Antitumor Agents

Comparison Between Polypyridyl and Cyclometalated Ruthenium(II) Complexes: Anticancer Activities Against 2D and 3D Cancer Models

Huaiyi Huang, Pingyu Zhang, Hongmin Chen, Liangnian Ji, and Hui Chao[a]

Abstract: The aim of this study was to illustrate the dramatically different anticancer activities between coordinatively saturated polypyridyl (1a–4a) and cyclometalated (1b–4b) ruthenium(II) complexes. The cyclometalated complexes 1b–4b function as DNA transcription inhibitors, exhibiting switch-on cytotoxicity against a 2D cancer cell monolayer, whereas the polypyridyl complexes 1a–4a are relatively inactive. Moreover, complexes 1b–4b exhibit excellent cytotoxicity against 3D multicellular tumor spheroids (MCTSs), which serve as an intermediate model between in vitro 2D cell monolayers and in vivo 3D solid tumors. The hydrophobicity, efficient cell uptake, and nucleus targeting ability, as well as the high DNA binding affinity of complexes 1b–4b, likely contribute to their enhanced anticancer activity. We surmise that cyclometalation could be a universal approach to significantly enhance the anticancer activity of substituted polypyridyl Ru complexes. We also suggest that 3D MCTSs may be a more practical platform for anticancer drug screening than 2D cancer monolayer approaches.

Introduction

Traditional in vitro cancer cell monolayers offer a simple and convenient approach for anticancer drug development. However, they also have significant limitations in reproducing the complexity and pathophysiology of in vivo solid tumors.[1–4] Moreover, the discovery of multidrug resistance (MDR) in solid tumors has highlighted the differences between 2D and 3D cell cultures.[5] Multicellular tumor spheroids (MCTSs) are heterogeneous cellular aggregates that have been gradually accepted as a valid 3D cancer model to mimic the features of in vivo solid tumors. MCTSs more accurately reflect the tumor microenvironment in terms of cellular heterogeneity and they provide insight into metabolic properties similar to solid tumor profiles, such as nutrient and oxygen gradients, hypoxic/neocrotic regions, cell-cell matrix interactions, and gene expression.[6–10] MCTSs bridge the gap between the oversimplified structure of the 2D cell monolayer and the highly complex nature of solid tumors. In this respect, MCTSs optimize anticancer drug screening and increase the accuracy of predicting in vivo anticancer activity with a reduced need for expensive animal studies.

One widely used clinical chemotherapy drug is cisplatin, which forms a Pt-DNA adduct and ultimately inhibits cellular DNA transcription and replication.[11] However, it also suffers from unprepossessing side effects such as drug resistance and severe toxicity such as nephrotoxicity and neurotoxicity.[12] During the last 20 years, ruthenium complexes have emerged as promising alternatives to platinum-based drugs for chemotherapy-based treatment.[13] So far, three ruthenium complexes: [ImH][Ru(Im)(Me2SO)Cl4] (NAMI-A; Im = imidazole), [IndH]+[Ru- (Ind)2Cl4] (KP1019; Ind = indazole), and [Ru(Im)3Cl] (ICR; Im = imidazole) have gone through Phase I clinical trials for treating metastatic cancer.[14] However, the novel Ru-based therapies are also associated with some limitations, such as poor water solubility and unimpressive cytotoxicity.[15]

In recent years, cyclometalated Ru complexes, such as Ru-diamine[16–19] and RAPTA,[20–22] have emerged as promising candidates as antitumor or antibiotic drugs. Pfeiffer’s group reported a variety of ruthenium-derived compounds (RDCs), some of which exhibited cytostatic and cytotoxic effects on cancer cells.[23–26] Turro’s group showed that coordinatively saturated Ru dyes displayed high cytotoxicity towards cancer cells.[27] While most of the above drug screenings were performed against 2D cell monolayers, to the best of our knowledge only one application of MCTSs in ruthenium-based anticancer drug development has been reported.[28] Our group has focused on the use of transition metal complexes as anticancer drugs or luminescent biological probes.[29,30] The aim of this work is to investigate structure–activity relationships (SARs) of polypyridyl and cyclometalated Ru complexes against both 2D cell monolayer and 3D MCTS cancer models. We merely replaced an N,N-chelating ligand bipy (2,2'-bipyridine) in polypyridyl complexes 1a–4a by a C,N-chelating ligand pp (2-phenylpyridine) to generate cyclometalated complexes 1b–4b (Scheme 1). Complexes 1b–4b were found to exhibit dramatic
Polypyridyl complexes 1a–4a and the cyclometalated RuII complexes 1b–4b.

Results and Discussion

Synthesis and characterization

Polypyridyl complexes 1a–4a[31–33] and the cyclometalated complex 4b[34] have been synthesized previously. Complexes 1b–3b were synthesized by reacting [Ru(ppy)(bpy)(NCCH$_3$)$_2$]PF$_6$[35] with an equimolar amount of the requisite aromatic ligand in refluxing ethanol. All of these complexes were purified by column chromatography and characterized by ES-MS, 1H NMR, and elemental analysis (Figures S1–S6). Important ly, the coordinatively saturated cyclometalated RuII complexes were completely stable in aqueous solution containing a small amount of DMSO (1%, v/v) over extended periods, as monitored by UV/Vis spectrophotometry (Figure S7). These two series of RuII complexes exhibited different photophysical properties. Significantly, cyclometallation significantly lowered the energy of the triplet metal-to-ligand charge-transfer state (3MLCT) state[34] (Figure S8). The MLCT absorptions of complexes 1b–4b showed significant bathochromic shifts relative to those of complexes 1a–4a. Unlike the polypyridyl complexes 1a–4a, which possess long-lived excited states, cyclometalated complexes 1b–4b exhibit weakly emissive 3MLCT states and the excited states decay rapidly (Figure S9 and Table S1). As a result, 1b–4b show no emission in aqueous solution. Moreover, complexes 1b–4b show much less positive RuIII/RuII potentials relative to complexes 1a–4a (Table S2). This significant cathodic shift may be associated with a decreased positive charge on the Ru center following coordination by the α-donor carbamion ppy ligand as compared to coordination by the neutral bpy ligand.

Octanol/water partition coefficients

Octanol/water partition coefficients (log $P_{o/w}$) provide a measure of drug lipophilicity, which indicates the ability of the molecule to pass through cell membranes.[26, 36] Apparently, cyclometalated RuII complexes 1b–4b are more hydrophobic than polypyridyl RuII complexes 1a–4a. After mixing with octanol and water, complexes 1a–4a were distributed mostly in the water phase, whereas complexes 1b–4b were distributed mostly in the octanol phase (Figure 1). Complexes 1a–4a gave negative log $P_{o/w}$ values, showing them to be hydrophilic in nature. In contrast, complexes 1b–4b proved to be highly hydrophobic, showing positive log $P_{o/w}$ values. The greater lipophilicity of complexes 1b–4b may facilitate their cell uptake and enhance their activities against cancer cells.

DNA binding

Because complexes 1a–4a have been shown to be efficient DNA intercalators,[31–33] the interaction between DNA and the cyclometalated RuII complexes was investigated. Complexes 2a and 4a were found to significantly impede the migration of a 5000 kb DNA marker (Figure 2). Cyclometalated RuII complexes were less efficient than polypyridyl RuII complexes in the gel retardation assay, which may have been due to the lower positive charge of these complexes. Significant hypochromic effects and bathochromic shifts were observed upon adding calf thymus DNA (CT-DNA) to solutions of complexes.


1b-4b, illustrating an intercalative DNA binding mode (Figure S10 and Table S3). Since 1b-4b were non-emissive under the experimental conditions, fluorescence-based competitive binding experiments with ethidium bromide (EB) and Hoechst 33258 were carried out to assess the interactions between these complexes and DNA. EB is a planar cationic dye that can intercalate into the DNA double helix. EB is weakly fluorescent in aqueous solution, but the EB-DNA adduct emits strong red fluorescence. Displacement of EB from its binding site by a non-emissive competitor leads to a significant decrease in the fluorescence intensity. As can be seen in Figure S11, there was a decrease in the fluorescence intensity when increasing amounts of 1b-4b were added to an EB-DNA mixture, showing that these complexes can compete with EB for DNA binding sites. It is well known that Hoechst 33258 binds to the DNA minor groove in low dye-to-DNA ratios, leading to a significant increase in its fluorescence intensity. When 1b-4b were added to Hoechst 33258/CT-DNA solution, decreases in the fluorescence were observed (Figure S12). This suggests that 1b-4b could interact with DNA in a non-selective intercalative mode. This intercalative binding mode of complexes 1b-4b could change the three-dimensional structure of duplex DNA, which may impede DNA replication or transcription processes.

**Cytotoxicity against 2D cell monolayer cancer models**

To evaluate the cytotoxicity of polypyridyl complexes 1a-4a and cyclometalated complexes 1b-4b against cancer cell lines, we used the fast and reproducible CellTiter-Glo Luminescent Cell Viability kit (Promega) with cisplatin as the positive control. Against 2D cancer cell monolayers, polypyridyl RuII complexes 1a-4a exhibited unimpressive therapeutic profiles and unclear structure–activity relationships (Figure 3a and Table S4). The IC50 values of complexes 1a, 2a, and 3a were typically above 100 μM, even when the incubation time was extended to 72 h. Complex 2a was only slightly more active than complex 1a. Complex 4a, the most lipophilic of the polypyridyl complexes investigated (Figure 1), exhibited relatively higher anticancer activity than complexes 1a-3a. However, compared with those of cisplatin, the cytotoxicities of complexes 1a-4a were not impressive. In contrast, the cyclometalated complexes 1b–4b exhibited significantly enhanced cytotoxicities and were more potent than cisplatin against all of the cancer cell lines screened. After 24 h incubation with HeLa cells, the IC50 values of complexes 1b–4b were less than 10 μM, whereas that of cisplatin was approximately 30 μM (Table S4). On extending the incubation time to 72 h, the IC50 values of complexes 1b–4b decreased toward 1.0 μM and that of cisplatin was approximately 10 μM. Notably, compared to cisplatin, complexes 1b–4b were also highly active against the cisplatin-resistant cancer cell line A549R. Most importantly, all of the cyclometalated RuII complexes exhibited modest cytotoxicity towards normal cells, whereas cisplatin exhibited comparable cytotoxicities towards cancer cells and normal cells. These results demonstrated that the complexes 1b–4b exhibited higher cytotoxicity towards cancer cells but lower toxicity towards normal cells compared to cisplatin, indicating improved selectivity for cancer therapy. Small changes in the structure of the RuII complexes led to a dramatic difference in cytotoxicity, which encouraged us to further study the mode of action of complexes 1b–4b.

**Stability in plasma**

Anticancer compounds must remain stable in plasma to ensure effective transport to cancer cells to perform their pharmacological function.[37] To further investigate the anticancer behavior of these cyclometalated complexes, the stabilities of 1b–4b in plasma were assessed. As is clearly evident from the LC-UV traces (Figure S13), complexes 1b–4b showed no discernible decomposition in plasma, even after three days, suggesting that they should be stable under physiological conditions. This result also indicates that intact complexes 1b–4b were responsible for the anticancer activity.

**Cellular uptake of RuII complexes**

Based on the results of the cytotoxicity assay, it is clear that the cyclometalated complexes 1b–4b exhibit superior anticancer activity compared to the polypyridyl complexes 1a–4a. Because the biological activity of a compound is highly correlated with its ability to enter cells, we used flow cytometry and inductively coupled plasma–mass spectrometry (ICP-MS) to analyze the cell uptake ability of these two classes of RuII complexes. Flow cytometry has been recommended to examine the cellular uptake efficiencies of luminescent transition metal complexes.[36] Since 1a–4a emit strong luminescence in aqueous solution, flow cytometry could be used to detect their cell uptake abilities. As shown in Figure 3b, this technique revealed that complexes 1a and 3a had no ability to penetrate HeLa cells because the signal did not change relative to the control sample. However, a small amount of complex 2a did enter the HeLa cells, and complex 4a seemed to enter the cells more easily, which may have been due to its relatively higher hydrophobicity compared to complexes 1a–3a. This assay could not be used to assess the cell uptake abilities of non-fluorescent complexes.
Instead, we used ICP-MS to compare the uptakes of these two series of Ru(II) complexes. ICP-MS can provide quantitative data on the cell uptake efficiencies of both fluorescent and non-fluorescent metal-based complexes. Complex 4a showed a time-dependent cell uptake ability, whereas the amount of ruthenium in the cells derived from complexes 1a–4a (5 μM) remained quite low, which is consistent with the results of the flow cytometry assay (Figure 3c and Table S5). Notably, the cyclometalated complexes 1b–4b showed higher cellular uptake efficiency than the poly(pyridyl) complexes 1a–4a. Indeed, the cellular Ru concentrations of cyclometalated complexes 1b–4b after 1 h of incubation were higher than that of complex 4a after 6 h of incubation. The concentrations of complexes 1b–4b in the cells increased with increasing incubation time. We further analyzed the distributions of complexes 1b–4b within the HeLa cells (Figure 3d and Table S6). The majority of the ruthenium was found in the nucleus, whereas the amounts in the cytoplasm, cytomembrane, and cytoskeleton were low. The cell nucleus contains most of the cellular genetic material, such as DNA. Because complexes 1b–4b have the ability to intercalate between DNA bases, their accumulation in the nucleus may lead to inhibition of cellular DNA transcription and replication. Moreover, the significant difference in cell uptake abilities between the cyclometalated complexes 1b–4b and the poly(pyridyl) complexes 1a–4a may, in part, account for the much higher anticancer activity of the former compared to that of the latter.

Figure 3. a) IC_{50} values of cisplatin and complexes 1a–4a and 1b–4b after incubation with different cancer cell lines and the normal cell line LO-2 for 48 h. b) Cell uptake properties of complexes 1a–4a (5 μM) as detected by flow cytometry. c) Amounts of ruthenium within HeLa cells after incubating with complexes 1a–4a (5 μM) and 1b–4b (5 μM) for 1, 3, and 6 h. d) Percentage distributions of ruthenium in cell membrane, cytoskeleton, cytosol, and nucleus after incubating with complexes 1a–4a (5 μM) and 1b–4b (5 μM) for 6 h.
Antiproliferation and apoptosis assay

An antiproliferation assay was carried out to confirm the inhibition of cellular DNA transcription. The traditional approach for monitoring DNA replication and cell proliferation relies on the incorporation of BrdU (5-bromo-2'-deoxyuridine) into nascent DNA by cellular DNA polymerases. However, this method requires harsh cell permeabilization conditions and nucleic acid denaturation procedures. In recent years, the use of EdU (5-ethynyl-2'-deoxyuridine) has been proposed as a simpler and quicker protocol compared to that with BrdU. EdU is a thymidine analogue that can be metabolized by mammalian cells and incorporated into replicating DNA. Thus, EdU serves as a sensitive fluorescence marker of DNA synthesis or rapidly proliferating cells.\textsuperscript{40–42} As shown in Figure 4a, 15 μM of cisplatin only slightly delayed DNA synthesis in HeLa cells, but significant suppression of DNA replication occurred in the presence of 30 μM of cisplatin under the same conditions. With the cyclometalated complexes 1b–4b, 2 μM of each was seen to significantly hinder HeLa cell proliferation. These data, together with the results of the cell uptake assays, lead us to conclude that the binding of complexes 1b–4b with DNA within cancer cells results in the inhibition of cellular DNA transcription.

Next, we assessed the cellular response after treatment with the Ru\textsuperscript{II} complexes using the acridine orange/ethidium bromide (AO/EB) dual-staining assay (Figure 4b). AO is a vital dye and can stain both live and dead cells. EB stains only cells that have lost their membrane integrity. Under a fluorescence microscope, live cells appear green. Necrotic cells are stained red but have a nuclear morphology resembling that of viable cells. Apoptotic cells appear green, and morphological changes such as cell blebbing and formation of apoptotic bodies can be observed. Living cells are uniformly stained green, early apoptotic cells are densely stained greenish-yellow, late apoptotic cells are densely stained orange, and necrotic cells are stained orange-red.\textsuperscript{40} After a 48 h treatment, the complexes 1a–3a (10 μM) did not induce cell apoptosis because the cells were only stained by AO (green), whereas a small amount of cells showed apoptosis after incubation with complex 4a (10 μM). In contrast, cells treated with the complexes 1b–4b (1 μM) became round in shape and the number of cells stained by EB (red) increased rapidly, indicating that the integrity of the cell membrane had been compromised. Intense orange fluorescence confirmed that cell apoptosis had occurred after treatment with the cyclometalated complexes 1b–4b.

Generation and analysis of MCTSs

According to literature data, some drugs may only be effective against 2D cancer cell monolayers and not against 3D solid tumors, which may reflect reduced drug penetration or pathophysiological differences in response to cellular hypoxia or relatively slower cell cycling in the solid tumor. In recent years, 3D multicellular tumor spheroid (MCTS) cell culture systems have received increasing interest because they can restore the in vivo-like extracellular matrix (ECM). Moreover, MCTSs can...
mimic therapeutic problems associated with the metabolic and proliferative gradients found in solid tumors, such as the altered responsiveness of chronically hypoxic tumor cells, as well as multidrug resistance to apoptosis-inducing agents. In this respect, MCTSs serve as a useful tool for negative anticancer drug selection and reduce the need for animal testing. Several methods have been designed to generate tumor spheroids, among which agarose-coated liquid-overlay 96-well plate culture provides an easy-to-handle protocol for generating single MCTSs. This method exhibits several desirable characteristics: (i) 96-well suspension culture, (ii) a single spheroid per well, (iii) high reproducibility, and (iv) simple harvesting for further analysis. As shown in Figure 5a, HeLa cells tended to aggregate near the center of the well on day 1. On day 2, a loose cell-cell net formed and gradually coalesced. After a 96 h incubation period, single MCTSs clearly appeared at the center of the well. As the incubation time was extended, the MCTSs became larger and thicker.

Cytotoxicity against 3D MCTSs cancer model

Tumor cells are generally less sensitive to chemotherapeutics in solid tumor models than in cultured cell monolayers. Small MCTSs with diameters up to 200 μm are sufficient to reflect in vivo-like cell-cell and cell-matrix interactions and are frequently used for anticancer drug testing. However, MCTSs with diameters of approximately 400 μm have been suggested to be a better choice than small MCTSs because they better resemble the pathophysiological conditions of solid tumors, such as the specific hypoxic areas in the center and proliferation gradients. On this basis, we decided to test the cytotoxicities of the cyclometalated Ru complexes 1b–4b against MCTSs with diameters of approximately 400 μm. The IC_{50} value of cisplatin against 400 μm MCTSs (60 μM) is almost an order of magnitude higher than that against a cancer cell monolayer (7.2 μM), indicating significant multicellular drug resistance. Under the same conditions, the IC_{50} values of complexes 1b–4b were approximately 2–5 μM, just slightly higher than those against the cancer cell monolayer. In contrast, complexes 1a–4a proved to be inactive against MCTSs (above 100 μM, Figure 5b). These data indicate that cyclometalated Ru complexes 1b–4b exhibit similar anticancer activities towards both cancer cell monolayers and MCTS cultures.

Growth inhibition of MCTSs

To evaluate the effect of cyclometalated complexes 1b–4b on the growth kinetics of 3D MCTSs, MCTSs were pre-treated with Ru compounds for 72 h, and then 50% of the culture medium containing Ru compounds was replaced with fresh culture medium. In this way, loss or disruption of the MCTSs was avoided and a halving of the residual drug concentration was obtained. The concentration of Ru compounds in the culture medium would be further halved every two days during

Figure 5. a) 2D HeLa cancer cell monolayer and generation of 3D multicellular tumor spheroids. b) IC_{50} values of cisplatin and complexes 1a–4a and 1b–4b against HeLa MCTSs after 72 h drug exposures. c) Routine monitoring of growth and regrowth inhibition on drug-treated MCTSs over 14 days. d) Calcein AM and EthD-1 dual-staining on drug-treated (cisplatin, 30 and 60 μM; complexes 1a–4a, 60 μM; complexes 1b–4b, 2 μM) HeLa MCTSs. A: bright field; B: calcein AM channel; C: EthD-1 channel; D: overlay of B and C. Scale bar = 200 μm.
14 days incubation. This assay allowed measurement of dynamic effects of anticancer activity over a period of time, which is a significant advantage compared with the standard endpoint assays used with cell monolayers. Complexes 1b–4b significantly delayed the growth of MCTSs during the first 72 h of drug treatment (Figure 5c). The volumes of the MCTSs were apparently smaller than those of untreated MCTSs, which meant that complexes 1b–4b inhibited the proliferation of cancer cells therein. Cisplatin exhibited significant growth inhibition activity at a concentration of 60 μM, which was much higher than those of the cyclometalated RuII complexes. Complexes 1b–4b also exhibited concentration-dependent inhibition of MCTS growth and regression (Figure S14). As the drug concentration was increased, visible inhibition of MCTS growth occurred, as evidenced by gradually diminishing volume of the MCTSs during a 14 day culture period. We also noted that the growth of MCTSs was not affected upon incubation with polyppyridyl RuII complexes 1a–3a (Figure S15). Only 4a exhibited slight growth delay activity against MCTSs. Thus, we could conclude that cyclometalated RuII complexes 1b–4b effectively inhibited the growth and regrowth of MCTSs, whereas polyppyridyl RuII complexes 1a–4a were ineffective.

Live/dead assay

The live/dead viability/cytotoxicity assay is a two-color fluorescence assay used to identify live and dead cells. The virtually non-fluorescent cell-permeant calcein AM is converted into green fluorescent calcein by intracellular esterases within living cells. In contrast, EthD-1 can only enter dead cells and emits green fluorescent calcein by intracellular esterases within living cells. In contrast, EthD-1 can only enter dead cells and emits green fluorescent calcein by intracellular esterases within living cells. The live/dead assay

Table 1. Cytotoxicities of related polyppyridyl RuII complexes against 2D HeLa cell monolayers.

<table>
<thead>
<tr>
<th>Biological activity</th>
<th>Cellular distribution</th>
<th>IC50 [μM]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Ru(bpy)2(PAIDH)]2+</td>
<td>inhibits DNA transcription</td>
<td>n.d. [51]</td>
<td>490 [52]</td>
</tr>
<tr>
<td>[Ru(bpy)2(bfip)]2+</td>
<td>topoisomerase inhibitor</td>
<td>n.d.</td>
<td>392 [53]</td>
</tr>
<tr>
<td>[Ru(bpyp)(ipadi)]2+</td>
<td>topoisomerase inhibitor</td>
<td>n.d.</td>
<td>26 [54]</td>
</tr>
<tr>
<td>[Ru(bpyp)(PITPHI)]2+</td>
<td>topoisomerase poison</td>
<td>n.d.</td>
<td>70 [55]</td>
</tr>
<tr>
<td>[Ru(bpyp)(PAIDH)]2+</td>
<td>generation of ROS mitochondrial</td>
<td>50 [56]</td>
<td></td>
</tr>
<tr>
<td>[Ru(bpyp)(pscl)]2+</td>
<td>topoisomerase poison</td>
<td>cytoplasm</td>
<td>77 [57]</td>
</tr>
</tbody>
</table>

[a] No data available.

Conclusion

Coordinatively saturated polyppyridyl and cyclometalated RuII complexes have been prepared for in vitro 2D cell monolayer and 3D MCTSs-based anticancer drug development. The cyclometalated RuII complexes 1b–4b were found to exhibit higher lipophilicity than the corresponding polyppyridyl RuII complexes 1a–4a. The cytotoxic effects of complexes 1b–4b were almost an order of magnitude higher than that of cisplatin against cancer cells of different sizes, with IC50 values similar to that against the cell monolayer. Notably, complexes 1b–4b restrained the growth and regrowth of MCTSs and induced cell death therein. We hope that these results will prove of value in further understanding the cellular uptake of RuII complexes and the resulting transcription inhibition, as well as laying a foundation for the discovery of new antitumor agents.
Experimental Section

Materials: All solvents were of analytical grade. All buffer components were of biological grade and were used as received. Cisplatin, acridine orange (AO), and ethidium bromide (EB) were obtained from Sigma-Aldrich. 5-Ethynyl-2′-deoxyuridine was obtained from Life Technologies. CT-DNA was purchased from Sigma. Stock solutions of cisplatin (2 mM) were prepared in sterile saline solution, and solutions of Ru(II) complexes (1 mM) were prepared in DMSO, which proved to be stable for at least 48 h at room temperature, as monitored by UV/Vis spectrophotometry. All stock solutions were stored at −20 °C, thawed, and diluted with culture medium prior to each experiment.

Physical measurements: Microanalysis (for C, H, and N) was carried out using a Vario EL elemental analyzer. 1H NMR spectra were recorded at room temperature on a Bruker AVANCE AV 400 NMR spectrometer using (CD)3SO as solvent and TMS as an internal standard. Electrospray mass spectra (ES-MS) were recorded on an LCQ system (Finnigan MAT, USA). The spray voltage, tube lens offset, capillary voltage, and capillary temperature were set at 4.50 kV, 20.00 V, and 200 °C, respectively, and the quoted m/z values are for the major peaks in the isotope distribution. UV/Vis spectra were recorded on a Perkin-Elmer Lambda 850 spectrophotometer. Emission spectra were recorded on a Perkin-Elmer LS 55 spectrophotofluorometer at room temperature. Cyclic voltammetric measurements were performed on a CHI 660A Electrochemical Workstation at room temperature. The supporting electrolyte was 0.1 M TBAP (tetrabutylammonium perchlorate; Fluka, electrochemical grade). All solvents were of analytical grade. All buffer components were of biological grade and were used as received. Cisplatin (2 mM) was used in place of IP. Yield: 51.2 mg, 65%; elemental analysis calculated (%) for C19H19N2Ru: C 65.21, H 3.91, N 15.21; found: C 65.14, H 3.86, N 15.05; ES-MS: m/z: 693.4 (M+, 100%); 1H NMR (400 MHz, [D6]DMSO): δ = 9.47 (d, J = 6.6 Hz, 1H), 9.30 (d, J = 8.1 Hz, 1H), 9.15 (d, J = 3.3 Hz, 2H), 8.45 (d, J = 5.4 Hz, 1H), 8.40–8.32 (m, 2H), 8.02 (d, J = 7.8 Hz, 1H), 7.91 (d, J = 8.1, 5.1 Hz, 2H), 7.84 (t, J = 8.7 Hz, 1H), 7.78 (t, J = 8.7 Hz, 1H), 7.68 (dd, J = 8.1, 5.4 Hz, 1H), 7.62 (m, 2H), 7.51 (d, J = 5.7 Hz, 1H), 7.32–7.21 (m, 7H), 7.18 (d, J = 7.2 Hz, 1H), 7.07 (t, J = 7.8 Hz, 1H), 6.94 (t, J = 7.8 Hz, 1H), 6.89 (t, J = 7.5 Hz, 1H), 6.77 (J = 7.8 Hz, 1H), 6.53 ppm (d, J = 7.5 Hz, 1H).

Absorption titration experiments: CT-DNA was prepared as a 5 mM stock solution according to the published procedure. Absorption titration experiments on Ru(II) complexes were performed at a fixed complex concentration (10 mM) until the absorbance showed no further change with increasing CT-DNA. The complex DNA solutions were allowed to incubate for 5 min before recording the absorption spectra. The intrinsic binding constants Ks to DNA were determined using Equations (1) and (2), in which [DNA] is the concentration of DNA in terms of base pairs, and the apparent absorption coefficients εm, εp, and εs correspond to Aapp/[Ru], the extinction coefficient for the free ruthenium complex, and the extinction coefficient for the ruthenium complex in the fully bound form, respectively. Ks is the equilibrium binding constant in M−1, C is the total metal complex concentration, and s is the binding size.

\[
(b - b_1 - b_2)/b_2 = 1 + Ks \cdot C/[DNA]/2s
\]

Ethidium bromide displacement experiments: In ethidium bromide (EB) fluorescence displacement experiments, 3 μL aliquots of a solution containing 100 μM CT-DNA and 4 μM EB (saturated binding level) in 50 μM Tris-HCl buffer at pH 7.4 were titrated with concentrated solutions of the respective complexes 1b–4b, thus producing solutions with varied molar ratios of 1b–4b to CT-DNA. After each addition, the solution was stirred at the requisite temperature for 5 min prior to measurement. Fluorescence spectra of the solutions were obtained by excitation at 520 nm and measuring the emission spectra in the 530–750 nm range, using 10 nm slits. Complexes 1b–4b were added to the solutions in 2.5 μM increments.


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Hoechst 33258 displacement experiments. In the Hoechst 33258 fluorescence displacement experiments, 3 mL aliquots of a solution containing 20 μM CT-DNA and 2 μM Hoechst 33258 in 50 mM Tris-HCl buffer at pH 7.4 were titrated with concentrated solutions of the respective complexes 1b–4b, thus producing solutions with varied molar ratios of 1b–4b to CT-DNA. After each addition, the solution was stirred at the requisite temperature for 5 min prior to measurement. Fluorescence spectra of the solutions were obtained by excitation at 338 nm and measuring the emission spectra in the 390–650 nm range, using 10 nm slits. Complexes 1b–4b were added to the solutions in 2.5 μM increments.

Cells and cell line culture: Human cancer cell lines, including breast cancer cell line MCF-7, cervical carcinoma HeLa, and hepatocellular carcinoma HepG2, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Hepatic LO2 cells and lung carcinoma cisplatin-resistant cell line A549 and cisplatin-resistant cell line A549R were obtained from the Cell Bank (Cell Institute, Sinica Academy Shanghai, Shanghai, China). All cell lines were cultured in 25 cm² culture flasks in either DMEM (Dulbecco's Modified Eagle's Medium; Gibco, Gaithersburg, MD) or culture media supplemented with 10% fetal bovine serum (Hyclone, Walton, MA), 100 units per mL of penicillin, and 50 units per mL of streptomycin in a humidified incubator with an atmosphere of 95% relative humidity and 5% CO₂ at 37 °C. Cells were grown to a stable state and in the logarithmic growth phase, unless otherwise specified.

Cytotoxicity of Ru²⁺ complexes against 2D cancer cell monolayer: 1 × 10⁴ cells/well were seeded in a 96-well flat-bottomed multiwell plate supplemented with culture medium (180 μL/well) and then incubated with 5% CO₂/95% air at 37 °C for 24 h. After the addition of 20 μL of medium containing serially diluted solutions of the Ru²⁺ complexes, the cells were incubated for a further 48 h. The cytotoxicity of the ruthenium complexes was measured on the basis of ATP concentration with a CellTiter-Glo Luminescent Cell Viability kit (Promega).

Stability in plasma: The stability of the complexes in plasma was assessed using a procedure analogous to that recently reported.[8] An aliquot (25 μL) of a solution containing the respective cyclometalated complexes 1b–4b (final concentration 20 μM) and diazepan (final concentration 10 μM) in DMEM was added to human plasma solution (975 μL) to give a total volume of 1000 μL. The resulting mixture was incubated for 72 h at 37 °C with continuous and gentle shaking (ca. 300 rpm). The reaction was stopped by the addition of acetonitrile (2 mL), and the mixture was centrifuged for 45 min at 10000 g at 4 °C. The acetonitrile was evaporated, and the residue was suspended in acetonitrile/H₂O (1:1, v/v; 200 μL). The suspension was filtered and the filtrate was analyzed by HPLC-UV. A 0.1 μL aliquot of the solution was injected into an HPLC system (Thermo, USA) connected to a UV/V is spectrophotometer. A Hypersil Gold Dim (100×2.1 mm, Thermo, USA) reversed-phase column was used at a flow rate of 0.5 mL/min. The runs were performed with a linear gradient of A (acetonitrile; Sigma-Aldrich, HPLC grade) in B (distilled water containing 0.1% HCOOH).

Flow cytometric analysis: HeLa cells at a density of 1 × 10⁶ cells/mL were incubated with 5 μM each of the respective Ru²⁺ complexes 1a–4a for 6 h at 37 °C. The cells were then washed with three times with 1× PBS and trypsinized. Thereafter, they were centrifuged and then washed with 1× PBS (1 mL) and finally resuspended in PBS (0.5 mL). The uptakes of complexes 1a–4a were quantified by flow cytometry on an FACSCanto II apparatus (BD Biosciences, USA) and analyzed with FlowJo 7.6.1.

Cell uptake of Ru²⁺ complexes: For the uptake studies, HeLa cells were plated at a density of 1 × 10⁵ cells per mL in DMEM (5 μL) and the respective ruthenium complexes 1a–4a and 1b–4b (5.0 μM) were added to the culture medium and incubated for varying durations at 37 °C. After digestion, the HeLa cells were counted and divided into two portions; for the first portion, the nuclei were extracted using a nucleus extraction kit; for the second portion, the cytoplasm was extracted using a cytoplasm extraction kit (Shanghai Sangon Biological Engineering Technology & Services Co. Ltd.). The samples were digested in 60% HNO₃ at room temperature for 24 h. Each sample was then diluted with doubly-distilled water to obtain 2% HNO₃ sample solutions. The ruthenium concentrations in the two portions were determined by ICP-MS (Thermo Elemental Co., Ltd.). Data are reported as the mean ± standard deviation (n = 3).

Cell treatment with EdU: To measure the anti-proliferation activities of the Ru²⁺ complexes, a Click-iT EdU Imaging Kit (Invitrogen) was used. A slightly higher drug concentration was used due to a shorter incubation time. Complexes 1a–4a (20 μM) and 1b–4b (2.0 μM) and cisplatin (15 and 30 μM) were each incubated with HeLa cells for 12 h in 5% CO₂/95% air at 37 °C. After incubation, the HeLa cells in the 96-well plate were washed with 1× PBS. Then, 200 μL of medium containing 10 μM EdU was added to each well. The cells were incubated overnight, then washed with 1× PBS, and fixed with 4% polyphosphoric acid (PFA). After 15 min of incubation, the cells were washed twice with 3% BSA, permeabilized with 0.5% Triton X-100 in 1× PBS, and incubated for a further 20 min. The cells were then washed twice more with 3% BSA and then the Click-it reaction mixture (200 μL) was added. After incubation for 30 min, the cells were washed once with 3% BSA. For nucleus staining, 200 μL of Hoechst 33342 solution was added to each well, and the cells were incubated for a further 30 min and washed with 1× PBS (2×200 μL). Finally, the cells were imaged under an inverted fluorescence microscope (Zeiss Axio Observer D1, Germany).

AO/EB dual-staining assay: A monolayer of HeLa cells was incubated in the absence or presence of complexes 1a–4a (10 μM) or 1b–4b (1.0 μM) at 37 °C in 5% CO₂ for 48 h. After treatment, the cells were stained with AO/EB solution (100 μg/mL A, 100 μg/mL EB). Samples were washed three times with 1× PBS and observed under an inverted fluorescence microscope (Zeiss Axio Observer D1, Germany).

Generation and analysis of MCTSs: MCTSs were cultured using the liquid overlay method.[46] HeLa cells in the exponential growth phase were dissociated by a trypsin/EDTA solution to obtain single-cell suspensions. About 2500 diluted HeLa cells were transferred to 1% agarose-coated transparent 96-well plates with DMEM (200 μL) containing 10% serum. The single cells generated MCTSs approximately 400 μm in diameter at day 4 with 5% CO₂ in air at 37 °C. After the formation of MCTSs in a 96-well plate, they were each imaged with a phase-contrast microscope (Zeiss Axio Observer D1, Germany) using a 10× objective to monitor their integrity, diameter, and volume. The radius of each tumor spheroid was used to calculate its volume (μm^3); V = πr^3/6.

Cytotoxicity of Ru²⁺ complexes towards 3D MCTSs: MCTSs were generated as mentioned above. At day 4, 50% of the culture medium containing MCTSs of diameters around 400 μm were carefully replaced by drug-supplemented fresh culture medium using an 8-channel pipettor. In parallel, for the untreated MCTSs, 50% of the culture medium was replaced by fresh medium. Four MCTSs were treated for each set of conditions and drug concentration, and the volume of DMSO was less than 0.5% (v/v). The MCTSs were stained with AO/EB (100 μg/mL A, 100 μg/mL EB) and analyzed with the same microscope. Data are reported as the mean ± standard deviation (n = 3).
were then allowed to incubate for a further 72 h. The cytotoxicities of the ruthenium complexes were assessed on the basis of ATP concentration with a CellTiter-Glo Luminescent Cell Viability Kit (Promega). After 20 min of incubation, the MCTSs were carefully transferred into black-bottomed, flat-bottomed 96-well plates (Corning) and pipette mixed for luminescence measurement on an Infinite M200 PRO apparatus (TECAN).

MCTSs growth and regrowth inhibition: MCTSs were generated as described above, and those of diameter approximately 400 μm were incubated with the respective ruthenium complexes 1a–4a (1.0–50 μM) and 1b–4b (0.1–20 μM) or cisplatin (1.0–60 μM) for 72 h. The medium was then renewed, and thereafter 50% medium exchange was performed every 48 h using an 8-channel pipettor for further culturing. Control MCTSs were treated with fresh medium. Image analysis was performed with a microscope (Zeiss Axio Observer D1, Germany) on days 4, 6, 8, 10, 12, and 14, as described above. Growth delay was defined by the comparing volume of MCTSs at different time intervals with the volume of MCTSs at day 4.

Live/dead viability/cytotoxicity assay: The live/dead assay of MCTSs was performed using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Life Technologies). Live cells were distinguished by the presence of ubiquitous intracellular esterase activity, as determined by enzymatic conversion of the virtually non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein (ex = 495 nm, em = 515 nm). EthD-1 could enter cells with damaged membranes and showed a 40-fold enhancement in fluorescence upon binding to nucleic acids, thereby producing a bright-red fluorescence in dead cells (ex = 635 nm). The determination of cell viability was based on these physical and biochemical cell properties. After treatment with the complexes, the MCTSs were incubated with calcein AM (2 μM) and EthD-1 (4 μM) solutions for 30 min and imaged directly using an inverted fluorescence microscope (Zeiss Axio Observer D1, Germany).

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Ru–C more effective than Ru–N: A small change in the structure of an RuII complex induces dramatically different anticancer activities (see graphic). Cyclo-
metalated RuII complexes 1b–4b function as DNA transcription inhibitors and exhibit significantly improved anticancer activities compared with polypyridyl RuII complexes 1a–4a.