Self-assembled ruthenium and osmium nanosystems display a potent anticancer profile by interfering with metabolic activity


We disclose novel amphiphilic ruthenium and osmium complexes that auto-assemble into nanomedicines with potent antiproliferative activity by inhibition of mitochondrial respiration. The self-assembling units were rationally designed from the [M(η6-cymene)(1,10-phenanthroline)Cl]PF6 motif (where M is either Ru or Os) with an appended C16 fatty chain to achieve high cellular activity, nano-assembling and mitochondrial targeting. These amphiphilic complexes block cell proliferation at the sub-micromolar range and are particularly potent towards glioblastoma neurospheres made from patient-derived cancer stem cells. A subcutaneous mouse model using these glioblastoma stem cells highlights one of our C16 Os nanomedicines as highly successful in vivo. Mechanistically, we show that they act as metabolic poisons, strongly impairing mitochondrial respiration, corroborated by morphological changes and damage to the mitochondria. A genetic strategy based on RNAi gave further insight on the potential involvement of microtubules as part of the induced cell death. In parallel, we examined the structural properties of these new amphiphilic metal-based constructs, their reactivity and mechanism.

Introduction

Anticancer metal complexes have been given continuous attention since the discovery of cisplatin, and platinum-based drugs are still widely used today. Lack of selectivity leads to side effects and resistance mechanisms are still of major concern. The success of platinum drugs has led to novel metal-based candidates and turned attention to neighboring transition elements. Among these, ruthenium, osmium, iridium, rhodium, titanium, rhenium, and gold are under study.

In the development course of Ru anticancer complexes, octahedral Ru complexes with anti-metastatic properties such as NAMI-A, KP1019 and NKP-1339 were initially advanced. NAMI-A was the first Ru compound to enter clinical trials, in combination with gemcitabine for treatment of lung cancer, but failed in phase II due to low efficacy. NKP1339 completed a phase I dose escalation study to treat advanced solid tumors in 2016 and was considered successful.

Development of pseudo-octahedral Ru organometallic complexes, mostly from the Sadler and Dyson groups, has come to prominence in recent years. Two major types of compounds were advanced: (1) metal derivatives with the general formula [Ru(η6-biphenyl)(N,N′-bidentate)(X)]+ from Sadler et al. and (2) [Ru(η6-toluene)Cl2(pta)][RAPTA-T] and RAPTA-type derivatives from Dyson et al. Known as RM175, has shown in vitro and in vivo activity on primary tumors and cisplatin-resistant cell lines at the low micromolar range; the latter have limited activity in vitro but good anti-metastatic properties in vivo.

Osmium analogues naturally follow as the next row element to ruthenium. These low-spin d6-complexes are more kinetically inert than their lighter Ru analogues, as observed for the Os NAMI-A analogues. Anticancer activity of various Os complexes (OsII, OsIII, OsVI) has been reviewed, among them the OsII-arene complex FY026 bearing an azopyridine ligand.
exhibits submicromolar IC₅₀ on the ovarian and breast cancer cell lines A2780 and MCF-7, and is 50 times more potent than cisplatin on the 809 cancer cell line panel from the Sanger Institute Cancer Genome Project.³⁵,³⁶

We have prepared original nano-constructs made from Ruᴵᴵ and Osᴵᴵ pseudo-octahedral amphiphilic self-assembling units with a promising anticancer profile, including high cellular accumulation and metabolic targeting. Similarly to Lippard et al.,³⁷ we appended a C₁₆ alkyl chain on the metal ligands, in an effort to address their usual low potency.³⁸–⁴⁰ The added lipophilicity resulted in a 100- to 300-fold increase in the anti-proliferative potency,⁴¹–⁴³ with IC₅₀ values typically far below the micromolar range, which is around 10 times more potent than for cisplatin. The lipophilic chain enables the spontaneous formation of nanostructures, binding to human serum albumin (HSA) and mitochondrial targeting, in an attempt to develop novel metabolic nanomedicines that specifically impair cancer cell types that rely on mitochondrial respiration.⁴⁴ Self-formation of nanoparticles from bioactive metal complexes has been scarce in the literature;⁴⁵–⁴⁸ none were reported for osmium complexes.

This success led us to test these novel agents on glioblastoma stem cells (GSCs) and derived mouse models. Glioblastoma (GBM), the most common and lethal form of glioma, is associated with an extremely poor prognosis: the five-year survival rate is below 5% and the median survival is around 16 months.⁴⁹ GBM contains self-renewing, tumorigenic cancer stem cells that contribute to tumor initiation and therapeutic resistance to common treatments.⁵⁰ Our metal-arene nanoassemblies were highly effective on patient-derived GSC neurospheres. We demonstrate promising in vivo efficacy on a GBM mouse model, with 40% of the Os-treated mice as long-term survivors, and much slowed tumor growth for the others.

Our final efforts focused on the cellular mechanisms conveying this promising anticancer profile, through an in-depth mechanistic study. Detailed cellular investigation and electron microscopy images indicated extensive mitochondrial damage, while an interfering RNA (RNAi) genetic assay further pointed towards tubulin and microtubules, highlighting an original mechanism of action that may involve multiple targets beside the mitochondria, and also operate by different mechanisms than classical anticancer drugs.

Results and discussion

Synthesis and solution-state conformation

The metal complexes were synthesized from the dinuclear µ-chloro precursors and the C₁₆-functionalized N,N-bidentate ligands, through ester and amide linkages at the C₅ position (Scheme 1). This led to four amphiphilic assembling units (1–4) that differ by the metal center and the linking group. Parent compounds (5–6) were also prepared for validation of our design. All characterization data, including ¹H and ¹³C NMR, IR, HRMS, HPLC purity and UV-Vis spectra can be found in the provided ESI (Fig. S1–S33†). Suitable crystals for X-ray diffraction were unsuccessful despite numerous attempts, and we therefore investigated the ground-state geometries by DFT and NMR spectroscopy. This allowed determination of the relative position of the phenanthroline ligand to the arene moiety, and the conformation of the isopropyl group (Fig. S34A–E†). The most stable structure is found to have the chloride ligand pointing out in the same direction as the isopropyl group.

Self-assembling and binding to albumin

The amphiphilic design leading to self-assembling units was verified and aqueous solutions of 1–4 were examined by dynamic light scattering (DLS) to determine particle size and distribution, as well as the critical aggregation concentration (CAC). Hence, aggregation of 1–4 into nanoassemblies occurred at around 5 µM (Fig. S35At). The median diameter of particles formed above the CAC were found around 100 nm with a positive surface potential, indicating the cationic nature of the nanoparticles (Fig. S35B†). In addition to the requested size for enhanced permeation and retention at tumor sites,⁵¹ this appears beneficial for cancer targeting: in contrast to normal cells, cancer cells overexpress anionic surface molecules such as phosphatidylserine, O-glycosylated mucins, sialylated gangliosides and heparan sulfates.⁵²–⁵⁶ Solutions of 2 and 4 were further analyzed by transmission electron microscopy (TEM), showing self-assembling into spherical nanoparticles (Fig. 1A–C) with a median diameter of around 85 nm for 2 and 155 nm for 4 (see distribution frequencies in Fig. 1B–D), in agreement with the results obtained from DLS (Fig. S35B†).

Human serum albumin (HSA) is the most abundant plasma protein and is involved in the transport of drugs, metal ions, metal complexes and fatty acids through the bloodstream.⁵⁷ We supposed it might efficiently bind our C₁₆ complexes and provide, together with the self-assembling, bloodstream transportation and long circulating times.⁵⁸ We measured the binding parameters through the fluorescence quenching of a key Trp residue in the main drug binding site (Fig. S45†),⁵⁹,⁶⁰ using the modified Stern–Volmer equation (ESI eqn (1)†).⁶¹ Binding affinities were found two to three times higher for 1 (Kₐ = 4.6 × 10⁸ M⁻¹) and 2 (Kₐ = 5.6 × 10⁴ M⁻¹) than for compound 5 (Table S1†). Osmium complexes 3 and 4 (Kₐ = 4.6 × 10⁴ M⁻¹) bind slightly better to the protein than 6 (Kₐ = 3.1 ×
Compound 2 was subsequently docked into HSA binding site I to visualize the interaction (Fig. S44B†). Binding thermodynamics were determined from the van’t Hoff equation and the negative ΔG° obtained for all compounds indicate a spontaneous association between the protein and the metal complexes. The ΔH° and ΔS° for all compounds remained negative (Table S1†), indicative of van der Waals interactions and/or hydrogen bonding.62 Reactivity in aqueous solution

Aquation of halido complexes renders the metal center reactive towards DNA and other bionucleophiles.32,63 Experiments to examine the aquation of 5 and 6 have been previously published and reported experimental half-lives are 22 min (k = 1.8 h⁻¹) for 5 at 37 °C64 and 9 h (k = 0.073 h⁻¹) for 6 at 45 °C;13 Os complexes thus being more inert towards aquation than their Ru analogues. Aquation of 2 and 4 were followed in 7 : 3 DMSO-d₆/D₂O at 37 °C for 72 h. For compound 2, ³H-NMR revealed the formation of about 5% of the aqua complex (Fig. S36B†), indicative of van der Waals interactions and/or hydrogen bonding.62

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Glutathione (GSH) is a major reducing agent and responsible for cellular detoxification of ROS and transition metals. Cancer cells may become resistant to platinum drugs by increasing their GSH levels. The reaction of 2 in 7:3 DMSO-d6/D2O with GSH was thus monitored by 1H-NMR for 72 h at 37 °C. A new set of signals with small intensities in the metal-arene and phenanthroline regions indicates the formation of new species from 2. Incubation of 2 at 37 °C for 24 h with 10 equiv. GSH and 1.5 equiv. AgPF6 allowed the formation of the GSH adduct, that is observed in the MS spectrum in small quantities in comparison with the starting compound 2, and in agreement with 1H-NMR (Fig. S41 and S42†). The 7.04 ppm resonance (Fig. S41B†) is characteristic of the p-cymene release,68 which was also confirmed by MS with the presence of metal-GSH derivatives without their p-cymene and where the three coordination sites of the arene are bound to solvent molecules (Fig. S42†).69–71 Our Os analogue 4 was inert toward GSH (Fig. S41C†) but for compound 2, 1H-NMR and MS showed formation of GSSG, which could suppose a potential catalytic activity of 2 and 4 with GSH. Therefore, to highlight a potential role of GSH and thios in the detoxification of 2 and 4, cytotoxicity assays on A549 and Hs683 were incubated during 72 h with the cysteine precursor N-acetylcysteine (NAC) (Fig. S43†). In case of GSH depletion by the metal complexes 2 and 4, NAC would restore its levels, act as a ROS scavenger and lower the antiproliferative activity of the complexes.72 We observed a weak but significant IC50 increase on the A549 cell line but not for Hs683, which suggests that GSH-depletion either by a direct or indirect mechanism may play a small role but is not a major mechanism involved in the activity.

Reaction with DNA nucleobases were finally considered and complexes 2 and 4 were incubated with 10 equiv. of 9-methylguanine (9MeG), 1.5 equiv. AgPF6 at 37 °C for 24 h and analyzed by MS. Similarly to the aquation and GSH reactions, the 9MeG adduct was only observed for the Ru complex 2, and in small quantity (Fig. S42C†).

Theoretical and experimental evidence thus showed slow aquation and limited reactivity with S-donors. The reactivity of complexes 2 and 4 toward bionucleophiles (aquation, glutathione, 9-methylguanine and human serum albumin) can here be considered as low and may not fully explain the potent activity. Despite differential reaction kinetics towards hydrolysis and GSH, our RuH and OsH amphiphilic complexes exhibited similar IC50 on a panel of cancer cell lines, which turned our attention to another possible mechanism of activation, comprising arene loss. We show oxidative loss of the arene moiety under physiological conditions in the presence of GSH, and this suggests it as a possible mechanism of reaction with intracellular targets, probably beside others.73 Figures and experimental details are given ESI (Fig. S36–S43†).

Cytotoxicity profiles and cellular accumulation

The in vitro anticancer activity was first determined by means of MTT assays (Table 1 and Fig. S46†) on five cancer cell lines; the non-small cell lung cancer cell line A549 (human lung carcinoma), MCF-7 (human breast adenocarcinoma), Hs683 (human glioma), B16F10 (murine skin melanoma) and M109 (murine lung carcinoma). The antiproliferative effects were compared to the parent phenanthroline compounds 5-6,41,43,64 with IC50 values in the range of 50–100 µM. In contrast, the C16 amphiphilic complexes 1–4 showed a dramatic increase of activity with IC50 values around 0.25 µM (Table 1). This sub-micromolar growth inhibition is rare, and only a few half-sandwich Ru and Os complexes with such activities were reported to date.38,71,74 It is also worth noting the improved antiproliferative activity of complexes 1–4 compared to cisplatin, notably 5- to 25-fold enhancement in human cancer cell lines.

We hypothesize that the amphiphilic nature of these assembling units enhances cellular accumulation to improve the anticancer effect. The intracellular metal contents were thus determined for all compounds by ICP-MS on the A549 and B16F10 cell lines. This revealed that the alkyl chain induced a 10- to 100-fold increase on the penetration rate of the compounds (Fig. 1E and F). Experimental log P for the six complexes were determined using the shake-flask method and correlated to both the MTT IC50s and the cellular accumulation

Table 1 In vitro growth inhibition as determined by MTT assays on five cancer cell lines (IC50, µM)

<table>
<thead>
<tr>
<th>Entry</th>
<th>A549 (µM)</th>
<th>Hs683 (µM)</th>
<th>MCF-7 (µM)</th>
<th>B16F10 (µM)</th>
<th>M109 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.35 ± 0.05</td>
<td>0.35 ± 0.04</td>
<td>0.26 ± 0.13</td>
<td>1.18 ± 0.45</td>
<td>2.99 ± 0.74</td>
</tr>
<tr>
<td>2</td>
<td>0.31 ± 0.07</td>
<td>0.25 ± 0.04</td>
<td>0.54 ± 0.11</td>
<td>3.04 ± 1.67</td>
<td>15.0 ± 6.20</td>
</tr>
<tr>
<td>3</td>
<td>0.28 ± 0.03</td>
<td>0.26 ± 0.11</td>
<td>0.22 ± 0.07</td>
<td>0.88 ± 0.52</td>
<td>8.98 ± 1.33</td>
</tr>
<tr>
<td>4</td>
<td>0.69 ± 0.07</td>
<td>1.58 ± 0.81</td>
<td>1.01 ± 0.33</td>
<td>2.33 ± 0.23</td>
<td>2.96 ± 0.08</td>
</tr>
<tr>
<td>5</td>
<td>65.4 ± 13.1</td>
<td>55.2 ± 16.3</td>
<td>75.2 ± 3.8</td>
<td>70.6 ± 16.5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>6</td>
<td>82.8 ± 3.4</td>
<td>77.9 ± 10.4</td>
<td>73.1 ± 5.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.72 ± 0.16</td>
<td>1.12 ± 0.20</td>
<td>8.75 ± 0.60</td>
<td>9.38 ± 0.75</td>
<td>0.97 ± 0.19</td>
</tr>
</tbody>
</table>
profiles (1.49 ± 0.06 for 1, 1.31 ± 0.06 for 2, 1.57 ± 0.08 for 3, 0.54 ± 0.05 for 4, −0.90 ± 0.02 for 5 and 0.29 ± 0.01 for 6, Fig. 1G), increasing with the addition of the C16 chain and inversely correlating to the IC50. They are similar to those observed for lipophilic PtIV prodrugs.

Cellular effects
First, changes in cell motility and mitotic function upon treatment were followed using quantitative video-microscopy (QVM). After 24 h treatment with 1–4, A549 cells elongated without entering mitosis or apoptosis, suggesting a cytostatic activity (Fig. S47†). Additional details from the QVM experiments are given in ESI (Fig. S48†).

Apoptotic cell death and cell cycle arrest at the G2/M phase are known to occur following treatment with DNA-targeting platinum compounds.73 Considering that apoptosis was also induced by some Ru and Os compounds,76,77 we decided to investigate it.78 Compounds 1–4 revealed no apoptosis after 72 h at their IC50 (annexin V/propidium iodide staining [PI], Fig. S49A†), which confirmed observations from QVM. No changes in the cell cycle profile were either observed, suggesting a DNA-damage independent activity (Fig. S49B†). We thus looked at DNA damage through histone phosphorylation and γH2AX levels to confirm the DNA-damage independent activity. Low levels of γH2AX were observed with 2 and 4, unlike gene-mediated cytotoxic immunotherapy (GMCI) which is known to induce DNA damages,79 used here as positive control (Fig. 1I).

It is now well understood that metallodrug-induced cytotoxicity is associated to increased ROS generation, therefore we turned to oxidative damage,36,80 and ROS were measured at 15, 24 and 48 h after treatment. A significant increase in ROS production was observed for the OsⅡ compounds 3 and 4, with respect to RuⅡ complexes 1 and 2 at 24 h, and all compounds increased ROS production at 48 h (Fig. 1H). Similar observations were made on the Hs683 glioma cell line but ROS reached their highest level after 24 h (Fig. S50C and D†).

Numerous metal-based compounds have been reported for their ability to modulate autophagy,81 which is a promising alternative strategy that we intended to explore for our amphiphilic compounds. Acridine orange (AO) staining after 48 h treatment with RuⅡ compounds 1 and 2 revealed critical accumulation of acidic vesicular organelles in the perinuclear region when compared to untreated cells (Fig. S51†), suggesting an autophagic process.82 Cells treated with 3 and 4 showed lower levels of AO when compared to the Ru complexes, and did not adopt the spherical shape, indicating of lower critical damage and weaker cell toxicity. Positive AO staining and lack of apoptotic cell led us to examine LC-3b, a specific marker of autophagy. G9pCDH GSCs treated with compounds 2 and 4 however did not display changes in LC-3b expression (Fig. 1J).

We also sought to highlight morphological changes induced by our amphiphilic compounds to cancer cells. Untreated A549 cells (Fig. 2A) have numerous lamellar bodies (LB) in the cytoplasm as well as functional mitochondria and endoplasmic reticulum (ER). Unlike controls, cells treated with 2 and 4 showed a significant decrease of LB, probably consecutive to the impaired metabolism and protein synthesis, which would arise from damaged mitochondria and deformed ER (Fig. 2B and C). In both treated conditions, the Golgi apparatus was kept intact. Treatment with 4 (Fig. 2C) showed similar damage with enlarged and elongated mitochondria, fragmented ER and the presence of multivesicular bodies. In contrast to cellular features observed under apoptotic conditions and by comparison with untreated cells, the integrity of the cytoplasmic and nucleic membranes were relatively well conserved following treatment.83 Similar observations were made with autophagy-inducing RuⅡ β-carboline complexes.84
Targeting mitochondrial respiration

Results obtained from this preliminary assessment allowed us to consider metabolism and the mitochondria as central in the mechanism of action of these new nano-assemblies made of amphiphilic complexes. We then treated A549 cells with compounds 2 and 4 via pneumatic injection in a Mito-stress assay and analyzed the changes in oxygen consumption rate (OCR). Known oxidative phosphorylation (OXPHOS) inhibitors were used: oligomycin, a complex V inhibitor, to view the basal OCR, FCCP (an uncoupler) used to observe the maximum OCR, and rotenone/antimycin A (Rot/AA), a complex I/III inhibitor respectively which then completely shuts down the electron transport chain (ETC). The addition of these inhibitors in sequential order allows for extrapolation of key bioenergetic parameters (Fig. 3A and B). We observed that compounds

Fig. 3  Seahorse XFe96 Cell Mito Stress profile for compounds 2 (A) and 4 (B). Bioenergetic parameters were extracted from the oxygen consumption rate (OCR) plot: spare respiratory capacity and ATP-linked OCR for compounds 2 (C and D) and 4 (E and F) on A549 cells. ATP-rate was extracted from the OCR/PER ratio to give contributions of glycolytic and mitochondrial ATP for compounds 2 (G) and 4 (I). Measurement of mtROS by flow cytometry (MitoSox dye) on A549 cell line with compounds 2 (H) and 4 (J) at their IC50 and twice their IC50 for 24 h. Mitochondrial membrane potential (MMP) measurement by flow cytometry (JC-1 staining) on A549 cell line with compounds 2 (K) and 4 (K) at their IC50 for 24 h. FCCP as positive control.
2 and 4 at concentrations as low as 300 nM significantly inhibited maximal respiration and spare respiratory capacity (Fig. 3C–E and S52A–C†). The acute loss of these two parameters signifies an immediate impact on mitochondria homeostasis. A significant loss of ATP production and a decrease in coupling efficiency were also observed for both compounds at 300 nM, suggesting that mitochondrial OXPHOS efficiency is acutely compromised (Fig. 3D–F and S52B–D†). Further bioenergetic parameters were extracted from the real time ATP-rate assay to give contributions of both drug concentrations (adjusted to achieve the IC₈₀, IC₈₅ and S₅₂B化合物 at 300 nM, suggesting that mitochondrial decrease in coupling efficiency is acutely compromised (Fig. 3D–F and S52†). Both compounds drastically reduced mitochondrial ATP production and at the IC₅₀, only glycolytic energy remains, indicative of a shift in metabolic dependency. We then sought to determine the level of mitochondrial ROS (mtROS) after exposure to compounds 2 and 4 at both their IC₅₀ and twice their IC₅₀ for 24 h (Fig. 3H–J and S54†). 2 and 4 significantly increase mtROS; up to three times compared to the control and up to twice compared to the known uncoupler (FCCP). Finally, we exposed A549 cells with compounds 2 and 4 at their IC₅₀ for 24 h to analyze the effect on the mitochondrial membrane potential (MMP, Fig. 3K). Both compounds cause severe loss of MMP, which is emphasized by the loss of red fluorescence (PE-A FACS channel, Fig. 3K). All together these results revealed a strong metabolic effect on bioenergetic health, which could directly target cancer cells and their high energy dependency, essential to sustain tumor proliferation, progression and to evade immunosurveillance. The rapid onset of mtROS production in conjunction with OXPHOS deficiency suggests a direct interaction with mitochondria and perturbation of the metabolic capacity of the cells.

RNA interference signatures

We performed multivariate genetic measurements to further probe underlying cellular mechanisms at play. This “ab initio” method, which makes no assumptions, is based on gene deletion effects that are compared to a vast set of known anticancer drugs, using supervised and unsupervised machine learning. K-Nearest neighbors (KNN), principal component analysis (PCA) and t-distributed stochastic neighbor embedding (t-SNE) clustering were then applied. To assess gene deletion effects on drug-induced cell death, murine lymphoma (Eμ-Myc) cell lines were separately infected with eight shRNA-GFP tagged vectors targeting critical proteins: p53, ATR, Chk1, Chk2, ATX, DNA-PK, BOK and Bim4 (Fig. S55†). A resistance index (RI; knocked down versus wild type, see ESI†) was then determined on the eight cell lines at three different drug concentrations (adjusted to achieve the IC₅₀, IC₈₅ and IC₉₀, Fig. S56A and B†). Cisplatin and doxorubicin were used as positive controls and were correctly classified as DNA cross-linking agents and topoisomerases II inhibitors, respectively. The obtained dendrogram (Fig. 4A) emphasizes three subgroups based on cellular responses: (1) cisplatin and doxorubicin, (2) complex 2 and (3) complexes 1, 3 and 4. Unlike cisplatin and doxorubicin, p53 and Chk2 are not involved in the antitumor activity of compounds 1, 3 and 4; 2 being the only complex in the series to be p53 and Chk2 dependent, but not as strongly as for cisplatin and doxorubicin. Compounds 1, 3 and 4 have negative RIs for the p53 knocked down cell line, in contrast to cisplatin, doxorubicin and 2 for which p53 mutations produce resistant phenotypes. We can here foresee an advantage at targeting p53-mutant tumors. The 2D t-SNE (Fig. 4B) distinctly highlights the close proximity of 2 to the central dogma disruptor category, that includes a wide variety of anticancer drugs such as DNA transcription and replication inhibitors, mTOR inhibitors and proteins synthesis inhibitors. The t-SNE map shows 1, 3 and 4 to be close to the mitotic spindle poisons, the histone deacetylases (HDAC) group and DNA methyltransferase (DNMT) inhibitors.

From KNN predictions, compounds 1, 3 and 4 are categorized as spindle poisons and compound 2 as a nucleic acid synthesis inhibitor, which partially correlates with the t-SNE predictions. However, a kernel density estimate determined insignificant p-values (<0.1) for compounds 1–4 (suggesting an alternative mechanism of action, different from those of “pure” mitotic spindle poisons for compounds 1, 3 and 4 and different than nucleic acid synthesis inhibitor for compound 2). Proper classification from supervised predictions is indeed obtained if p-values are significant and, if not, it would predict a novel or balanced mechanism of action.

Vinca alkaloids inhibit microtubule polymerization and mitotic spindle destabilizers have an inhibitory effect on the autophagy process by blocking maturation of autophagosomes,89,90 which in our case may explain the lack of induction of autophagy, low LC₃-B levels (Fig. 1J) and low abundance of late autophagic structure (Fig. 2B and C). Similarly, Sadler et al. reported in 2013 a series of osmium iminopyridine complexes sharing similar cancer cell selectivity patterns to vinblastine, using the COMPARE algorithm, but with no direct effect on tubulin polymerization.91 More recently, Ru¹¹ poly(pyridyl) complex (RPC2) appears to act as microtubule stabilizers in vitro but not as taxane derivatives do. The same study also revealed intriguing similarities with our series of amphiphilic complexes, without disruption of the cell cycle at the IC₅₀ and the induction of mitochondrial damage.92 Ru¹¹ arene complexes targeting plectin, a cytolinker protein associated to non-mitotic microtubules, were recently synthesized and studied by means of proteomics.93

According to the shRNA data that pointed out microtubules as a potential target, we sought to determine the effect of 2 and 4 on cytoskeleton dynamics (Fig. 4C). Compounds 2 and 4 seem to have a slight stabilizing effect on tubulin polymerization, yet much lower than the positive control paclitaxel. V₅₀ values of 2 and 4 (3.4 and 3.6 OD per min respectively) are closer to the negative control V₅₀ (0.9 OD per min) than to paclitaxel (12.3 OD per min). V₅₀ is increased by four with paclitaxel and decreased approximatively by a 3.5-fold with colchicine. Complementary to the tubulin polymerization assay, immunostaining experiments were able to provide additional information. Untreated cells revealed the spreading of microtubules around the nucleus and throughout the cytoplasm. In
contrast, microtubules in cells treated with 4 appeared more
condensate around the nucleus and stretched out through
their cytoskeleton extremities, a morphology change that was
already observed from QVM. Cells treated with 2 adopted a
spherical shape indicative of cell death (Fig. 4D), emphasizing
the higher toxicity of RuII compound 2 compared to the OsII
compound 4, as seen in the AO staining experiment. These
results support the idea of an alternative mechanism of action
in which microtubules could be involved but probably not as
the main target, that would remain disruption of cellular
metabolism and mitochondrial respiration.

**Glioblastoma models**

Glioblastoma remains one of the most aggressive and challen-
ging forms of cancer. The presence of glioblastoma initiating
cells in the tumor microenvironment provides self-renewing
populations of tumor cells bearing enhanced tumorigenic
properties. Current chemotherapies, such as temozolo-
mide (TMZ) and cisplatin, have been shown to be less efficient
towards these populations and despite reducing the tumor
mass, would allow cancer relapse from resistant GSCs. Owing
to the amphiphilic nature of the compounds, their metabolic
disruptor profile, and our recent work on glioblastoma and GSCs, we anticipated that they could be potent in
this model. This assumption was supported by the evidence
that GSCs are highly dependent on the OXPHOS machinery
compared to more differentiated GBM cells to assure their
proliferation. Thereby, targeting CSGs seems particularly
appropriate for our energy disruptor nanoassemblies. The use
of 3D-spheroid models made of GSCs is insightful to better
depict in vivo characteristics and therefore these models are
powerful tools for in vitro drugs screening assays. Hence, we
used the growth in low attachment assay (GILA), to evaluate
the antiproliferative activity of complexes 1 and 3 on two GEP-
expressing patient-derived GSCs, i.e. G9pCDH and G30pCDH.

The IC50 obtained on G9 GSCs for 1 and 3 were 0.5 µM and
2.4 µM respectively, which is far more potent than TMZ, the
standard of care for glioblastoma (IC50 = 7 µM) (Fig. 5A). The
amphiphilic complexes, reached the potency of cisplatin (IC50
around 1 µM) and surpassed recently reported OsVI nitrido
compounds, being at least 10 times more potent.

Compounds 1 (IC50 = 1.1 µM) and 3 (IC50 = 2.0 µM) display
similar antiproliferative potency to cisplatin (IC50 = 0.8 ±
1.2 µM) on the more resistant G30-derived spheroids, but they
appear more active than TMZ (IC50 = 11 ± 3 µM, Fig. 5B). We
can note that at 5 µM there were no surviving cells and that
compound 1 was more potent than its osmium analog 3 on
both GSC lines.
Nude mice were implanted subcutaneously with G9pCDH cells and the fluorescence was monitored together with tumor size. Compounds 2 and 4 were injected intratumorally at the indicated timepoints (Fig. 5C–E). Tumor volume and fluorescence markedly increased in the control group, unlike the Os-treated group that showed no tumor growth over time (Fig. 5C–E), confirming its promising in vitro antiproliferative activity and neurosphere potency. 40% of the mice from the Os-treated group were long-term survivors without visible signs of remaining tumor cells. The group initially treated with the Ru compound 2 was discarded due to the appearance of edema after injection and the apparent lack of efficacy. These in vivo experiments thus highlight again a relative weaker toxicity of the OsII compound 4 in comparison to the RuII compound 2, which caused edema and necrosis at the injection site, thereby limiting the frequency of injection, and was therefore not able to prevent tumor growth.

We have thus developed here antiproliferative metal-arene units that spontaneously assemble into nanostructures under physiological conditions, resulting in particles of the requested size for good permeation and retention at tumor sites. The four newly synthesized amphiphilic organometallic complexes...
bear high antiproliferative activity on human cancer cell lines A549, MCF-7 and Hs683, owing to the G_{16}-modification of the N,N-bidentate ligand. This lipophilic tail dramatically enhanced the cell penetration of the complexes, conferring previously scarce in vitro potency to metal-arene pseudo-octahedral Ru and Os compounds. Unaltered cell cycle and QVM suggest that extensive DNA damage is not responsible for the activity. The increase in ROS contents in the A549 and Hs683 cancer cell lines indicates some degree of cellular stress, but rather as a secondary effect. Genetic assays based on shRNA knocked downs pointed out similarities between cellular responses triggered by mitotic spindle poisons and our amphiphilic constructs, but apparently without direct interaction with tubulin. From the t-SNE maps, we also noticed spatial proximity with HDAC/DNMT inhibitors, and our amphiphilic complexes could therefore share similitudes in terms of cellular response with some epigenetic modulators.

The in vitro efficacy of our metal-organic nanoassemblies surpassed that of cisplatin and although cancer stem cells are usually less sensitive to anticancer drugs,^{50,96} 1 and 3 have demonstrated promising activity on the GSC neurospheres, which are known to be resistant to conventional chemotherapies. Glioma cells are highly dependent on the OXPHOS machinery for ATP production,^{192,103} and detailed in vitro investigations on a subcutaneous glioma model confirmed the efficacy of the osmium compound 4, that achieved complete tumor regression. TEM revealed mitochondrial damage, and a detailed in vitro assay showed direct OXPHOS inhibition. We indeed demonstrate potent disruption of cell metabolism and mitochondrial function, with loss of mitochondrial ATP, increase of mtROS and mitochondrial membrane depolarization. Recent reports on CuII complexes functionalized with a mitochondria-penetrating peptide showed activity on breast cancer stem cell mostly through induction of mitochondrial structure and function, being particularly potent on aggressive breast cancer cell line. All together, this suggests that metallo drugs bearing a positive charge and balanced lipophilicity can accumulate inside mitochondria, offering promising alternatives to tackle tumor cells via non-nuclear or genomic pathways.

**Conclusion**

To summarize, we disclosed here a novel series of nano-assembling and highly potent Ru and Os metal-arene complexes that are active on in vitro and in vivo glioblastoma stem cells models, through multi-targeted cellular disruptions that comprise poisoning of the cytoskeleton and mitochondrial processes. This original metabolic and multi-target profile from self-assembling nanoparticles, active on cancer stem cells, will be profitable to avoid resistances and relapse of tumor growth after treatment, warranting further in vivo studies on cancer models.

**Conflicts of interest**

The authors declare no conflicts of interest.

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**References**


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