Increasing the Cytotoxicity of Ru(II) Polypyridyl Complexes by Tuning the Electronic Structure of Dioxo Ligands

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ABSTRACT: Due to the great potential expressed by an anticancer drug candidate previously reported by our group, namely, Ru-sq ([Ru(DIP)i(sq)][PF6]) (DIP, 4,7-diphenyl-1,10-phenanthroline; sq, semiquinonate ligand), we describe in this work a structure–activity relationship (SAR) study that involves a broader range of derivatives resulting from the coordination of different catecholate-type dioxo ligands to the same Ru(DIP)i core. In more detail, we chose catechols carrying either an electron-donating group (EDG) or an electron-withdrawing group (EWG) and investigated the physicochemical and biological properties of their complexes. Several pieces of experimental evidences demonstrated that the coordination of catechols bearing EDGs led to deep-red positively charged complexes 1–4 in which the preferred oxidation state of the dioxo ligand is the uninegatively charged semiquinonate. Complexes 5 and 6, on the other hand, are blue/violet neutral complexes, which carry an EWG-substituted dinegatively charged catecholate ligand. The biological investigation of complexes 1–6 led to the conclusion that the difference in their physicochemical properties has a strong impact on their biological activity. Thus, complexes 1–4 expressed much higher cytotoxicities than complexes 5 and 6. Complex 1 constitutes the most promising compound in the series and was selected for a more in-depth biological investigation. Apart from its remarkably high cytotoxicity (IC50 = 0.07–0.7 µM in different cancerous cell lines), complex 1 was taken up by HeLa cells very efficiently by a passive transportation mechanism. Moreover, its moderate accumulation in several cellular compartments (i.e., nucleus, lysosomes, mitochondria, and cytoplasm) is extremely advantageous in the search for a potential drug with multiple modes of action. Further DNA metalation and metabolic studies pointed to the direct interaction of complex 1 with DNA and to the severe impairment of the mitochondrial function. Multiple targets, together with its outstanding cytotoxicity, make complex 1 a valuable candidate in the field of chemotherapy research. It is noteworthy that a preliminary biodistribution study on healthy mice demonstrated the suitability of complex 1 for further in vivo studies.

INTRODUCTION

The worldwide approval of the anticancer drug cisplatin and later of carboplatin and oxaliplatin has made platinum-based drugs the leading compounds in the field of medicinal inorganic chemistry.12–14 However, the side effects and resistance associated with these compounds have spurred numerous investigations into other metal-based drugs as potential chemotherapeutic agents against cancer.15–20 Ruthenium complexes are presently regarded as the successors/alternatives to the aforementioned platinum compounds.7–14 Three ruthenium complexes were (i.e., NKP-1019 and NAMI-A, Figure 1) or are (i.e., IT-139, Figure 1) in clinical trials as chemotherapeutic agents. In addition, a Ru(II) polypyridyl complex, namely, TLD-1433 (Figure 1) has just entered phase II of clinical trials as a photosensitizer for photodynamic therapy against bladder cancer.15–20 Ru(II) polypyridyl complexes have also been extensively studied for their cytotoxicity and showed great potential as chemotherapeutic agents.21–26 Recently, we reported a detailed study on a very promising Ru(II) polypyridyl complex, namely [Ru(DIP)i(sq)][PF6] (Ru-sq, Scheme 1a), where DIP is 4,7-diphenyl-1,10-phenanthroline and sq is a semiquinonate ligand.27–29 Semiquinonate (sq) is the product of the first one-electron oxidation of catecholate (cat) and can be further oxidized to quinone (q) (Scheme 1b).30–32 Catecholate and its redox congeners semiquinone and 1,2-benzoquinone are pivotal examples of the class of "noninnocent" ligands.33 This
Figure 1. Chemical structures of ruthenium complexes that were (i.e., NKP-1019 and NAM-I-A) or are (i.e., IT-139 and TLD-1433) in clinical trials.

Scheme 1. (a) Syntheses of Complexes 1–6 and (b) Catecholate (cat) and Its Oxidized Forms, Semiquinonate (sq) and Quinone (q)

\[
\begin{align*}
\text{Ru(DMSO)}_3\text{Cl}_2 & \quad \rightarrow \quad \text{Ru-sq} \\
1: R_1 = R_2 = R_3 = R_4 = H & \quad R_5 = \text{OCH}_3 \\
2: R_2 = R_3 = R_4 = H & \quad R_1 = \text{CH}_3 \\
3: R_1 = R_2 = R_3 = H & \quad R_4 = \text{CH}_3 \\
4: R_1 = R_2 = R_3 = H & \quad R_4 = \text{Cl} \quad \text{(C6H}_6\text{Cl}_3) \\
5: R_1 = R_2 = R_3 = R_4 = \text{Br} & \quad R_5 = \text{NO}_2 \\
6: R_1 = R_3 = R_4 = H & \quad R_2 = \text{NO}_2
\end{align*}
\]

\[(i) \text{DIP}, \text{LiCl}, \text{DMF}, \text{reflux}, 24 \text{ h}, 78\%; (ii) \text{(i) NaOH, 3-methoxycatechol (1), 3-methoxycatechol (2), 4-methoxycatechol (3), or 4-tert-butylcatechol (4) 2-propanol, reflux, 24 h (i) air, 2 h (ii) NH}_3\text{PF}_6, \text{2-propanol/H}_2\text{O (1:8), 19\% (Ru-sq), 23\% (1), 24\% (2), 29\% (3), 16\% (4). (iii) (i) NaOH, tetrabromocatechol (5) or 4-nitrocatechol (6), 2-propanol, reflux, 24 h (i) air, 2 h (ii) NH}_3\text{PF}_6, \text{2-propanol/H}_2\text{O (1:8), 54\% (5), 27\% (6).}
\]

Definition more appropriately refers to a metal ion/ligand pair, both of which are redox-active and whose frontier orbitals are strongly mixed, defying an unambiguous assignment of redox states to either component.\(^{3}\) Catechols are also considered to be pan-assay interference compounds (PAINS) due to their chelating and redox properties.\(^{3}\)

Ru-sq was, to the best of our knowledge, the first Ru(II) polypyridyl complex carrying a semiquinonate moiety, which was investigated in depth as an anticancer drug candidate. We could demonstrate that it is a valuable option as a chemotherapeutic agent, both in vitro and in vivo. Interestingly, its mechanisms of action involve more than one cellular target. This finding could potentially be a key feature to
overcoming resistance, which is an inherent problem for platinum-based anticancer drug candidates. Driven by the promising properties unveiled for Ru-sq, we undertook a structure–activity relationship (SAR) study, keeping the same Ru(II) polyppyridyl core (i.e., Ru(DIP)) but substituting the catechol-type dioxo ligand. Over the years, many studies have been performed that focus on the noninnocent character of different metal complexes from both experimental and theoretical points of view. In 2006, Wada and co-workers demonstrated how the oxidation state of the dioxo ligand in a given metal-colligand environment depends on the nature of its substituents. More specifically, the authors investigated a series of [Ru(OAc)(dioxolene)(terpy)] complexes with dioxo substituents. In 2012, more specifically, the authors investigated a structure–activity relationship (SAR) study, keeping the same considered to be a limitation for in vivo applications. However, chemo therapeutic treatments, which is the occurrence of resistance. Moreover, as common for most complexes of this kind, complex 1 displays poor water solubility. Recently, we reported the formation of colloids of Ru-sq and complex 1 in a water–DMF (1:1 v/v) mixture, which could have been easily mistaken for solutions if no appropriate characterization had been performed. Poor aqueous solubility is usually considered to be a limitation for in vivo applications. However, in this study, we could demonstrate that with an appropriate formulation, complex 1 is able to be distributed in healthy BALB/c mice, which renders it a suitable candidate for further in vivo studies.

### RESULTS AND DISCUSSION

**Synthesis and Characterization of 1–6.** The synthesis of compounds 1–6 was achieved by adapting a previously reported procedure. Ru(DIP)₂Cl₂ was obtained starting from the known Ru(DMSO)₂Cl₂ as previously described. This precursor complex was then refluxed under a nitrogen atmosphere with the corresponding catechol derivative in the presence of NaOH in 2-propanol overnight. Subsequently, the reaction vessel was opened to air for 2 h to allow for the final oxidation step. Interestingly, oxidation of the catecholate ligand to a semiquinonate occurred only for those complexes bearing catecholate ligands with EDG groups (complexes 1–4 in Scheme 1a). When the synthesis involved catechol derivatives bearing EWG groups, neutral complexes (5 and 6) were generated. The drastic change in the electronic properties of the complexes studied in this work is also reflected by their color. EDG-containing derivatives are deep-red solids, while EWG-containing derivatives are blue/violet.

![UV/vis/NIR spectrum in DMF solution of complexes 1-6 and their precursor Ru(DIP)₂Cl₂](image)

Figure 2. UV/vis/NIR spectra in DMF solution of complexes 1–6 and their precursor Ru(DIP)₂Cl₂. This confirms the assignment of the EDG-substituted ligands as semiquinonates in that compounds 1–4 display a band at ca. 885–900 nm. This absorption is characteristic for ruthenium-bis(dimine) complexes containing a semiquinonate ligand and is due to a Ru(II) → sq transition. A set of bands, which is responsible for the visual color impression of these compounds, is found in the range of 470 to 525 nm. On the basis of literature data on similar compounds, this band can be assigned as Ru(II) → sq transition.

The paramagnetic nature of compounds 1–4 was indirectly confirmed by their characteristically broadened ^1^H NMR.
spectra (Figure S1). In contrast, compounds 5 and 6, whose purity was confirmed by elemental analysis, are neutral and formally diamagnetic. However, the proton NMR spectra also provided severely broadened resonances. The addition of zinc dust to the NMR tube slightly improves the resolution of NMR signals, which suggests the formation of paramagnetic species in solution (Figure S1 (5 and 6)). All complexes were characterized by ESI-MS, and their purity was verified by HPLC (Figure S2) and elemental analysis.

**EPR, (Spectro)electrochemistry, and Electronic Structures.** The oxidation state of the catecholate/semiquinone ligand of complexes 1-6 was further investigated by electron paramagnetic resonance (EPR) spectroscopy, cyclic voltammetry (CV), rotating disc electrode voltammetry (RDE), and UV/vis/NIR spectroelectrochemistry and supported by density functional theory (DFT) calculations. Compounds 1-4 are, as obtained from synthesis, EPR-active. At room temperature, they display a rather broad, featureless, isotropic signal (Figure 3a), which becomes slightly anisotropic at lower temperatures and in the frozen glass (Figure S3). The substituents on the dioxo ligand and their positions seem to have only a small influence on the locus and the distribution of the unpaired spin density. Thus, $g_{zz}$ values of 1.9893, 1.9891, 1.9872, and 1.9870 for 1-4, respectively, are all close to the free-electron value of 2.0023 and differ only slightly from each other.

These data are also confirmed by the spin density distributions computed for compounds 1 and 3 reported in Table 1.

### Table 1. Computed Mulliken Spin Densities on the Different Fragments of the Cationic Forms of the Complexes

<table>
<thead>
<tr>
<th>Fragment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>dioxo ligand</td>
<td>0.8200</td>
<td>0.8268</td>
<td>0.6276</td>
<td>0.6003</td>
</tr>
<tr>
<td>ancillary1</td>
<td>-0.0090</td>
<td>-0.0091</td>
<td>-0.0077</td>
<td>-0.0062</td>
</tr>
<tr>
<td>ancillary2</td>
<td>-0.0093</td>
<td>-0.0089</td>
<td>-0.0078</td>
<td>-0.0098</td>
</tr>
<tr>
<td>Ru</td>
<td>0.1983</td>
<td>0.1913</td>
<td>0.3880</td>
<td>0.4157</td>
</tr>
</tbody>
</table>

Table 1 and graphically depicted in Figure 4 (see the Experimental Section for computational details). Indeed, roughly 82% of the spin density is localized on the dioxo ligand with only limited delocalization onto the Ru center (0.19%e-1). Consequently, the computed $g$ tensors (reported as Table S2 in the SI) show very small anisotropies and shifts with respect to the free-electron value. These results are consistent with the assumption that the 1,2-dioxo ligand can be formally represented by its semiquinone form in the case of complexes 1-4.

To further investigate the electrochemical properties of compounds 1-4 and to obtain better insight into the effect of the substituent's position and nature on the redox behavior of the complexes, cyclic voltammetry (CV) and rotating disc electrode (RDE) analyses were performed. Complexes 1-4 display reduction as well as oxidation features in line with what
Figure 4. Isodensity plots of computed spin densities. Positive spin density corresponds to blue lobes.

Figure 5. UV/vis/NIR spectroelectrochemistry data for compounds 1 and 2.

we have reported in our previous work on Ru-sq, carrying the nonsubstituted semiquinonate ligand. Figure 3 shows the RDE and cyclic voltammograms of compound 1, while those of 2–4 are available in the SI (Figure S4). Like all other complexes bearing EDG-substituted dioxo ligands, complex 1 exhibits four well-defined reversible redox processes. The RDE experiment shows four features with very similar limiting currents, which indicates that the same number of electrons is exchanged during every process. On the basis of our previous results, the redox process at +0.595 V vs MeSO\textsubscript{4} can be attributed to the oxidation of Ru(II) to Ru(III), while the first reduction process at −0.201 V vs MeSO\textsubscript{4} can be attributed to the sq/cat redox process. UV/vis/NIR spectroelectrochemistry further supports this hypothesis. As evident from Figure 5ab, reducing complexes 1 and 2 at sufficiently negative potential yields spectra whose general band structures closely resemble those of compounds S and 6. The observed red shift with respect to the latter complexes is the result of the higher-lying donor orbitals (cat) and (Ru(II)), which is due to the electron-rich nature of the dioxo ligand in 1. Oxidation to the corresponding dications (Figure 5cd) 1\textsuperscript{2+} and 2\textsuperscript{2+} (denoted as 1\textsuperscript{2+} and 2\textsuperscript{2+} in Figure S5), leads to a general blue shift in the vis/NIR bands. The first visible transition is now found at 675 nm for both complexes. Compared to the [Ru(II)(bpy)\textsubscript{3}(q)]\textsuperscript{2+} reference, this corresponds to a red shift of 810 cm\textsuperscript{-1}. Adhering to the assignment of a Ru(II) → diimine MLCT transition, this effect can be explained through the presence of a more extended π-system in the DIP ligand as compared to bpy. We note, however, that a Ru(III)-sq scenario resulting in a dioxo ligand-to-metal charge transfer (LMCT) is also possible. The strong electronic coupling between these two redox-active entities prohibits a clear assignment of redox states without additional experimental support or quantum chemical calculations.
Comparing the results, it is clear shown in Figure 6a, while the voltammograms of compound 6 selected for this experiment.

Semiquinonate ligand. (See full data in Table S3 of the Supporting Information.)

A 1:1 mixture of DMF and water containing 20 mM NaHCO3 at 70 °C, strong suggesting that compounds 1–4 can at least be partially reduced to their neutral forms inside the cell (Figure S5). Slightly alkaline conditions were necessary in addition, the measurements underline the charge transfer nature of all transitions in the Vis/NIR region as all corresponding absorptions experience a blue shift with an increase in solvent polarity, i.e., these complexes display negative solvatochromism.

Remarkably, the presence of one or more EWGs on the dioxo ligand causes a distinct change in the electronic structure of the complex. The CV and RDE voltammograms of 6 are shown in Figure 6a, while the voltammograms of compound 6 are available in the SI (Figure S4). The voltammograms of 5 were recorded over two distinct potential ranges, from −1.0 to −2.0 V and from −1.0 to 1.0 V, to avoid the adsorption of the complex on the electrode (Figure S4).

Complexes 5 and 6 are EPR-silent from 20 °C down to −1.50 °C, which confirms that the dioxo ligand is present in its catecholate form as found by UV/vis/NIR spectroscopy and elemental analysis. To provide an attribution of the observed voltammetric features in the positive potential range, further EPR experiments were conducted on the monooxidized forms of 5 and 6 (5ox and 6ox, respectively, in Scheme 2). Complexes 5 and 6 were converted to their monocationic forms by treatment with an excess of ferrocenium hexafluorophosphate (FcHPF6, E1/2 = 0.450 V vs SCE in DMF/0.1 M NaBu4PF6).

5ox and 6ox are EPR-active, giving slightly anisotropic signals at −140 °C with g-value values of 2.019 for 5ox and 2.032 for 6ox (Figures 4 and 6b). The absence of an EPR signal at room temperature for the oxidized complexes is explained by the electron-poor nature of the perboronated- and nitro-substituted ligands, which increases the contribution of the Ru center to the spin density in the case of the oxidized complexes at the expense of the dioxo ligand.40 Indeed, as reported in Table 1, the spin density on the Ru center increases to 0.46 e− in the cases of 5ox and 6ox with a corresponding spin density on the dioxo ligand of roughly 0.43 e−. Therefore, 5ox and 6ox have their spin densities nearly equally shared between the Ru center and the dioxo ligand, corresponding to strong orbital mixing. This can be represented as the two resonance structures in Scheme 2, where the contribution of the Ru(III)/cat form is nearly as important as that of the Ru(II)/sq form. A higher metal contribution to the overall spin densities is also indicated by the distinct anisotropy of the g tensor, which is more clearly evident for 6ox (i.e., showing the larger computed spin density) with individual g tensor components of g∥ = 1.870, g∥ = 2.025, and g⊥ = 2.190 as determined by the simulation of the experimental spectrum (Figure S3 in the SI). Tables 3 and S4 list the potentials related to the redox processes observed for compounds 5 and 6.

To shed additional light on the impact of the substituent on the dioxo ligand, complex 5 was also subjected to UV/vis/NIR spectroelectrochemical investigations in the positive potential range. As evident from Figure 7a, the generation of the monocation furnishes a structured, broad band with a maximum at 940 nm, while the features characteristic of the neutral forms disappear. The significant red shift of this band as compared to compounds 1–4 is a clear token of the electron-poor nature of the corresponding semiquinonate
Figure 6. (a) RDE and cyclic voltammograms of complex 5 (from -2.0 to -1.0 V and from -1.0 to +1.0 V) at a glassy carbon electrode in DMF (1 mM) containing N\textsubscript{Bu\textsubscript{4}}PF\textsubscript{6} (100 mM) as the supporting electrolyte. Data were recorded versus SCE at a scan rate of 100 mV/s and recalculated versus the Me\textsubscript{10}Fc' potential value (-0.001 and -0.002 V for RDE and CV, respectively). (b) EPR spectra of complexes 5ox and 6ox at -140 °C.

Scheme 2. Structures of 5, 6 and 5ox, 6ox Represented as Two Resonance Structures*  

**Ru oxidation states +II and +III are marked in green and red, respectively.**

ligand, which lowers the energy difference between the Ru(II) donor and the dioxo ligand acceptor orbitals. The relatively larger loss of electron density experienced by the ruthenium atom and the rather electron-poor nature of the electro-generated semiquinonate ligands in oxidized 5ox are further underlined by the blue shift of the Ru → DIP MLCT and sq → DIP LL'CT bands in the visible range to 465 and 410 nm instead of 510 and 470 nm observed for 1. Further oxidation to the dication, in agreement with the CV studies, leads to decomposition as reduction after electrolysis yielded spectra that differed considerably from those of their monocationic or neutral forms (Figure 7b).

The change from an EDG to an EWG at the sq/cat ligand has two effects. First, the overall redox potential of the molecule is increased in such a manner that, by applying the same synthesis procedure and under identical conditions, no longer a paramagnetic cationic but rather a neutral diamagnetic complex is obtained. Second, the spin density in the monocationic form presents distinctly larger contributions from the ruthenium center when EWGs are present. This occurrence results in a slight increase in EPR signal anisotropy.
Stability in DMSO and Human Plasma. Next, the stability of compounds 1–6 was investigated in DMSO and in human plasma. DMSO stability is a key factor to consider in medicinal chemistry as this solvent, in some cases, was found to be problematic for biological experiments for its coordinative property.51-53 The stability in DMSO was monitored by \(^1\)H NMR spectroscopy over 96 h at room temperature. Overall, all complexes are stable under these conditions as no significant new peaks appear in the spectra (Figure S6). Only complex 3 shows a certain degree of degradation after 96 h, displaying a conversion of about 15% of the initial complex. The spectrum of complex 3 shows new, more resolved peaks which are a clear indication of the formation of a nonparamagnetic product. Additionally, to estimate the stability under physiological conditions, the stability of complex 1 (the most promising of the series) was investigated in human plasma. Complex 1 was incubated over 96 h in human plasma at 37 °C in the presence of an internal standard (caffeine). Figure S7 reveals a linear decrease in concentration of complex 1 over time and a half-life of 48 h.

Cytotoxicity Studies. The first step toward the biological investigation of complexes 1–6 was the evaluation of cell viability in monolayer cultures of the HeLa (human cervical adenocarcinoma), A2780 (human ovarian carcinoma), A2780 cis (human cisplatin-resistant ovarian carcinoma), A2780 ADR (human doxorubicin-resistant ovarian carcinoma), CT-26 (mouse colon adenocarcinoma), CT-26 LUC (mouse colon adenocarcinoma stably expressing luciferase), and RPE-1 (human normal retina pigmented epithelial) cell lines using a fluorometric cell viability assay (single graphs available in Figure S8).54 Doxorubicin and cisplatin as well as the ligands and the Ru(DIP)_2Cl_2 precursor were tested in the same cell lines as positive and additional controls (Table 4 and Table S5).54,56 Table 4 displays the IC_{50} (the half maximal inhibitory concentration) values of the tested compounds and previously reported Ru-sq.55 Ru-sq is the analogous complex carrying the unsubstituted semiquinonate ligand. The comparison between Ru-sq and the derivatives carrying an EDG- and EWG-substituted diropeptide (complexes 1–4, respectively) reveals how the electron density on the organic moieties impacts the observed cytotoxicity in the tested cell lines. In general, compounds 1–4 present higher cytotoxicity in most of the cell lines tested. In contrast, complexes 5 and 6 show much lower cytotoxicity with IC_{50} in the micromolar range for all of the cell lines tested. Of particular interest is complex 1 with an IC_{50} in the low nanomolar range (0.07 µM) against the cisplatin-resistant cell line, which makes it 10 times more active than doxorubicin and around 200 times more active than cisplatin (0.54 and 18.33 µM for doxorubicin and cisplatin, respectively). Overall, complexes 1–4 display a cytotoxicity which is comparable to that of doxorubicin and much higher than that of cisplatin. The Ru(DIP)_2Cl_2 precursor (Table 4) and the different catechols (Table S4) display much lower activity, suggesting that the great activity shown by complexes 1–4 is a consequence of the

<table>
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<tr>
<th>IC_{50} (µM)</th>
<th>HeLa</th>
<th>A2780</th>
<th>A2780 ADR</th>
<th>A2780 cis</th>
<th>CT-26</th>
<th>CT-26 LUC</th>
<th>RPE-1</th>
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<tr>
<td>cisplatin</td>
<td>9.28±0.20</td>
<td>4.00±0.76</td>
<td>6.32±0.71</td>
<td>18.33±2.92</td>
<td>2.60±0.18</td>
<td>2.42±0.23</td>
<td>50.24±5.11</td>
</tr>
<tr>
<td>doxorubicin</td>
<td>0.34±0.02</td>
<td>0.19±0.03</td>
<td>5.84±0.58</td>
<td>6.36±0.57</td>
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<td>6.65±0.95</td>
<td>3.13±0.07</td>
</tr>
<tr>
<td>Ru(DIP)_2Cl_2</td>
<td>5.09±0.93</td>
<td>6.76±0.44</td>
<td>4.13±0.2</td>
<td>4.55±0.43</td>
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<td>0.90±0.04</td>
</tr>
<tr>
<td>Ru-sq</td>
<td>0.50±0.03</td>
<td>6.76±0.44</td>
<td>4.13±0.2</td>
<td>4.55±0.43</td>
<td>1.00±0.03</td>
<td>1.51±0.14</td>
<td>0.90±0.04</td>
</tr>
</tbody>
</table>

Values are taken from ref 29. Note that these experiments were performed on the same days.
Values are taken from ref 29. Note that these experiments were performed on the same days.

Coordinated electron-rich sq ligands to the Ru(II) polypyridyl core.

Complex I was found to be the most promising candidate among the series of complexes investigated in the 2D model. Due to its remarkably high cytotoxicity and its great activity toward resistant cell lines, its cytotoxicity was explored in a multicellular tumor spheroids (MCTS) model. In 3D spheroids, proper cell-to-cell and cell-to-environment interactions as well as the cellular morphology and polarity are maintained. Additionally, the growth pattern, metabolism, and gene expression mimic the complexity of the initial stages of solid tumors. These features allow for a good estimation of in vivo antitumor activity, qualifying MCTS as a more reliable model than monolayer cell cultures in cancer research. In addition to the complex of interest (1), the Ru(DIP)Cl2 precursor, the 3-methoxycatechol ligand, and positive controls (cisplatin and doxorubicin) were tested via a luminescent cell viability assay in HeLa MCTS (single graphs are available in Figure S9). Moreover, Ru-sq was tested for comparative purposes. Table 5 shows the IC50 values after a 48 h treatment for all of the compounds tested. Complex I displays high cytotoxicity toward HeLa MCTS with IC50 ≈ 21 µM. This value indicates an activity which is almost double that of current drugs cisplatin and doxorubicin (IC50 ≈ 46 and 39 µM, respectively). Nevertheless, the previously reported Ru-sq still shows a slightly higher cytotoxicity (IC50 ≈ 14 µM). 3-Methoxycatechol proved to be nontoxic, and the Ru(DIP)Cl2 precursor showed a cytotoxicity comparable to that of cisplatin.

To evaluate the time-dependent effect on the growth of MCTS treated with complex I, an additional experiment was performed. HeLa MCTS (400 µm) was treated with a range of different concentrations of I. Every 3 days, half of the medium in the wells was exchanged (and the treatment concentration consequently decreased by half), and pictures of the spheroids were taken (Figure 8a). HeLa MCTS had a reduced diameter when treated with concentrations higher than the IC50 (20, 25, and 30 µM) of complex I. Additionally, Figure 8 indicates that this effect was maintained even after 13 days for the highest concentration, while slow regrowth was observed for 20 and 25 µM treatments. These findings are similar to the one previously reported for the Ru-sq complex.

Overall, the outstanding activities shown by complex 1 in the monolayer model are confirmed by an MCTS model. These findings represent a powerful motivation for the further investigation of complex 1 as a potential chemotherapeutic agent.

Cell Death Mechanism. Many of the novel or existing chemotherapeutic agents are developed to trigger cell death through apoptosis. This is considered to be a carefully regulated and energy-dependent type of cell death in contrast to necrosis, which is considered to be a rapid, unregulated, energy-independent mode of death. The mode of cell death induced by the treatment with complex 1 was investigated in the HeLa cell line via flow cytometry using the Annexin V and propidium iodide (PI) staining method. Figure 9a shows the obtained dot plots at t = 24 h. (See Figure S10 for the dot plots at each time point.) Figure 9b represents the percentage of cell population in different stages of cell death, at different time points in comparison to staurosporin (apoptosis inducer, positive control) and colchicine. Collected data demonstrated that after 24 h of treatment a large population of HeLa cells was in the late apoptotic/necrotic stage. It is worth noting that apoptosis or necrosis is induced by different pathways and mechanisms, and it is highly unlikely for a cell to undergo necrotic death after several hours of the early apoptotic stage. Hence, these data indicate that complex 1 most probably induces cell death through apoptosis.

Cellular Uptake, Biodistribution, and DNA Metallation. The high cytotoxicity of complex 1 in the tested cell lines encouraged its further biological studies. First, cellular
Figure 9. (a) Flow cytometry dot plots of Annexin V and PI staining in HeLa cells treated with complex 1 (10 µM) and staurosporine (1 µM) for 24 h. (b) Percentage of cell population in different stages of cell death for staurosporin (positive control) and the complex.

Figure 10. Cellular uptake (a), cellular fractionation (b), and DNA metalation (c) of HeLa cells after treatment with tested compounds (5 µM, 2 h). Data are presented as the mean ± SD of at least three technical replicates. All data related to Ru-sq were previously reported by our group.29 We, however, note that these experiments were performed on the same days.

uptake, the mechanism of uptake, and the intracellular distribution were tested using inductively coupled plasma-mass spectrometry (ICP-MS). Working concentrations and incubation times were chosen accordingly to avoid extended cell mass loss due to the high cytotoxicity of the complexes but considering a final ruthenium amount that allowed for the determination of the metal content. Nevertheless, the working conditions (5 µM treatment for 2 h) allowed for a minor accumulation of the drug cisplatin, which was used as a control.65-67 Complex 1 shows higher cellular accumulation than the positive control cisplatin and the Ru-sq analogue previously reported69 (Figure 10a). To clarify whether the mechanism of uptake involves passive or active mechanisms, additional experiments were performed. HeLa cells were kept at low temperature (4 °C) or were pretreated with different uptake pathways inhibitors. Thus, 2-deoxy-o-glucose and oligomycin were used to block cellular metabolism, chloroquine or ammonium chloride were used to impede endocytic pathways, and tetrathylammonium chloride was used to block cation transporters. After pretreatment, cells were incubated with the test compounds (2 h, 5 µM). The amounts of ruthenium found in cells were then quantified using ICP-MS. Low temperature slightly inhibited the uptake of complex 1 while none of the other conditions (regulating active transportation mechanisms) affected the total uptake (Figure S11). These findings strongly suggest that the internalization of complex 1 is due only to a passive, energy-independent mechanism, unlike Ru-sq, whose mechanism of uptake involves both active and passive transports.69 The intracellular distribution among the cytoplasm, mitochondria, and nucleus was determined by isolating pure cellular compartments. Most of the compounds accumulate in the nucleus and lysosomes and to a smaller extent in the mitochondria and cytoplasm (Figure 10b). To verify whether the accumulation in the nucleus leads to a direct interaction with DNA, the genetic material was extracted from treated cells and the metal content was analyzed via ICP-MS. Figure 10c shows that complex 1 successfully binds DNA to a much higher extent when compared to cisplatin, which results are in perfect agreement with what was previously reported.67 Even though the accumulation in the nucleus is lower, the DNA metalation
slightly decreases the mitochondrial membrane potential in staining method. JC-1 is considered to be the most reliable shows comparable results for complex 1 and Ru-sq, which can and the loss of the ability to restore proton balance after FCCP analyzer was used. The performed Mito stress test pointed to an increase in the process of oxidative phosphorylation, further experiments were performed. For this purpose, a Seahorse XF analyzer was used. The performed Mito stress test pointed to very low basal respiration levels, inhibited ATP production, and the loss of the ability to restore proton balance after FCCP treatment (Figure 11b and Figure S12). These data suggest that the mitochondrial processes are defective in HeLa cells incubated with complex 1. On the contrary, the same effects were not observed during treatment with the Ru(DIP)Cl₂ precursor or the 3-methoxycatechol ligand. Furthermore, no influence of compound 1 on the cytosolic process of glycolysis was detected (Figure S13). Unfortunately, no direct impact on three main fuel pathways could be determined due to very low oxygen consumption rates (Figure S14).

Metabolic studies confirm that the effects of complex 1 on mitochondrial respiration can contribute to cell death, leading to multiple modes of action involving at least the nucleus and mitochondria as possible targets.

**Preliminary In Vivo Biodistribution Studies.** In this study, complex 1 was demonstrated to be of great interest as a potential chemotherapeutic agent. However, one of its main drawbacks is its scarce water solubility, which could limit its potential use in vivo. Analogue Ru-sq, previously reported by our group, showed potential as an anticancer drug in immunocompetent mice bearing Ehrlich tumors. Nevertheless, its clinical interest is limited by its poor water solubility, which prevented intravenous administration. Anticancer drugs such as paclitaxel and doxorubicin have faced the same limitation but reached the clinic. Following these examples, we successfully developed a formulation of compound 1 using polysorbate 80, a nonionic surfactant generally recognized as safe and already used for doxorubicin. Using the film rehydration method, usually applied to the preparation of polymeric surfactant micelles and liposomes, up to 0.84 ± 0.06 mg/mL of compound 1 could be dissolved in 50 mg/mL of polysorbate 80, with an encapsulation efficiency of 95 ± 3%. Liver and kidneys are the main routes by which drugs and metabolites leave the body, so the effect of complex 1 on them was evaluated. Complex 1 was tested in TIB-75 (mouse, epithelial liver) and HEK 293 (human, embryonic kidney) cell lines, showing cytotoxicity in both cases (Table 6, single graph available in Figure S8).

![Figure 11](https://example.com/figure11.png)

*Figure 11.* (a) Fluorescence signal of the JC-1 dye detected in HeLa cells treated for 24 h with different concentrations of complex 1 (from 0.05 to 0.25 µM). The bar marked in red indicates the IC₅₀ concentration (0.2 µM), FCCP is used as a positive control, and cisplatin and DMSO (1%) are used as negative controls. (b) Mito stress test profile after 24 h of treatment; the oxygen consumption rate changes after treatment with specific electron-transport chain inhibitors. Oligomycin (inhibitor of ATP synthase (complex V)), FCCP (uncoupling agent), antimycin A (complex III inhibitor), and rotenone (complex I inhibitor).

**Table 6. IC₅₀ Values for Cisplatin, Complex 1, Ru(DIP)Cl₂, and 3-Methoxycatechol in the HEK293 and TIB-75 Cell Lines**

<table>
<thead>
<tr>
<th>IC₅₀ (µM)</th>
<th>cisplatin</th>
<th>1</th>
<th>Ru(DIP)Cl₂</th>
<th>3-methoxycatechol</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293</td>
<td>6.60 ± 1.49</td>
<td>0.07 ± 0.005</td>
<td>5.42 ± 0.31</td>
<td>18.96 ± 0.88</td>
</tr>
<tr>
<td>TIB-75</td>
<td>2.83 ± 0.08</td>
<td>0.02 ± 0.0001</td>
<td>5.06 ± 0.32</td>
<td>19.16 ± 1.15</td>
</tr>
</tbody>
</table>
These results, together with the ones shown in Table 4, point out the nonselectivity of complex 1 between cancerous and noncancerous cell lines. This shortcoming is often faced in medicinal chemistry, and it could be improved by the introduction of a targeting moiety. Therefore, we decided to pursue the biodistribution study with the developed formulation to verify the ability of the drug to distribute in the organism upon administration, despite its low aqueous solubility. A preliminary biodistribution study was performed on healthy BALB/c mice after intravenous injection of the formulation at a dose of 5 mg/kg of compound 1. After 30 min and 1 and 2 h, mice were sacrificed and the ruthenium content was analyzed via ICP-MS in relevant tissues (brain, liver, kidneys, spleen, intestine, lungs, and blood). We note that the formulation was well tolerated, and no sign of pain or acute toxicity was observed over the course of the experiment.

According to data shown in Figure 12, compound 1 seems to accumulate preferentially in the liver and the kidneys and, more surprisingly, in the lungs and the spleen. This phenomenon has already been described in the case of doxorubicin formulated in polysorbate 80 and was attributed to this carrier. The low level of ruthenium detected in the blood at all time points suggests a fast distribution in the tissues, possibly associated with fast renal and biliary elimination, supported by the increase of the ruthenium content in the intestine over time.

These preliminary results show compound 1’s ability to distribute in the body, despite its hydrophobicity, using a formulation strategy. While promising, the formulation with polysorbate 80 has also been associated with side effects. To overcome this drawback, a more biocompatible excipient could be used, as already successfully achieved in the case of docetaxel.72

**CONCLUSIONS**

In this work, we performed a structure–activity relationship study (SAR) based on the promising activity expressed by the Ru-sq complex recently reported by our group. More specifically, we explored the coordination of differently substituted catechol-type dioxo ligands to the Ru(DIP)2 core. Electrochemical, EPR, and electronic structure studies allowed us to conclude that the two classes of dioxo ligands tested, carrying either electron-donating (EDG) or electron-withdrawing (EWG) groups, gave Ru(II) complexes with either the semiquinonate (sq, for EDG-modified dioxo ligands in complexes 1–4) or catecholate ligand (cat, for EWG-modified dioxo ligands in complexes 5 and 6). Complexes 1–4 are deep-red solids and carry an overall positive charge due to the monoanionic sq ligand. Complexes 5 and 6 are blue/violet in color and overall neutral due to the dianionic charge associated with the catecholate. Both classes of complexes were found to be stable in DMSO, and complex 1 displayed a half-life of 48 h in human plasma. Cytotoxicity studies using the monolayer model revealed that complexes 1–4 displayed much higher bioactivities than complexes 5 and 6. These findings clearly suggest that the high cytotoxicity that is observed is a direct consequence of the coordination of electron-rich semiquinonate ligands to the Ru(II) polypyridyl core. Precisely, complex 1 was found to be the most promising candidate of this series, with IC50 values in the low nanomolar range, and was chosen for more detailed investigations. First, its cytotoxicity was confirmed using a more reliable 3D model (MCTS), where it displayed an IC50 value of almost half that of cisplatin and doxorubicin. Complex 1 was found to be taken up by HeLa cells very efficiently through a passive transportation mechanism. Cellular fractionation studies revealed major accumulation in the nucleus, lysosomes, and, to a smaller extent, in the mitochondria and cytoplasm. DNA ruthenation, MMP determination, and mitochondria metabolism studies indicated that DNA and mitochondria are both cellular targets of complex 1. Multiple targets are essential to overcoming resistance, which is one of the main drawbacks associated with chemotherapy treatments nowadays. Moreover, despite poor water solubility, complex 1 is demonstrated to distribute well in vivo with the use of an appropriate formulation. These results together with the advantageous modes of action and the outstanding cytotoxicity displayed by complex 1 make it an interesting compound for clinical application in the search for potential chemotherapeutic agents against cancer.

**EXPERIMENTAL SECTION**

**Materials.** All chemicals were either of reagent or analytical grade and used as purchased from commercial sources without additional purification. Ruthenium trichloride hydrate was provided by FCNS; 4,7-diphenyl-1,10-phenanthroline, lithium chloride (anhydrous, 99%), 3-methoxy catechol, 4-methyl catechol, 4-tert-butyl catechol, and 4-nitrocatechol were provided by Aldrich; and tetrabromocatechol was provided by BOC Science. All solvents purchased were analytical or HPLC grade. When necessary, solvents were degassed by purging with dry, oxygen-free nitrogen for at least 30 min before use.

**Instrumentation and Methods.** Amber glass or clear glassware wrapped in tin foil were used when protection from light was necessary. Schlenk glassware and a vacuum line were employed when reactions sensitive to moisture/oxygen had to be performed under a nitrogen atmosphere. Thin-layer chromatography (TLC) was performed using silica gel 60 F254 (Merck) plates with the detection of spots being achieved by exposure to UV light. Column chromatography was used with silica gel 60–200 µm (VWR). Eluent mixtures are expressed as volume to volume (v/v) ratios. 1H and 13C NMR spectra were recorded on a Bruker Avance III HD 400 MHz or a Bruker Avance Neo 500 MHz spectrometer using the signal of the deuterated solvent as an internal standard. The chemical shifts δ are reported in ppm (parts per million) relative to tetramethylsilane (TMS) or signals from the residual protons of deuterated solvents. Coupling constants J are given in Hertz (Hz).
Samples were prepared and analysed using a Thermo Fisher (Carlo Erba) Flash 2000 elemental analyser, configured for %CHN. IR spectra were recorded with a Spectrum Two FTIR spectrometer (PerkinElmer) equipped with a Specac Golden Gate ATR (attenuated total reflection) accessory, applied as neat samples (1/4 in cm). A reproducible ATR measurement was performed using the following system: a 2 x 2 AgInSe2 1260 prepump system with an Agilent G1631A 1260 DAD detector equipped with an Agilent Pursuit XRS SC18 (100 Å, C18, 5 μm 2.5 x 46 mm) column and an Agilent G1346B 1240-FC fraction collector. The solvents (HPLC grade) were acetonitrile (0.1% TFA, solvent A) and water (solvent B). The flow rate was 4.6 mm (2.5 μm) and an Agilent G1361A 1260 DAD WRS detector, with a 4 nm slit.

Detection was performed at 215, 250, 350, 450, and 560 nm with a 4 nm slit.

**Synthesis and Characterization.** Ru(DMSO)2Cl2. Ru(DMSO)2Cl2 (0.30 g, 2.1 mmol) was added to 1 L of dichloromethane (DCM) and the solution was heated to reflux for 24 h. After cooling to room temperature, the mixture was stirred open to air for 2 h while still being protected from light, and the solvent was removed under vacuum. The residual solid was dissolved in 2-propanol (2.5 mL) and H2O (20 mL), and NH4PF6 (0.250 g, 1.5 mmol) was added. The mixture was heated to reflux for 30 min. After cooling to room temperature, the mixture was stirred open to air for 2 h while still being protected from light. The red solid was collected with dichloromethane and dried under vacuum to yield a clean product.

**Electrochemical Measurements.** The electrochemical experiments were carried out with a conventional three-electrode cell (solution volume of 1 mL) and a PC-controlled potentiostat/galvanostat (Princeton Applied Research Inc. model 263A).
working electrode was a vitreous carbon electrode from Oraglys (France) exposing a geometrical area of 0.071 cm² and mounted in Teflon. The electrode was polished before each experiment with 3 and 0.3 µm alumina paste, followed by extensive rinsing with ultrapure Milli-Q water. A platinum wire was used as the counter electrode, and a saturated calomel electrode, SCE, was used as the reference electrode. Electrolytic solutions and DMF containing 0.1 M tetrabutylammonium hexafluorophosphate (NBu4PF6, Aldrich, >99%) as the supporting electrolyte were routinely deoxygenated by bubbling. All of the potential values are given versus the saturated calomel electrode (SCE) and are recalculated versus the Methylene red/pararosanilin (E = 0.49 V vs. SCE).

Computational Details. All structural optimizations were performed using the Gaussian 06 suite of programs at the unrestricted Kohn–Sham (U) level. The nonrelativistic double-ζ LANL2DZ pseudopotential (effective-core-potential, ECP) was used for Ru and Br atoms, together with the Pople split-valence double-ζ basis set for C, N, O, S, and H except for the ruthenium atom, for which the def2-TZVP(d,f) basis set was used. The calculations were sped up by employing the chain-of-sphere (RIJCOSX) approximation along with the decontracted auxiliary basis set of def2-SVP(δ) coulomb fitting. Increased interaction between electrons (RI-MP2) and tight grids were used throughout the calculations. Solvent effects were included using a conductor-like screening model (COSMO) with a dielectric constant of 20.5. The closest value to the isopropanol environment was taken in the geometry optimization.

Electron Paramagnetic Resonance Spectroscopy. Electron paramagnetic resonance (EPR) experiments were performed on a MiniScope MS400 table-top X-band spectrometer from Magnettech. Simulations of the experimental EPR spectra were performed with the MATLAB EasySpin program. All samples were dissolved in dry and N2-saturated DCM at a concentration of ca. 1 mM. Oxidized forms were generated using ferrocenium hexafluoroadontinate (FcPF6, E1/2 = 0.450 V vs. SCE in DMF/0.1 M NBu4PF6). Calculations of g tensors were performed using the zeroth-order regular approximation (ZORA).

The calculations were sped up by employing the chain-of-sphere (RIJCOSX) approximation along with the decontracted auxiliary basis set of def2-SVP/δ coulomb fitting. Increased interaction between electrons (RI-MP2) and tight grids were used throughout the calculations. Solvent effects were included using a conductor-like screening model (COSMO) with a dielectric constant of 20.5. The closest value to the isopropanol environment was taken in the geometry optimization.

UV/Vis/NIR Spectroelectrochemistry. UV/Vis/NIR spectra were recorded on a FTIR TESCAN spectrometer (combined M5 UV/Vis/NIR and PUG NIR instrumentation) from HELMA quartz cuvettes with a 0.1 cm optical path length. The OTTLE (optically transparent thin layer electrolysis) cell used for spectroelectrochemical studies was laboratory-built according to the design by Hartl et al. and comprises a Pt-wire working electrode, a Pt-sheet counter electrode, and a Ag-sheet pseudo-reference electrode sandwiched between CaF2 windows. For regular absorption spectra, dry DMF was used as the solvent, while SEC experiments were conducted in a DMF electrolyte containing 0.1 M NBu4PF6. A BAS potentiostat was used to apply the necessary voltage to generate the reduced/oxidized species.

Stability Studies. The stability in DMSO-D6 at room temperature was assessed by 1H NMR over 96 h.

The stability of complex 1 in human plasma at 37 °C was evaluated following a slightly modified procedure already reported by our group. The human plasma was provided by Biowest. Caffeine (internal standard) was obtained from TCI Chemicals. Stock solutions of the complexes (10 mM in DMSO) and caffeine (10 mM in Milli-Q water) were prepared. An aliquot of the respective stock solutions was then added to the plasma solution (380 µL) to a total volume of 500 µL and final concentrations of 400 µM for the complexes and 2 mM for caffeine. The resulting plasma solution was incubated for either 0, 1, 3, 5, 16, or 24 h at 37 °C with continuous and gentle shaking (ca. 800 rpm). The reaction was stopped by the addition of 1 mL of methanol, and the mixture was centrifuged for 3min at 2000 rpm at room temperature. The methanolic solution was directly analyzed using HPLC with a total injection volume of 20 µL.

The HPLC conditions were as follows: HPLC: 0–3 min: isocratic 85% A (15% B), 3–7 min: linear gradient from 85% A (15% B) to 100% A (0% B), 7–22 min: isocratic 100% A (0% B). The mobile phase was isocratic 85% A (15% B), 3–7 min: linear gradient from 85% A (15% B) to 100% A (0% B), 7–22 min: isocratic 100% A (0% B). The resulting plasma solution was injected into the HPLC system for analysis.

Cytotoxicity Assay Using a 3Cellular Model. The cytotoxicity of the tested Ru complex was assessed by a fluorometric cell viability assay using Resazurin (Acros Organics). Briefly, cells were seeded in triplicate in 96-well plates at a density of 4 × 10³ cells/well in 100 µL. After 24 h, cells were treated with increasing concentrations of the ruthenium complexes and ligands. Dilutions for complexes 1–4 were prepared as follows: 1.25 mM stock in DMSO was diluted to 100 µM with medium and then filtered (0.22 µm filter). Dilutions of complexes 5 and 6 were prepared as follows: 1.25 mM stock in DMSO was diluted to 100 µM with medium and then filtered (0.22 µm filter). For Ru(DP)2Cl2, 2.5 mM stock in DMF was prepared, which was further diluted to 100 µM and filtered (0.22 µm filter). After 48 h of incubation, the medium was replaced, and 100 µL of complete medium containing resazurin (0.2 mg/mL final concentration) was added. After 4 h of incubation at 37 °C, the fluorescence signal of the resoruvin product was read (exc 540 nm, em 590 nm) in a SpectraMax M5 microplate Reader. IC₅₀ values were then calculated using GraphPad Prism software.

Generation of 3D HeLa MCTS. MCTS were cultured using ultra-low-attachment 96-wells plates from Corning (Fisher Scientific 1532974). HeLa cells were seeded at a density of 5000 cells per well in 200 µL. The single cells were cultured MCTS approximately 400 µm in diameter on day 4 at 37 °C in 5% CO₂.

Treatment of 3D HeLa MCTS. After 4 days of growing at 37 °C in 5% CO₂, MCTS were treated by replacing half of the medium in the well with increasing concentrations of compounds for 48 h in the dark. Untreated reference MCTS, half of the medium was replaced with fresh medium only. The cytotoxicity was measured via the ATP concentration with a CellTiter-Glo cell viability kit (Promega, USA).

HeLa MCTS Growth Inhibition. MCTS were grown and treated as described above. MCTS sizes were observed under a light microscope, and pictures were taken with an iPhone 6s thanks to a phone microscope adaptor. Before imaging, the plate was shaken, and half of the medium was exchanged to remove dead cells. Images were recorded before treatment (day 0) and on days 3, 6, 9, and 13 after treatment. Pictures were first processed using GIMP as a cross-platform image editor with a batch automation plug-in. The MCTS sizes were then calculated with SpheroidRuler, a MATLAB-based, open-source software application to measure the size of tumor spheroids automatically and accurately. Data analysis was done using GraphPad Prism software.
treatments. The medium was removed and replaced with a 10 μM solution of complex 1 or 1 μM staurosporine (positive control, ABCam cat no. 120056) and further incubated for 30 or 4 or 24 h. Cells were collected, washed twice with ice-cold PBS, and resuspended in 1X Anacronin V binding buffer (10X buffer composition: 0.1 M HEPES (pH 7.4), 1/4 M NaCl, 25 mM CaCl2). Samples were processed according to the manufacturer’s instructions (BD Scientific, cat. no. 564663 and 564619) and analyzed using a JES Biorad instrument at the Corometrics Platform, the ROCKLAND nuclear extract protocol was used. Data were analyzed using the FloVyo software.

**Sample Preparation for Cellular Uptake.** Cells were seeded at a density of 2 × 10^6. The next day, cells were treated with 5 μM 1 or RuCl₃(DIP)M. After 2 h, cells were collected, washed, and snap frozen in liquid nitrogen and stored at –20 °C. ICP-MS samples were prepared as follows: samples were digested using 70% nitric acid (1 mL, 60 °C, overnight) and then further diluted 1:100 (1% HCl solution). Samples were then further diluted 1:10 or 1:100 (1% HCl solution in MQ water) and analyzed using ICP-MS.

**Sample Preparation for Cellular Fractionation.** HeLa cells were seeded in three 15 cm² culture dishes so that on the day of treatment cells were 90% confluent. On the day of treatment, cells were incubated with the target complex at a concentration of 5 μM for 2 h. After that time, the medium was removed and cells were washed, collected, and counted. After resuspension in cold PBS, the organelles were isolated via different procedures. (One cell culture dish per isolation was used.)

**Mitochondrial Isolation.** To isolate mitochondria, a mitochondria isolation kit (cat. no. MitoIsol, Sigma-Aldrich) was used according to the manufacturer’s procedure for the isolation of mitochondria via a homogenization method.

**lysosome Isolation.** To isolate lysosomes, a lysosome isolation kit (cat. no. LYSIsol, Sigma-Aldrich) was used, according to the manufacturer’s procedure for the isolation of lysosomes via option C.

**Nuclear and Cytoplasmic Fractionation.** To isolate nuclear and cytoplasmic fractions was used. Briefly, cells were collected by centrifugation, resuspended in cytoplasmic extraction buffer, and incubated on ice. The tubes were centrifuged, and supernatant (CE) was removed. Pellets were washed with cytoplasmic extraction buffer without detergent and centrifuged. The pellet (NE) was resuspended in nuclear extraction buffer and incubated on ice. Both CE and NE were centrifuged. The supernatant from CE samples was indicated to be a cytoplasmic extract, whereas the pellet obtained from NE samples was indicated to be a nuclear extract.

ICP-MS samples were prepared as follows: isolated cellular fractions were lyophilized and digested using 5 mL of 70% nitric acid (60 °C, overnight). Samples were then further diluted (1:1000 for nuclear pellet samples and 1:100 for all the other samples) with MQ water (containing in 1% HCl solution) and analyzed using ICP-MS.

**Sample Preparation for Studies on the Mechanism of Cellular Uptake.** Samples were prepared as previously reported. Briefly, HeLa cells were seeded at a density of 2 × 10⁶ and the next day were pretreated with corresponding inhibitors or kept at specific temperatures for 1 h. Then, cells were washed with PBS and were incubated with 5μM complex 1 for 2 h. The low-temperature sample was kept at 4 °C. Afterward, cells were washed with PBS, counted, and snap frozen in liquid nitrogen. Pellets were stored at –20 °C. ICP-MS samples were prepared as follows: samples were digested using 70% nitric acid (1 mL, 60 °C, overnight), further diluted 1:100 (1% HCl solution in MQ water), and analyzed using ICP-MS.

**Metabolism of HeLa Cells.** Cells were seeded at a density of 2 × 10⁶. The following day, cells were treated with a 5 μM concentration of I or cisplatin. After 2 h, cells were collected, snap frozen in liquid nitrogen, and stored at –20 °C. The following day, DNA was extracted using a PureLink Genomic DNA Mini Kit (Invitrogen). The DNA’s purity was checked by absorbance measurements at 260 and 280 nm. Concentrations of genomic DNA were calculated assuming that one absorbance unit equals 50 μg/mL. ICP-MS samples were prepared as follows: samples were digested using 70% nitric acid (60 °C, overnight) in a 1:16 DNA to acid volume ratio. Samples were then further diluted 1:10 or 1:100 (1% HCl solution in MQ water) and analyzed using ICP-MS.

**ICP-MS Studies.** All ICP-MS measurements were performed on a high-resolution ICP-MS (Element II, ThermoScientific) located at the Institut de Physique du Globe de Paris (France). The monitored isotopes are ⁹⁹Ru and ¹⁰⁰Pd. Daily, prior to the analytical sequence, the instrument was first tuned to produce maximum sensitivity and stability while also maintaining low uranium oxide formation (UO₂/U ≤ 5%). The data were treated as follows: intensities were converted to concentrations using uFREASI (user-Friendly Elemental data processing). This software, made for the HR-ICP-MS users community, is free and available at http://www.ipgp.fr/~tharaud/uFREASI.html.

**ICP-MS Data Analysis.** Cellular Uptake Studies. The amount of metal detected in the cell samples was transformed from ppm to μg of metal. Data were subsequently normalized to the number of cells and expressed as μg/metal/number of cells.

**Cellular Fractionation.** The amount of detected ruthenium in the cell samples was transformed from ppm to μg of ruthenium. Values were then normalized to the number of cells used for specific extraction. Due to the low yield of lysosomae extraction (only 25%), the values obtained were multiplied by a factor of 4. Because of a low yield of mitochondrial extraction (50% of the cells were homogenized), the values obtained for that organelle were multiplied by a factor of 2. Extraction protocols allow for the isolation of pure subcellular fractions. Therefore, the total amount of metal found in the cells was calculated by summing the values obtained for the pure organelles.

**Mechanism of Uptake.** The amount of ruthenium detected in cell samples was transformed from ppm to μg of ruthenium, and the values obtained were normalized to the number of cells used for specific treatment. The value for the ruthenium found in the 37 °C sample was used as 100%.

**Cellular Metabolism.** The amount of ruthenium detected in cell samples was transformed from ppm to μg of ruthenium per g of tissue.

**JC-1 Mitochondria Membrane Potential Test.** HeLa cells were seeded at a density of 6000 cells/well in black 96-well plates (Costar 3516). The following day, cells were treated with different concentrations of 1 and RuCl₃(DIP). After another 24 h, cells were treated according to the instructions of the JC-1 Mitochondria Membrane Potential Assay Kit (Abcam, ab113850). The data were analyzed using GraphPad Prism software.

**Metabolic Studies.** HeLa cells were seeded in Seahorse XF96-well plates at a density of 30 000 cells/well in 80 μL of medium. After 24 h, the medium was replaced with fresh medium, and cisplatin (1 μM), doxorubicin (1 μM), 3-methoxycarbonyl-11 μM, complex RuCl₃(DIP) (1 μM), or complex 1 (1 μM) was added. After 24 h of incubation, the regular medium was removed, and cells were washed three times using Seahorse base media and incubated in a non-CO₂ incubator at 37 °C for 1 h.

**Mito Stress Test.** A Mito stress test was run using 1 μM oligomycin, 1 μM FCCP, and mixture of 1 μM antimycin-A/1 μM rotenone in ports A–C, respectively, using a Seahorse XF96 extracellular flux analyzer.

**Glycolysis Stress Test.** A glycolytic stress test was run using glucose (10 mM), oligomycin (1 μM), and 2-deoxyglucose (50 mM) in ports A–C, respectively, using a Seahorse XF96 extracellular flux analyzer.

**Mito Fuel Flux Test.** A fuel flux assay for the different fuel pathways, viz., glucose, glutamine, and fatty acid, was studied by measuring the basal oxygen consumption rates and that after the addition of the inhibitor of the target pathway in port A and a mixture of the inhibitors of the other two pathways in port B. This gave a measure of the dependency of the cell on the fuel pathway. To study the capacity of a certain fuel pathway, the sequence of addition of the inhibitors was reversed. In port A, the mixture of inhibitors for the other two pathways was added, and in port B, the inhibitor for the target
pathway was added. UK-5099 (pyruvate dehydrogenase inhibitor, 20 µM) was used as an inhibitor for the glucose pathway. BPTES (selective inhibitor of glutaminase GLS1, 30 µM) was used as an inhibitor for the glutamine pathway. Etoxim (O-carboxylalanine transferase-1 (CPT-1) inhibitor, 40 µM) was used as an inhibitor for the fatty acid pathway.

**Formulation of Compound 1 in Polysorbate 80.** Formulation Protocol. Compound 1 was formulated in polysorbate 80 using the film rehydration method. Briefly, compound 1 (2 mg, 2.2 µmol) and polysorbate 80 (100 mg) were dissolved in acetone (3 mL). The solvent was removed by rotary evaporation at 40 °C. The red film was rehydrated in PBS (2 mL) at room temperature. The solution was finally sterile-filtered on a 0.22 µm nylon membrane (Corning 431224) to yield a clear red solution.

**Compounds Concentration Determination.** The sample (50 µL) was diluted in 100 µL of acetonitrile, and the absorbance was recorded at 480 nm in 96-well plates from Corning (Fisher Scientific 15329740) using a Spectramax M5 microplate reader. The measurement was performed in triplicate, and the compound 1 concentration was determined using a calibration curve obtained under the same conditions (50 mg/mL polysorbate 80 in PBS/acetonitrile 1:3). The encapsulation efficiency was calculated by comparing the absorbance of the solution before and after filtration using the following equation:

\[
\text{encapsulation efficiency} = 100 \times \frac{\text{absorbance after filtration}}{\text{absorbance before filtration}}
\]

To ensure its repeatability, the procedure was performed in triplicate.

**Biodistribution Study.** This study was carried out in accordance with EU regulations and approved by the Ethical Commission of the Faculty of Pharmaceutical and Biological Sciences Paris-Desمرض (agreement no. 875-06-02).

Eight-week-old BALB/c mice (Janvier) were separated into three groups and injected intravenously with 5 mg/kg compound 1 formulated in polysorbate 80 (200 mg/kg) and PBS. After 30 min or 1 or 2 h, mice were sacrificed, and relevant organs including blood, liver, lungs, brain, intestines, spleen, and kidneys were harvested, weighed, and digested using 70% nitric acid (5 mL, 60 °C, 24 or 48 h for intestines), further diluted in 1:100 (1% HCl solution in MQ water), and analyzed using ICP-MS.

**ASSOCIATED CONTENT**

**Supporting Information**

- UV/Vis/NIR absorptions of the complexes in their native and electrochemically generated states in DMF; NMR spectra of complexes 1–6; HPLC traces at 450 nm of complexes 1–6; EPR spectra; computed and experimental g tensors; voltammograms recorded by CV and with the use of RDE for complexes 2–6; electrochemical data for complexes 1–4; UV/Vis/NIR spectroelectrochemistry data for complex 1 in the presence of reducing agent glutathione; chemical data for complexes 5 and 6; overlap of ^1^H spectra of complexes 1–6 in DMSO-d6, over 90 h, percentage concentration of complex 1 in human plasma; fluorometric cell viability assay; IC₅₀ values for catechols; CellTiter Glo viability test; cell death mechanism; cellular uptake mechanism of complex 1; oxygen consumption rates and different respiration parameters in HeLa cells alone or after treatment with various test compounds; extracellular acidification rate and different parameters during glycolysis in HeLa cells alone or after treatment with various test compounds; and fuel flex assay in HeLa cells (PDF)

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