ruthenium(II) complexes have also been studied for their binding to DNA model bases.[13,14,27,28] In general, the binding mode of ruthenium complexes is thought to be different from that of cisplatin.[3] Therefore, it is proposed that the here-reported series of ruthenium complexes might be able to avoid DNA repair mechanisms that are present in cisplatin-resistant cells or be unaffected by the higher levels of GSH.

Comparison of the cytotoxicity of the whole series of azpy compounds with cisplatin and carboplatin shows that α-Cl is the only compound far more active than cisplatin. The other compounds show an activity slightly below that of cisplatin, except for β-Cl, which is as active as cisplatin. All compounds show a higher activity than carboplatin and even β-NO_3 shows comparable activity, confirming the large potential of this series of compounds. Compound **1** shows a lower activity than the parent compound α-Cl in both cell lines. Nevertheless, compound **1** shows an activity in the A2780 cell line similar to that of carboplatin, and **1** is even far more active in the resistant cell line than carboplatin, showing an IC_{50} value of 4.9 μM compared to the value of 41.6 μM for carboplatin. This confirms once more that the activity of **1** is not influenced by the resistance mechanisms in the A2780CisR cell line (vide supra).

Together with the water solubity and stability, **1** appears to be a compound as interesting as carboplatin itself.

The cytotoxicity data of the here-tested complexes are better than other ruthenium polypyridyl complexes such as the antitumor-active $[Ru^{III}(terpy)Cl_3]$ with an IC_{50} value of 11.0 and 32.5 μM in A2780 and A2780cisR (data not shown), respectively. The IC_{50} values of the azpy compounds are in the same range as the reported[8] cytotoxicity in the A2780 cell line of the ruthenium(II) arene complexes. It is also interesting to see the huge difference in activity between the bis(2-phenylazopyridine)ruthenium complexes and a related noncytotoxic[5] complex, *cis*-$[Ru(bpy)_2Cl_2]$, which exhibits IC_{50} values exceeding the value of 130 μM in a series of human tumor cell lines.[29] In conclusion, the cytotoxicity data of the water-soluble ruthenium azpy complexes are promising in comparison to several other antitumor-active ruthenium and platinum compounds.

Activity after a Short Exposure Time To Investigate Influence of Uptake. In the cytotoxicity tests described above, the cells are exposed to the compounds for a relatively long time (3 days). This implies that all compounds are eventually able to reach DNA, or other biological targets, and act on this level. To investigate whether the activity of the compounds can be correlated to their ability to enter the cells, a short-exposure experiment has been performed in which the cells have been exposed to the drug for only 1 h in PBS. In this way, it may be possible to determine if the different ligands X in the α-$[Ru(azpy)_2X]$ complexes influence the ability of the compounds to enter the cells. In this experiment, only the α isomeric complexes have been investigated.

The concentrations used, i.e., 500, 50, and 5 μM, are higher than their IC_{50} values but are justified in this experiment by the short exposure time of only 1 h. Figure 6 shows the reduction of cell proliferation evaluated by the MTT test at 24 h after treatment at 50 and 5 μM. At the highest concentration, i.e., 500 μM (data not shown), all compounds show a statistically significant reduction of cell proliferation in normal and resistant cell lines when compared to the control group ((***) $p < 0.001$, Student−Newman−Keuls test). The compounds α-Cl and α-NO_3 maintain their significant activity even at 50 and 5 μM in the resistant (data not shown) and normal cell lines. At 50 μM, it is interesting to see that cisplatin and compound **3** are not active while **1**, **2**, α-Cl, and α-NO_3 still show a considerable reduction of cell proliferation in the A2780 cell line (Figure 6). With a decrease in the concentration to as low as 5 μM, the ruthenium carboxylato compounds and cisplatin are inactive while α-Cl and α-NO_3 still remain very active in both A2780 and A2780cisR (Figure 6). The activity shown in these graphs might thus be a combination of fast cell entering and rapid interaction with biological targets, probably DNA, leading to cell death. Preliminary atomic absorption data showing the amount of ruthenium inside the cells after 1 h of drug challenge show indeed that the activity in these kinds of short exposure experiments is correlated to the amount of ruthenium inside the cell.[30] The further mechanisms underlying the different activity of the α-Cl and α-NO_3 compounds compared to **1**, **2**, and **3** under these conditions are thus a subject of further research.[31]

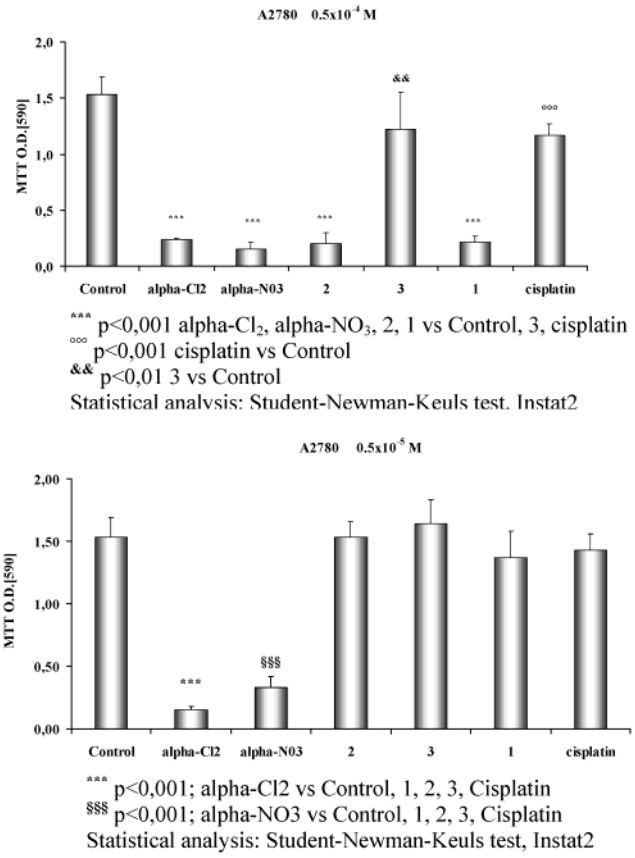

Figure 6. Reduction of proliferation in the A2780 cell line after the 1 h of PBS treatment at 50 μM (top) and 5 μM (bottom).

Concluding Remarks

The synthesis of the carboxylatobis(2-phenylazopyridine)ruthenium(II) complexes has resulted in 1,1-cyclobutanedicarboxylatobis(2-phenylazopyridine)ruthenium(II), α-$[Ru(azpy)_2(cbdca\text{-}O,O')]$ (**1**), oxalatobis(2-phenylazopyridine)ruthenium(II), α-$[Ru(azpy)_2(ox)]$ (**2**), and malonatobis(2-phenylazopyridine)ruthenium(II), α-$[Ru(azpy)_2(mal)]$ (**3**). The X-ray structures of **1** and **2** have unambiguously proven the chelating coordination of the carboxylato ligands. Its molecular structure shows **1** to be the first ruthenium compound in which the 1,1-cyclobutanedicarboxylato ligand is coordinated to ruthenium. Just like carboplatin versus cisplatin in **1,** the cis chloride ligands in the parent compound α-$[Ru(azpy)_2Cl_2]$ have been replaced by the carboxylato group to generate a water-soluble compound, which might be equally biologically interesting as carboplatin.

The here-reported complexes are water-soluble, but **2** and **3** are also inert to hydrolysis under physiological conditions. Compound **1** shows slow hydrolysis in both D_2O and phosphate buffer. The water-soluble α-NO_3 and carboxylato complexes **1**, **2**, and **3** display a cytotoxicity of a factor 10 less than the insoluble parent complex α-Cl in the A2780 and A2780cisR cell lines. Regarding the β isomeric complexes, β-Cl and β-NO_3 also show considerable activity in the same order as the other azpy complexes in this series, although β-Cl and β-NO_3 are less active than their α counterparts (i.e., α-Cl and

α-NO_3). In comparison to cisplatin and carboplatin, α-Cl is far more active than cisplatin. The water-soluble compounds display a slightly lower activity than cisplatin but a higher or comparable activity relative to carboplatin. In particular, it is interesting to focus on compound **1**, which is less active than the parent compound α-Cl (note also that carboplatin is less active than cisplatin). But interestingly, **1** displays in the A2780 cell line an activity comparable to that of carboplatin, and in the resistant cell line, **1** is even 10-fold more active than carboplatin. The cytotoxicity data of **1**, together with the water solubility and stability (only showing relatively slow hydrolysis), show that **1** might be interesting for further in vitro and in vivo studies.

The cytotoxicity data of this series of compounds indicate that structural differences such as isomeric structures (α and *β* backbones) and variation of the counterions influence the activity. However, the reason behind all factors influencing the activity of these compounds is yet far from understood, and more in vitro and in vivo studies are needed in the search for stucture−activity relationships for these kind of complexes.

Experimental Section

Instrumental Methods. ^{1}H NMR spectra were obtained at 300.13 MHz on a Bruker 300 DPX spectrometer and at 600.13 MHz on a Bruker 600 DMX where indicated. Spectra were recorded in $CDCl_3$ and D_2O and calibrated on the residual solvent peaks. All spectra were obtained at 25 °C. Elemental analyses were performed with the use of a Perkin-Elmer analyzer.

Cell Lines. A2780 (human ovarian carcinoma) and A2780 cisplatin-resistant cell lines were maintained in continuous logarithmic culture in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Invitrogen Corporation, The Netherlands) supplemented with 10% fetal calf serum (Perbio Science, Belgium), penicillin G sodium (100 units/mL, Duchefa Biochemie BV, The Netherlands), streptomycin (100 *μ*g/mL, Duchefa Biochemie BV, The Netherlands), and Glutammax 100x (Gibco BRL, The Netherlands). The cells were harvested from confluent monolayers.

Cytotoxicity Assay. For the cytotoxicity evaluation, 2000 cells/well were seeded in 100 *μ*L of complete medium in 96-multiwell flat-bottom microtiter plates (Corning Costar). The plates were incubated at 37 °C in 5% CO_2 for 48 h prior to drug testing to allow cell adhesion.

The stock solutions (1 mg/mL DMSO) of all tested compounds were freshly prepared and directly used for the dillutions. Because α-Cl and *β*-Cl are poorly water-soluble but are needed in the comparison with the water-soluble compounds, a DMSO solution was chosen. The dilutions (six-step dilutions except for the α-Cl for which two more dilutions were used) were prepared in complete medium, and the final tested concentrations were 0.01, 0.005, 0.001, 0.0005, and 0.0001 mg/mL (and in the case of α-Cl, the concentrations were also 0.00005 and 0.00001). Each concentration was tested in quadruplicate using 100 *μ*L/well added to the 100 *μ*L of complete medium. In the control group, only 100 *μ*L of complete medium containing 1% of DMSO was added. The range of concentrations used ended up with a concentration from 1% to 0.01% DMSO (α-Cl up to 0.001%). Parallel experiments showed that no difference in cell proliferation was observed in control groups with or without 1% DMSO. The plates were incubated for 72 h, and the evaluation of cell proliferation was performed by the MTT colorimetric assay.[32] A 50 *μ*L MTT solution (5 mg/mL in PBS, Sigma Chemical Co.) was added to each well and incubated for 1 h. Formazan crystals were solubilized with 100 *μ*L of DMSO. Optical density was measured by microplate reader (Bio Rad) at 590 nm. IC_{50} values were obtained by GraphPad Prism software, version 3.05, 2000. Statistical analysis was done with the Student−Newman−Keuls test, Instat2.

Short Exposure Time Experiment by 1 h of PBS Treatment. For the 1 h of incubation in PBS, 10 000 cells/well were seeded in 100 *μ*L of complete medium in 96-multiwell flat-bottom microtiter plates (Corning Costar). The plates were preincubated for 48 h to allow cell adhesion. The tested concentrations were 500, 50, and 5 *μ*M.

The stock solution of all compounds (50 mM in DMSO) was freshly prepared. The first concentration (i.e., 500 *μ*M) was obtained by a 1:100 dilution in PBS from the stock solution. Other concentrations were obtained by a sequence of two 1:10 dilutions in PBS of the 500 *μ*M solution.

Before treatment, the complete medium was removed and the cells were washed with 100 *μ*L of PBS. For treatment, 100 *μ*L of the desired concentration was added to the wells, each concentration in eight wells. The control group consisted of PBS added with 1% of DMSO (as for the 500 *μ*M solution). The plates were incubated for 1 h at 37 °C under 5% CO_2. At the end of the incubation time, the cells were washed twice with PBS and o grew for an additional 24 h in 100 *μ*L of complete medium. The MTT test was performed at 24 h after treatment (see above). Statistical analysis was done with the Student−Newman−Keuls test, Instat2.

Crystal Structure Determination and Refinement of 1 and 2. Diffraction data were collected with a Nonius Kappa CCD diffractometer on a rotating anode (Mo Kα radiation, λ = 0.710 73 Å). Structures were solved with direct methods[33] and refined[34] on F2. Non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms were included in calculated positions in riding mode. Final data for **1**: $C_{28}H_{24}N_6O_4Ru$, M_r = 609.60, black, needle-shaped crystal (0.02 mm × 0.03 mm × 0.20 mm), monoclinic, space group $P2_1/c$ (No. 14) with a = 9.6537(10) Å, b = 18.397(2) Å, c = 16.492(2) Å, β = 119.875(9)°, V = 2539.7(5) Å^3, Z = 4, D_c = 1.594 g cm^{-3}, 49 729 reflections measured, 5830 independent, 1.6° < θ < 27.5°, T = 150 K, 352 parameters, wR2 = 0.0725, R1 = 0.0365 (for 5830 reflections with $I > 2\sigma(I)$), S = 1.003, −0.48 < $\Delta\rho$ < 0.79. Final data for **2**: $C_{24}H_{18}N_6O_4Ru$·solvent, M_r = 555.51, purple, needle-shaped crystal (0.05 mm × 0.05 mm × 0.35 mm), monoclinic, space group $C2/c$ (No. 15) with a = 24.073(3) Å, b = 17.195(2) Å, c = 16.479(3) Å, β = 129.536-(12)°, V = 5260.7(12) Å^3, Z = 8, D_c = 1.403 g cm^{-3}, 43 692 reflections measured, 4770 independent, 1.6° < θ < 25.3°, T = 250 K, 317 parameters, wR2 = 0.1396, R1 = 0.0675 (for 2904 reflections with $I > 2\sigma(I)$), S = 1.041, −0.68 < $\Delta\rho$ < 0.56. The unit cells contains four symmetry-related voids of 240 Å^3 each, filled with disordered solvent (probably diethyl ether, 45.5*e* per void) and incorporated in the final model via the squeeze procedure.[35]

A CIF file for compounds **1** and **2** is available from the authors.

Syntheses. Oxalic acid dihydrate (Merck) ($H_2ox(H_2O)_2$), malonic acid (Merck-Schuchardt) (H_2mal), and 1,1-cyclobutanedicarboxylic acid (Janssen Chimica) (H_2cbdca) were obtained commercially and used without purification. 2-(Phenylazo)pyridine[36] and the complexes[37,38] α-[$Ru(azpy)_2Cl_2$] and *β*-[$Ru(azpy)_2Cl_2$]·$CHCl_3$ were synthesized according to published methods. The compounds α-[$Ru(azpy)_2(NO_3)_2$] and *β*-[$Ru(azpy)_2(NO_3)_2$]·$CHCl_3$ were prepared as described before.[13,14]

α-[Ru(azpy)$_2$(cbdca-*O,O′*)] (1). 1,1-Cyclobutanedicarboxylic acid (0.027 g, 0.19 mmol) was added to a solution of α-[$Ru(azpy)_2(NO_3)_2$] (0.100 g, 0.17 mmol) in 30 mL of acetone. A KOH solution (3.4 mL, 0.1 M) was added, and the mixture was stirred at 40 °C for 4 days. The blue-purple solution was filtered and evaporated to dryness by rotary evaporation. The solid was dissolved in 30 mL of absolute ethanol, and the solution was then filtered. The solution was evaporated and dissolved in 30 mL of acetone. Dropwise addition of ca. 40 mL of diethyl ether resulted, after 24 h, in crystals suitable for X-ray analysis. Yield: 0.040 g (39%). Anal. Calcd for $RuC_{28}H_{24}N_6O_4$: C, 55.2; H, 3.97; N, 13.8. Found: C, 54.8; H,

4.49; N, 13.8. ESI-MS *m/z*: 611 [M + H]. ^{1}H NMR (300 MHz, chloroform-*d*): δ 8.64 (d, 2H), 8.45 (d, 2H), 8.05 (t, 2H), 7.41 (t, 2H), 7.31 (t, 2H), 7.11 (t, 4H), 6.72 (d, 4H), 2,22 (m, 2H), 1.93 (m, 2H), 1.70 (m, 2H).

α-[Ru(azpy)$_2$(ox)] (2). To a solution of α-[Ru(azpy)$_2$(NO$_3$)$_2$] (0.100 g, 0.17 mmol) in 30 mL of acetone was added oxalic acid dihydrate (0.017 g, 0.013 mmol). The procedure used was the same as that for **1**, and recrystallization from absolute ethanol and ether resulted in crystals suitable for X-ray analysis. Yield: 0.080 g (85%). Anal. Calcd for $RuC_{24}H_{18}N_6O_4$: C, 51.9; H, 3.27; N, 15.1. Found: C, 49.1; H, 2.99; N, 14.3. ESI-MS *m/z*: 556 [M + H]. ^{1}H NMR (300 MHz, chloroform-*d*): δ 8.68 (d, 2H), 8.33 (d, 2H), 8.10 (t, 2H), 7.48 (t, 2H), 7.34 (t, 2H), 7.10 (t, 4H), 6.75 (d, 4H).

α-[Ru(azpy)$_2$(mal)] (3). The synthesis was analogous to the synthesis of **1** and **2**. Yield: 0.026 g (54%). Anal. Calcd for $RuC_{25}H_{20}N_6O_4$: C, 52.7; H, 3.45; N, 14.8. Found: C, 51.1; H, 3.38; N, 14.4. ESI-MS *m/z*: 570 [M + H]. ^{1}H NMR (300 MHz, chloroform-*d*): δ 8.68 (d, 2H), 8.47 (d, 2H), 8.10 (t, 2H), δ 7.46 (t, 2H), 7.30 (t, 2H), 7.09 (t, 4H), 6.72 (d, 4H), 3.01 (s, 3H).

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Supporting Information Available: Tables of atomic coordinates, bond lengths and angles, and displacement parameters for complexes **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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