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Exploring the cytotoxicity, uptake, cellular response, and proteomics of mono- and dinuclear DNA light-switch complexes.

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Drug resistance to platinum chemotherapeutics targeting DNA often involves abrogation of apoptosis, and has emerged as a significant challenge in modern, non-targeted chemotherapy. Consequently, there is great interest in the anti-cancer properties of metal complexes - particularly those that interact with DNA - and mechanisms of consequent cell death. Herein we compare a parent cytotoxic complex $[Ru(phen)_2(tpphz)]^{2+}$ [phen = 1,10-phenanthroline, tpphz = tetrapyridyl [3,2-a:2',3'-c:3'',2''-h:2''',3'''-j] phenazine], with a mononuclear analogue with modified intercalating ligand, $[Ru(phen)_2(taptp)]^{2+}$,[taptp = 4,5,9,18-tetraazaphenanthreno[9,10-*b*] triphenylene], and two structurally related di-nuclear, tpphz-bridged, heterometallic complexes, RuRe and RuPt. These changes result in a switch from intercalation to groove binding DNA interaction, concomitant reduction in cytotoxic potency, but no significant change in relative cytotoxicity toward platinum-resistant A2780CIS cancer cells, indicating that DNA interaction mode is not critical for the mechanism of platinum resistance. All variants exhibited a light-switch effect, which for the first time, was exploited to investigate timing of cell death by live cell microscopy. Surprisingly, cell death occurred rapidly as a consequence of oncosis, characterized by loss of cytoplasmic volume control, absence of significant mitochondrial membrane potential loss, and lack of activation of apoptotic cell death markers. Importantly, a novel, quantitative proteomic analysis of the A2780 cell genome following exposure to either mononuclear complex reveals changes in protein expression associated with global cell responses to oxidative stress, and DNA replication/repair cellular pathways. This combination of a multiple targeting modality and induction of a non-apoptotic death mechanism makes these complexes highly promising chemotherapeutic cytotoxicity leads.

Introduction

The spectacular success of cisplatin as an anticancer therapeutic has led to huge growth in research into the interaction of metal complexes with biomolecules. Initial work focussed on Pt^{II}-based analogues of cisplatin, [1-8] although these studies have broadened out to include other metal centres. [9-12] These systems are genotoxic as they irreversibly bind to DNA. In the case of cisplatin, its most prominent effect is to generate platinated intrastrand lesions through coordination at adjacent G-sites, [13-15] triggering DNA repair mechanisms [1, 16-17] that ultimately lead to mitochondrial-mediated apoptosis. Despite a consistent rate of initial responses, cisplatin treatment often results in the development of chemoresistance, leading ultimately to therapeutic failure. Consequently, there is a considerable interest in the development of systems which may be used to overcome this challenge.

As a chemotherapeutic that induces apoptosis, [1, 16-17] cisplatin is not unusual, most anti-cancer therapeutics are pro-apoptotic. [18-19] This has implications for the treatment of therapeutically-resistant cancers as mechanisms of intrinsic or acquired chemotherapy resistance often involve abrogation of apoptotic pathways. [20-24] Approximately one third of all solid cancers respond poorly to current treatments due to insensitivity to pro-apoptotic signalling. [25]

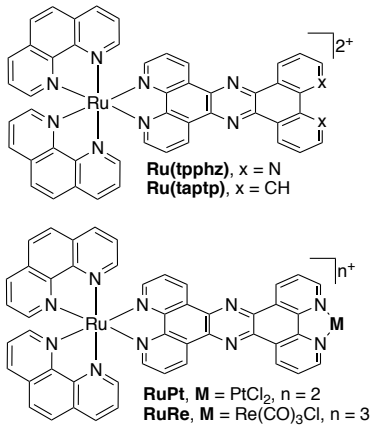
The primary cause of most cancer-related mortality, metastasis, arises from the avoidance of cell death induced by loss of anchorage, or anoikis. [26] Anoikis is a form of apoptosis initiated by detachment from the extracellular matrix or neighbouring cells. [27-28] Consequently, tumour cells that evade apoptosis are not only likely to be chemoresistant, but more liable to survive to metastasize and produce secondary tumours. As a result of these issues, molecules that induce non-apoptotic cell death[29-30] are actively being sought as new and effective treatments for therapeutically resistant and metastatic cancers. [25, 31-33]

Although cisplatin and its analogues irreversibly bind to DNA, research into metal complexes that bind reversibly has also burgeoned. A large amount of this work has involved photoactive systems, [34] and in particular polypyridyl Ru^{II} complexes. [35-37] Much attention was prompted by the properties of the original DNA light-switch system, $[Ru(bpy)_2(dppz)]^{2+}$ (bpy = 2,2'-bipyridine, dppz = dipyrido[2,3-*a*:3′,2′−*c*]phenazine); [38-39] this complex is non-emissive in aqueous solutions but displays bright, $Ru^{II}\rightarrow$dppz ^{3}MLCT-based luminescence on intercalation into DNA. Although the parent complex is not taken up into cells, localization into the nucleus and binding to chromatin has been accomplished through changes in ancillary ligands[40-41] or attachment to targeting moieties.[42]

Most of these studies have been aimed at creating live-cell imaging probes, however some recent reports have shown that specific Ru^{II}(dppz) derivatives can act as novel sensitizers for photodynamic therapy. [43-45] In related work, we have been investigating the biological properties of Ru^{II}(tpphz) systems, where tpphz = tetrapyridyl [3,2-a:2',3'-c:3'',2''-h:2''',3'''-j] phenazine.[46]

Previous studies have investigated the DNA binding properties of both mono- and dinuclear Ru(tpphz) complexes. For example, it is established that $[Ru(bpy)_2(tpphz)]^{2+}$ binds to duplex DNA with affinities comparable to its dppz analogues [47] and although it acts as a DNA light-switch, its emission output can be "switched off" by

metal ion binding at its second coordination site. [48] While studies on its dinuclear analogue $[\{Ru(bpy)_2\}_2(tpphz)]^{4+}$ have revealed that it displays a light-switch effect when it binds to DNA despite interacting though non-intercalative groove binding. [49] Our work has led to the identification of $[\{Ru(phen)_2\}_2(tpphz)]^{4+}$ (phen = 1,10-phenanthroline) as a luminescent probe for nuclear DNA in live cells. This DNA groove-binding complex, which is photostable and displays low toxicity even after 24 hours exposure, is spontaneously taken up by cells through an energy-dependent, non-endocytic mechanism.



Scheme 1. Complexes investigated in this study

Like the dinuclear system, the mononuclear intercalating complex $[Ru(phen)_2(tpphz)]^{2+}$, **Ru(tpphz)**, is taken up by cells, and shows cellular DNA binding;[50] however, it also displays cytotoxicity comparable to cisplatin and its potency is retained even in cisplatin-resistant cell lines. [50] Unsurprisingly given its mode of interaction with DNA, **Ru(tpphz)**, interferes with DNA replication[51] and it is a potent radiosensitizer. [51] Yet, a more detailed understanding of the cellular responses it induces, including cell death mechanisms, is still to be developed. Given the lack of cross-resistance and distinctly different DNA binding mode of **Ru(tpphz)** compared to cisplatin, we decided to investigate the biological action of this system in more detail and compare its properties to three new structurally related complexes, **Ru(taptp)** and the dinuclear complexes **RuPt** and **RuRe** – Scheme 1.

Results and Discussion

Synthesis and characterization of complexes

Complexes **Ru(tpphz)** [50] and **Ru(taptp)** [52] were synthesized through previously reported methods. We initially attempted to synthesize complex **RuPt** by reacting **Ru(tpphz)** with $Pt(DMSO)_2Cl_2$; however, this produced intractable mixtures. It was found that the target complex could be successfully synthesized through the direct reaction of **Ru(tpphz)** with $K_2(PtCl_4)$. Using a similar method **RuRe** can be synthesized through the direct reaction of **Ru(tpphz)** with $Re(CO)_5Cl$.

By comparison with the previously reported spectrum of **Ru(tpphz),** [53] and with the aid of COSY, the ^{1}H-NMR spectra of all the other complexes were assigned. For example: in the spectrum of complex **RuRe** the signals at 10.12 ppm and 9.60 ppm which were assigned to protons a and c of the tppz ligand correlate with a signal at the center of a complex multiplet found at 8.34-8.17 ppm, which is assigned to proton b. A similar pattern of connectivities is found for the other tpphz based signals d, e, and f. The phenanthroline-based signals can also be assigned through similar cross-peak patterns – Figure 1.

It should be noted that even in a coordinating solvent such as MeCN, no changes in the spectra of **RuPt** and **RuRe** were observed, suggesting that in these conditions, the chlorido ligands on the Pt and Re centres of these complexes do not undergo ligand exchange. This is consistent with many previous studies [33, 54-55] in which chlorido extraction from these inert metal centers is only accomplished using Ag+. [56] As it is known that the ^{1}H NMR spectrum of **Ru(tpphz)** is concentration-dependent due to stacking of the tppz unit, the effect of concentration on the spectra of the two new dinuclear complexes was also investigated.

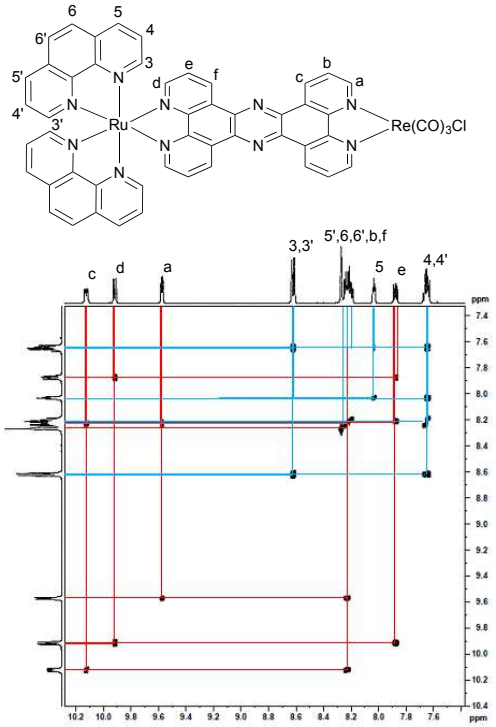


Figure 1. Details of the ^{1}H NMR COSY spectrum of $[RuRe](PF_6)_3$ in MeCN

While complex **RuPt** shows distinctive changes that are consistent with the previously reported stacking interactions involving the tpphz ligand, [56] **RuRe** displays no concentration dependent shifts in its ^{1}H NMR spectrum (Figure S1). This difference is attributed to the different coordination geometries of the metal centers attached to the Ru^{II}(tpphz) fragment. As the Pt^{II} unit is square planar, and essentially linear, stacking between individual **RuPt** complexes, involving extended tpphz moieties, is still possible. In contrast, **RuRe** units do not stack upon one another as the 3-D structure of the octahedral Re^{I} unit prevents such interactions; an observation that is consistent with previous reports on the NMR spectra of homo-dinuclear Ru^{II} complexes such as $[\{Ru(bpy)_2\}_2(tpphz)]^{4+}$.

DNA binding studies

The complexes were converted to water-soluble chloride salts for their biophysical and cellular studies. The cell-free DNA binding properties of RuPt and RuRe were studied in aqueous buffer solutions.

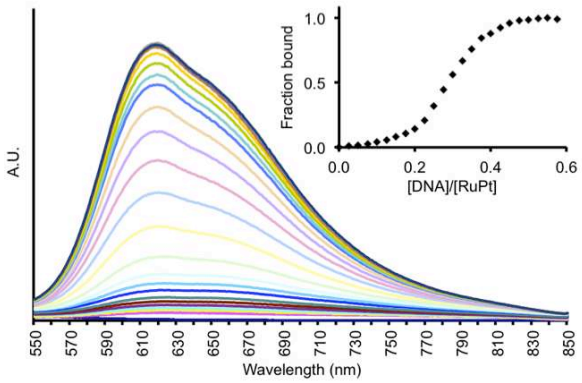


Figure 2. Increase in luminescence of $[RuPt]Cl_2$ on addition of calf thymus DNA (λex = 450 nm). Inset: derived binding curve from these data.

To aid comparison, corresponding data for **Ru(tpphz)** and **Ru(taptp)** and the dinuclear complex $[\{Ru(bpy)_2\}_2(tpphz)]^{4+}$ in the same conditions were also reassessed. As expected, all the complexes displayed light-switch effects on the addition of CT-DNA, as illustrated by the response of **RuPt** shown in Figure 2.

Fits of these data to the non-cooperative McGhee-von Hippel model yielded the binding parameters summarized in Table 1. The estimated binding affinity of **Ru(tpphz)** is very similar to that previously reported values, whereas the value for **Ru(taptp)** is higher than that for the closely related complex $[Ru(bpy)_2(taptp)]^{2+}$, [52] but the two sets of data were collected using different conditions and fitted to different models. A comparison of the binding affinities of **RuPt** and **RuRe** with $[\{Ru(bpy)_2\}_2(tpphz)]^{4+}$ reveals some interesting differences.

As shown before, the homo-dinuclear system displays a very high affinity for duplex DNA ($>10^7$ M^{-1}), consistent with the fact that it is a confirmed groove binder. Site sizes less than 1 bp for the hetero-dinuclear complexes suggest that these complexes also display external stacking modes and imply that the estimated K_b values are actually lower limits for affinities.

Table 1 Summary of DNA binding parameters for all complexes.[a]

Complex	K_b ($\times 10^6$ M^{-1})	Site Size (b.p.)
Ru(tpphz)	0.45	1.8
Ru(taptp)	7.2	1.7
RuPt	80	0.8
RuRe	1.6	0.8
$[\{Ru(bpy)_2\}_2(tpphz)]^{4+}$	45	2.2

[a] As chloride salts in 5 mM Tris, 25 mM NaCl, pH 7.4.

Strikingly, the estimates for **RuPt** indicate a binding affinity approaching 10^8 M^{-1}, a figure that is extremely high for a single-site binding, non-threading, metal complex. In contrast, **RuRe** displays a lower K_b comparable to the figures for **Ru(tpphz)** and **Ru(taptp)**.

The higher binding affinity of **RuPt** compared to **RuRe** and $[\{Ru(bpy)_2\}_2(tpphz)]^{4+}$ is striking, as this complex is less charged than the other two dinuclear systems; therefore electrostatics will make a lower contribution to the interaction with DNA. [57]To gain a second estimate of K_b, through a non-optical method equilibrium dialysis experiments were also carried out and these led to a calculated K_b for **RuPt,** of 2.8 x10^7 M^{-1}, which is in good agreement with luminescence based data (Figure S2). Nevertheless, as the NMR data described above also show that **RuPt** aggregates and it is known that such interactions can greatly affect estimates of binding parameters, [58] this figure must be treated with some caution. On the other hand, the reduced binding affinity of **RuRe** compared to the other dinuclear groove binders is also notable.[59] In our previous studies on Ru^{II} systems we have found that, due to specific unfavourable contacts within a groove, even a single functional group can reduce DNA binding affinities by several orders of magnitude. Given that the square planar Pt^{II} moiety of **RuPt** does not negatively affect binding, it seems that the axial ligands of the $Re^{I}(CO)_3Cl$ unit of **RuRe** must be responsible for similarly unfavourable groove contacts.

Although **Ru(tpphz)** is an established intercalator,[46,47] viscosity experiments showed that, unlike the well-known intercalator ethidium bromide (which induces large positive viscosity changes), progressive addition of each of the three new complexes to buffered CT-DNA solutions produce virtually no change in relative viscosity - Figure 3.

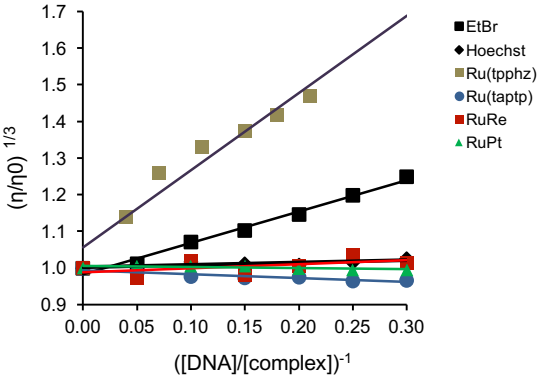


Figure 3. Changes in the relative viscosity $(\eta/\ \eta^0)^{1/3}$ of an aqueous DNA solution after addition of Ru(tpphz), Ru(taptp), RuRe and RuPt (all chloride salts). The intercalator ethidium bromide (EtBr) and groove-binder Hoechst 33258 are included for comparative purposes.

As these effects are almost identical to the minor groove binder Hoechst 33258, these data confirm that both mononuclear **Ru(taptp)** and the new dinuclear complexes **RuPt** and **RuRe** are also groove binders. These observations are consistent with our recent report on a mononuclear, tetracationic, derivative of **Ru(tpphz)**; this complex, in which the uncoordinated site of the tpphz ligand has been converted into a dicationic ethylene-bipyridyldiylium unit, also groove binds to duplex DNA,[60] confirming previous reports that even fused and rigid polycyclic aromatic systems like tpphz derivatives can groove bind if their curvature and charge distribution is complementary to that of duplex DNA. [61-63]

Cell studies: Cytotoxicity

To determine whether the new complexes have potential as anticancer agents, and, if so, which structural properties maximize their potency, and any ability to counteract cisplatin-resistance, they were tested for cytotoxicity against the human ovarian carcinoma cell line A2780 and its cisplatin-resistant daughter cell line A2780CIS. Cells were exposed to a concentration gradient of each complex for 48 h and resultant cell population viabilities were assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [64] and these data were compared with the anticancer drug cisplatin, and **Ru(tpphz)** as controls. IC_{50} values, the concentration at which cell viability is reduced by 50% compared to untreated control, were determined from the curves of percentage cell viability against complex concentration. Figure 4 shows the cell viability curves produced by this analysis, and the IC_{50} values for each complex are summarized in Table 2. In addition, the resistance factor, RF, the ratio IC_{50}(A2780CIS)/IC_{50}(A2780), was determined for each complex.

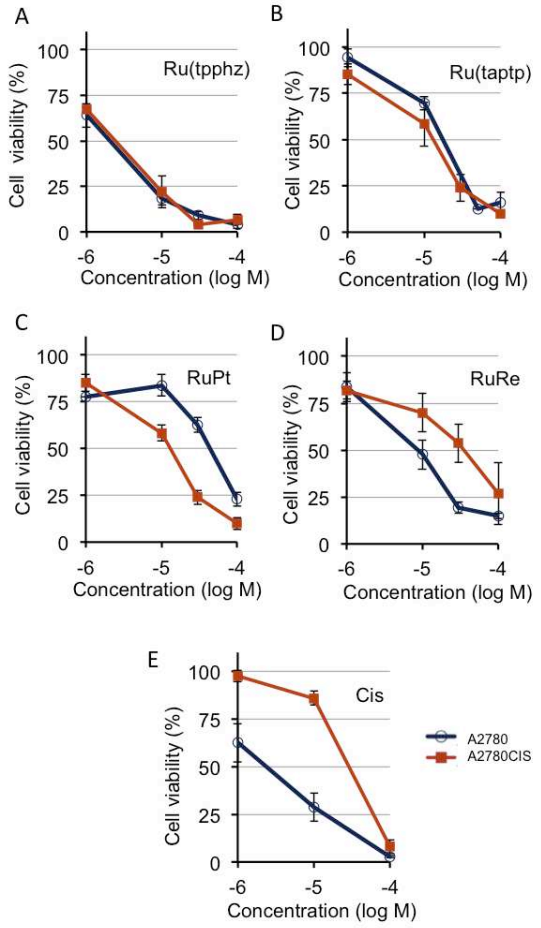


Figure 4. Cell viability data for A2780 and A2780CIS cells treated with the indicated complexes and cisplatin (Cis), analysed by MTT assay (experiments performed in triplicate and data displayed as an average of at least 2 independent experiments

The RF for cisplatin, an eleven-fold reduction in potency between A2780CIS compared to A2780, is in good agreement with literature values.[50] Consistent with our previous reports,[50] **Ru(tpphz)** displays cytotoxicity with potency equivalent to cisplatin in A2780 cells, but importantly, this compound remains equipotent against A2780CIS cells (Figure 4A). Although **Ru(taptp)** is not as potent as **Ru(tpphz)**, it still displays significant cytotoxicity against A2780 cells, and importantly shows no loss of potency in A2780CIS cells (Figure 4B). Interestingly, despite incorporating a Pt^{II} centre, **RuPt** displays the lowest overall cytotoxicity in both A2780 and A2780CIS cell lines - although, like **Ru(taptp)**, there is no cross-resistance observed in cisplatin-resistant cells. **RuRe** has the second highest potency against A2780 cells, but – apart from cisplatin itself – displays the highest RF of this series of compounds.

Table 2. IC_{50} values for all complexes and cisplatin

	IC50 values μM (S.D.)				
Cell Line	Cisplatin	Ru(tpphz)	Ru(taptp)	RuPt	RuRe
A2780	2 (1)	2 (1)	22 (4)	44 (5)	9 (4)
A2780CIS	22 (4)	4 (2)	13 (5)	36 (3)	27 (10)
RF	11	2	0.6	0.8	3

Resistance factor (RF) defined as IC_{50}(A2780CIS)/IC_{50}(A2780).

Given the striking absence of cross-resistance with either **Ru(tpphz)** or **Ru(taptp)** in the A2780 ovarian cancer model system, these two complexes were selected for further study and comparison to cisplatin cytoxicity in additional cancer cell models (MCF7 breast carcinoma, T24 bladder carcinoma as well as two non-cancer cell lines (HEK293 human embryonic kidney cells and MRC5 lung fibroblasts). The cell viability curves are shown in Figure S3, and the IC_{50} values for each complex are summarized in Figure S4. In all cases IC50 values obtained with cisplatin were consistent with literature values. [65-67]

Interestingly in MCF7 cells, **Ru(taptp)** was found to be significantly more cytotoxic (IC50 = 7.2 μM) than cisplatin (IC50 = 19.2 μM) and substantially more effective than **Ru(tpphz)** (IC50 >100 μM). Bladder carcinoma is known to be particular sensitive to platinum based drugs [68]. Consistent with this, T24 bladder cancer cells were most sensitive to cisplatin (IC50 = 1.5 μM) with reasonable toxicity observed with **Ru(taptp)** (IC50 = 7.6 μM). As with MCF7cells, T24 cells were relatively insensitive to **Ru(tpphz).** The MRC5 non-cancerous cell line showed similar sensitivities to both **Ru(taptp)** and cisplatin (IC50 = 15.8 μM and 11.3 μM respectively) while once again, sensitivity to Ru(tpphz) was not detected over the concentrations examined. In addition, both complexes are only active in the HEK293 non-tumor line at concentrations that are over two orders of magnitude higher than the values for even the therapeutically resistant ovarian cancer line (Figure S4). Overall, this study suggests **Ru(tpphz)** and **Ru(taptp)** exhibit the most promising cytotoxic properties as both display low IC_{50} values and RFs, and markedly lower cytotoxic activity against non-cancer cells. Next the cellular uptake of each complex was investigated.

Cellular uptake

Although each mononuclear species displays intrinsic cytotoxicity and lacks cross-resistance with cisplatin, there is a significant difference in potency between them in A2780 cell lines, despite similarity in structure (Figure 4, Table 2). This could be the consequence of two possibilities – either cytotoxicity reflects differential uptake efficiency [5, 69-70] with the same intracellular mode of action

for both complexes, or each complex utilizes a distinct intracellular mechanism to provoke cell death.

To examine whether the differing cytotoxicity profiles could be attributed to differences in uptake, inductively coupled plasma mass spectrometry (ICP-MS) was used to determine the relative cellular accumulation of all 4 complexes in either A2780 or A2780CIS cells, by measuring the ruthenium content of extracts derived from cells after 1hour exposure (Figure S5). Cells were treated with all complexes at equipotent (IC_{50} values; Table 2) concentrations and results are expressed as the molar intracellular Ru concentration.

Interestingly, treatment of A2780 cells with either **Ru(tpphz)** or **Ru(taptp)** resulted in comparable intracellular content after 1 hour (46 and 61 μM respectively), whilst both **RuPt** and **RuRe** were actually internalized at slightly higher concentrations (103 μM and 83 μM respectively). Nevertheless, all compounds showed substantial cellular accumulation, from ~9-fold (RuRe) to 20-fold (Ru(tpphz) above the medium concentration. As expected, cellular accumulation of all compounds in A2780CIS cells was significantly reduced, consistent with the notion that A2780CIS cell have acquired significant efflux capability. [71] In A2780 cells, the extent of accumulation of each complex followed the order **RuRe**>**RuPt** >**Ru(taptp)**>**Ru(tpphz)**, which does not correlate with the order of anti-proliferative capability (**Ru(tpphz)>RuPt>Ru(taptp)> RuRe)**. Similarly, the efficacy of accumulation in A2780CIS cells was in the order **RuRe**>**RuPt**>**Ru(tpphz)**>**Ru(taptp)** with anti-proliferative capacity being (**Ru(tpphz)>Ru(taptp)>RuPt>RuRe)**. Taken together, these data show that differential uptake is not responsible for the differences in cytotoxic potency seen across this series of compounds. In addition, the efficiency of accumulation of all compounds was significantly reduced in A2780CIS cells compared with the parental A2780 cells. Significantly this suggests that complex accumulation is not a limiting factor in the determination of cytotoxic potency.

Cell death

Pt-based complexes primarily target DNA, and provoke a genotoxic response, which induces cell death predominantly via apoptosis. [1] Other metal-based systems induce a range of cytotoxic responses, disrupting mitochondrial as well as genomic integrity [72-76], in addition to oxidative stress, [77-79] to bring about apoptotic cell death. Cell morphology changes characteristic of apoptosis include membrane blebbing, cell shrinkage, and pyknosis. [29] Some metal based systems have been reported to induce either non-specific necrosis, or ischemic cell death or oncosis. [75, 80]

Live cell imaging, using brightfield and fluorescence microscopy, was initially used to investigate mechanisms of cell death induced by each complex. To observe cell death in a period of hours, a complex concentration greater than the determined IC_{50} values, 50 μM, was chosen. Images were recorded at 2 minute intervals, with observations initiated after treatment in a live-cell microscopy chamber equilibrated to 37 °C in an atmosphere containing 5% CO_2.

Following treatment with each of the complexes, both A2780 and A2780CIS cells were observed to undergo a variable degree of rounding, manifested as an apparent decrease in cell circumference, followed by a period of dramatic swelling, with changes that were consistent with oncosis (Figure S6) rather than apoptosis.

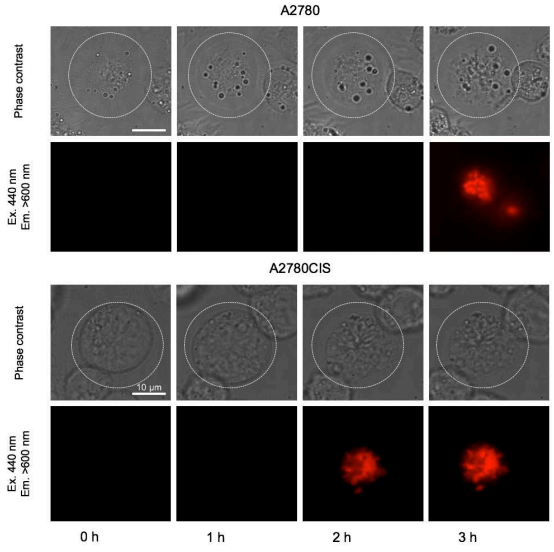


Figure 5. Morphology and luminescent imaging of live A2780 and A2780CIS cells following exposure to 50 μM RuRe for the indicated times.

These observations were quantified by manual measurements of cell diameter using ImageJ software (Figure S7). Simultaneously, luminescence intensity was monitored by imaging using an excitation wavelength of 405 nm and emission over the range 640 – 800 nm. Strikingly, cell swelling was followed by a rapid increase in nuclear luminescence, as loss of cell and organelle integrity, associated with cell death, facilitated unfettered access of each complex to chromosomal DNA, giving rise to a light-switch effect (Figure 5). We utilized this effect as an indicator of timing of cell death, and determined the time duration required to induce cell death for each of the complexes in individual A2780 and A2780CIS cells (Figure 6).

In A2780 cells, death-associated increase in luminescence was observed within 180 mins, with little difference observed between **Ru(tpphz)**, **Ru(taptp)** or **RuPt**, (mean time to death 103 +/- 5, 96 +/- 5, 110 +/- 5 mins respectively) while death in **RuRe**-treated cells occurred more slowly (mean time to death 138 +/- 9 mins). Interestingly, the variability in **RuRe**-treated cells reflected an increase in the dependence of time to death on initial cell size (Figure S8), prior to swelling. In A2780CIS cells, the mean time to death was shorter compared to A2780 cells for all 4 compounds (**Ru(tpphz):** 73 +/- 5 min, **Ru(taptp):** 74 +/- 5 min, **RuRe:** 103 +/- 13 min, **RuPt:** 107 +/- 6 min). Again, with **RuRe** treated cells, an increased dependence on initial cell size was observed (Figure S8).

Taken together, these cellular studies indicate that the mononuclear complexes **Ru(tpphz)** and **Ru(taptp)** display the most promising properties as therapeutic leads to address intrinsic or acquired platinum treatment resistance, as they display an excellent combination of higher potency, and low cross-resistance with cisplatin. In contrast, the dinuclear **RuRe** and **RuPt** complexes display lower overall potency or higher cross-tolerance.

Apoptosis is known to involve the initiation of a proteolytic enzyme cascade, following mitochondrial release of cytochrome C and loss of membrane potential, in which the caspase family of proteases are activated. [29] These induce the specific cleavage of a

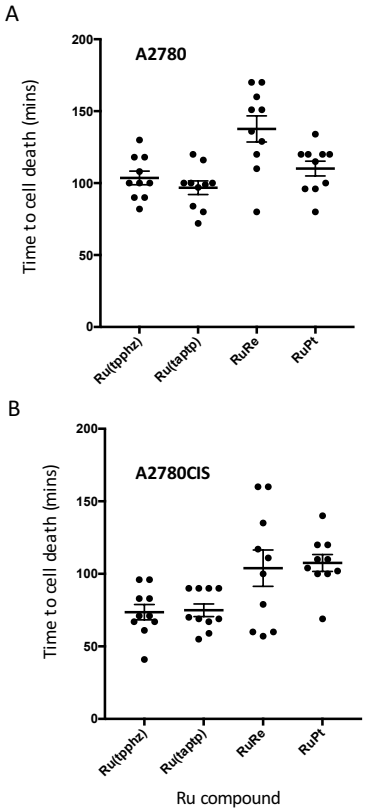


Figure 6. (A & B) Time elapsed until cell death of individual A2780 (A) and (B) A2780CIS cells exposed to the indicated compounds (50μM). Data indicate mean +/- SEM for 10 cells in each treatment.

number of intracellular protein targets including the DNA repair protein PARP-1 (Poly(ADP-ribose) polymerase-1). [29] In order to establish whether either **Ru(tpphz)** or **Ru(taptp)** activate any apoptotic response in cell populations, immunoblotting of whole cell lysates was undertaken to detect the apoptotic-specific cleaved form of PARP-1 in A2780CIS cells treated with either **Ru(tpphz)** or **Ru(taptp)** at an equipotent (IC_{50}) concentration. No evidence of apoptosis was observed in either **Ru(tpphz)** and **Ru(taptp)**-treated cells, in contrast to cisplatin treatment of either A2780CIS or HeLa cell lines (Figure 7).

Consistent with this observation, measurements of mitochondrial membrane potential in A2780CIS cells exposed to either **Ru(tpphz)** or **Ru(taptp)** for up to 24 hours at IC_{50} concentrations showed little or no significant change compared with untreated control cells, in contrast to the mitochondrial poison FCCP (Figure S9).

Taken together, the observations outlined above indicate that cell death induced by **Ru(tpphz)** and **Ru(taptp)** occurs via oncosis. Given the promising therapeutic characteristics of **Ru(tpphz)** and **Ru(taptp)** we sought to gain further insights into their mechanism of action and biomolecular targeting through a proteomic study.

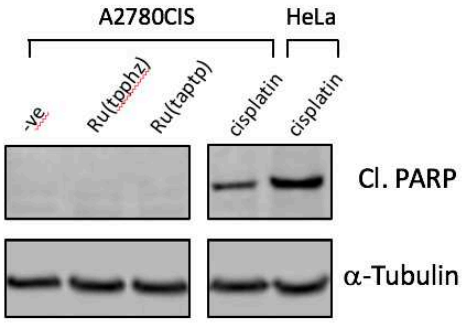


Figure 7. Absence of PARP cleavage by Ru(tpphz) or Ru(taptp). Immunoblotting analysis of lysates derived from A2780CIS cells treated with either Ru(tpphz), Ru(taptp) or cisplatin (Lanes 1-4) or Hela cells treated with cisplatin alone for the presence of cleaved PARP with α-tubulin as loading control. In A2780CIS cells, IC_{50} values (were used for Ru(tpphz) and Ru(taptp), and IC_{25} values for cisplatin (determined as described in SI). HeLa cells were treated with cisplatin (5 μM) for 48 h.

Proteomics

Systematic studies into the effect of biologically active metal complexes on the proteome are rare. A number of initial reports involved cisplatin and its derivatives; for example, mechanisms of acquired cisplatin resistance in ovarian cancer, [81] neuroblastoma, [82] and cervix squamous cell carcinoma cells have been explored. [83] More recently, studies on ruthenium-based systems have emerged. A proteomic study on the antimetastatic agents trans-$[RuCl_4(DMSO)(imidazole)]^-$ and $[Ru(\eta^6\text{-toluene})Cl_2$ (PTA)] (RAPTA-T), (PTA = 1,3,5-triaza- 7-phosphaadamantane) revealed that they induce changes in protein expression that are very different to cisplatin. [84] Further studies on RAPTA, including an affinity precipitation assay, revealed putative protein targets involved in cell cycle regulation, cell growth, histone modification, and ribosomal processing. [85]

As **Ru(tpphz)** and **Ru(taptp)** are kinetically inert reversible DNA binders, this suggests that their therapeutic action is distinctly different to both classical genotoxins like cisplatin and antimetastatics such as RAPTA-T, both of which contain kinetically labile chlorido ligands that are an intrinsic to their function. To explore this issue in more depth, the effect of treatment with **Ru(tpphz)** and **Ru(taptp)** on protein expression levels in A2780CIS cells was undertaken using Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC), [86] combined with LC-MS/MS analysis using a hybrid Orbitrap Elite mass spectrometer. This approach has the advantage of providing accurate determinations of change in protein expression by ratiometric determination of multiple peptides derived from any one protein. Additionally, it provides the opportunity to undertake subsequent informatics analyses to identify protein functional groups, thus providing insight into the global cellular response to any one compound.

This approach facilitates a direct comparison of individual protein expression levels following exposure of cells to a specific treatment compared to untreated control. Here, cells were grown either in standard culture medium containing isotopically normal ("light") amino acids or medium containing "heavy," $^{13}C_6$, and $^{15}N_4$ labelled, arginine and lysine. After treatment of the "light" cell population with the compound of interest, and control treatment of the

isotopically "heavy" cells, derived cell lysates are combined in a 1:1 mass ratio, and the mixture denatured and fractionated by SDS-PAGE.

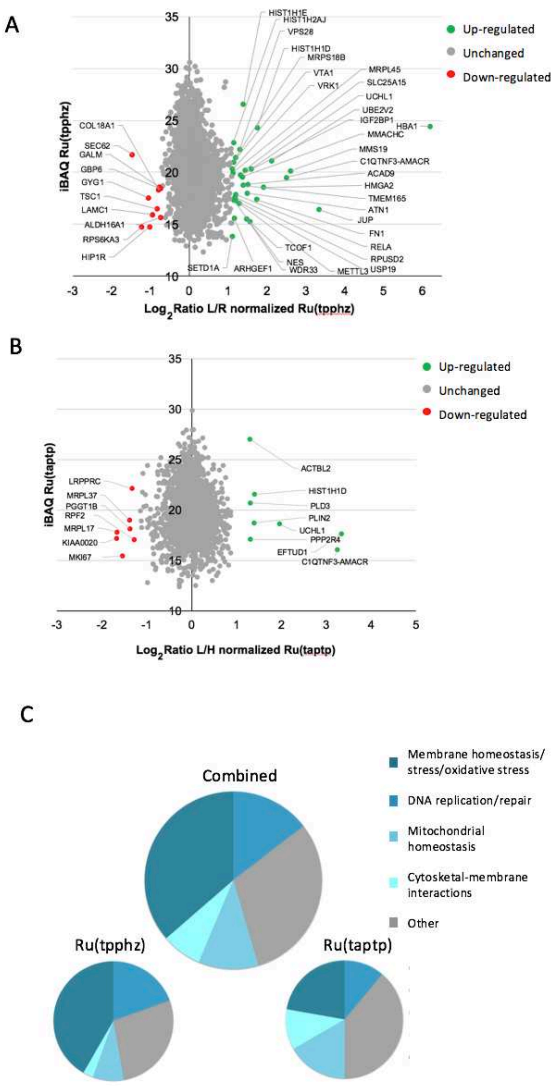


Figure 8. Analysis of protein expression change following treatment of A2780CIS cells for 48 hours with respective IC_{50} concentrations of (A) **Ru(tpphz)** and (B) **Ru(taptp).** Data are presented as the log ratio of expression in the presence versus the absence of the indicated compound plotted against intensity Based Absolute Quantitation for each protein. Significant changes (down-regulation: red, up-regulation green) in protein expression are annotated using Uniprot protein IDs. (C)Bio-informatics analysis of gene products whose expression is altered in A and B.

Proteins are proteolytically fragmented in situ, and resultant peptides extracted and subjected to LC-MS/MS (see SI). Changes in protein expression may be determined by ratiometric determination of signal intensity in pairs of chemically identical "light" and "heavy" peptides, revealing up- or down-regulation of specific proteins on treatment. Analysis of data was carried out as described in SI, using MaxQuant and Pegasus software. [87]

A2780CIS cells were left untreated, or treated with either **Ru(tpphz)** or **Ru(taptp)** at a concentration close to respective IC_{50} value (**Ru(tpphz)**: 4.4 μM; **Ru(taptp)**: 13 μM) of each complex for 48 hours. Following cell lysis, protein content determination, normalization, purification, in-gel digest, analysis and data processing, a list of identified proteins and the ratio of their expression between untreated and treated populations was generated. Over 3400 distinct proteins were identified and ratios of abundance in the absence and presence of each complex determined as described above. This generated 54 hits that met the selection criteria described in S.I. For each complex, the extent to which each protein was up- or down- regulated following exposure is shown in Figure 8A and B, and is expressed as the $\log_2$ [ratio of light/heavy isotopic species] (X-axis) plotted against intensity-based absolute quantification (IBAQ;Y-axis), which provides an indicator of the relative abundance of each identified protein. The summary of protein sequence and function included in Supplementary Figure 10 is from the open access resource UniProt.

In the case of cells treated with **Ru(tpphz),** the abundance of 42 proteins was significantly altered, with 32 up-regulated, and 10 proteins down-regulated. Of the 42 proteins identified, a significant fraction (9) is associated with the processes of DNA replication, DNA repair, or checkpoint signaling associated with replication stress (Fig 8C). Three of these are histone proteins, the levels of which are increased in cells undertaking or arrested in S phase.[88] Eight proteins are directly involved in a number of DNA repair processes. MMS19 is an essential component of the CIA (cytosolic iron-sulphur protein assembly complex), that mediates incorporation of iron-sulphur cluster in proteins involved in DNA metabolism including the helicases ERCC2/XPD, required for nucleotide excision repair, FANCJ, involved in DNA double strand break repair, and RTEL1, required for telomere maintenance and genome stability. [89] METTL3 encodes the catalytic subunit of N6-adenosine-methyltransferase, which methylates adenosine residues in RNA, and is absolutely required for the recruitment of DNA polymerase κ to nuclear sites of DNA damage for nucleotide excision repair and trans-lesional synthesis. [90] UBE2V2 is a non-canonical variant ubiquitin ligase-like protein that, as a heterodimer with UBE2N, catalyzes the synthesis of poly-ubiquitin chains that generate a location signal for the consequent recruitment of DNA repair proteins. [91] VRK1 is a nucleosomal associated protein kinase that phosphorylates the DNA damage marker histone H2AX as an essential step in the formation of nuclear DNA repair foci. [92]

Strikingly, HMGA2 is a member of high-mobility group A non-histone chromatin proteins that are involved in both activation of the mismatch repair response and base excision repair. [93] This protein group has been implicated in the cellular response to platinated DNA adducts:. [94] HMGA2 is required for the activation of the ATR/Chk1 checkpoint signalling system in response to the presence of guanine adducts [95] and consequently, suppression of apoptosis. [96] Elevated expression of SETD1A facilitates the binding of cyclin K [97] a cell-cycle regulated transcription factor, and binding is essential for the up-regulation of multiple genes involved in the DNA damage response. [98]

Taken together these data are consistent with the known DNA binding properties of **Ru(tpphz)** *in vitro* [50] (Figure 3) as well as its effect on DNA replication *in vivo*. [51] Importantly, they provide additional detailed insight into the nature of the cellular DNA damage response to **Ru(tpphz),** and suggest that, in addition to replication fork stalling, binding of **Ru(tpphz)** to DNA *in vivo* provokes a requirement for both nucleotide excision and DNA mismatch repair pathways.

Unlike cisplatin and its derivatives, a significant number of Ru(II) based systems that target DNA are, catalytically inert; fur-

thermore, a number of the systems that show significant cytoxicity in human cancer cells have been reported to generate reactive oxygen species. [99-102]

Levels of reactive oxygen species that surpass cellular anti-oxidant defenses result in oxidative stress, where the capacity of cells to repair bio-molecular oxidation of proteins, lipids, RNA and DNA is exceeded, resulting in persistent damage. Multiple cellular processes are triggered in response to oxidative stress. These include modulation of oxygen-sensitive signaling pathways, such as the nutrient and stress sensor, mTORC1, [103] the NFkB transcription factor family,[104] the hypoxia-inducible factor (HIF-1) transcription system, [105] in addition to checkpoint signaling pathways described above. These pathways are responsible for metabolic reprogramming [105] to increase anti-oxidant levels, activation of DNA repair [106-107], activation of the endoplasmic reticulum unfolded protein response (UPR), [108] elevation of organelle regeneration via autophagy - the process of damaged cell content recycling [109] - and response to lipid peroxidation.[110]

Interestingly the largest group of proteins whose expression level was altered in response to **Ru(tpphz)** treatment are associated with cellular or mitochondrial responses to reactive oxygen species and oxidative stress. 13 proteins associated with the cellular response to oxidative stress were up-regulated in response to cellular exposure to **Ru(tpphz)** in A2780CIS cells. Up-regulation of the ubiquitin carboxy-terminal hydrolyase USP19 as well as C1QTNF3, [111] mediates both the ROS-stimulated stabilization of the transcription factor HIF (hypoxia inducible factor) 1 [112] as well as the ERAD (endoplasmic-reticulum-associated degradation) or UPR (unfolded protein response) system.[113-114] USP19 cellular function in conjunction with UCHL1, another ubiquitin hydrolyase, results in HIF1-mediated metabolic reprogramming to increase levels of the anti-oxidant reduced glutathione (GSH). [115] Sec62 up-regulation facilitates resolution of the unfolded protein response by selectively delivering ER components to the autolysosomal/autophagy system for clearance and or recycling. Hamartin (TSC1) expression together with Tsc2 down-regulates mTORC1 which is a central regulator of autophagic flux. Reduced mTorc1 activity facilitates induction and nucleation of phagophores, which in turn leads to elevated autophagic flux. [109] VTA1 in association with components of the ESCRT complex [116] including VPS28, is involved in the multivesicular body-mediated protein transport in autophagy.[117] RPS6KA3 encodes ribosomal S6 protein kinase, which is an essential effector of mTORC signaling. [103]

Elevated levels of the NF-kB transcription factor family component RelA plays a role in cell growth and survival in response to oxidative stress, by increasing transcription of genes involved in reducing levels of ROS. [118] Although its precise function is unknown, RPUSD2, a member of the pseudouridiidine synthase family, is a known target of NFkB. [119] TCOF1 encodes Treacle, a nucleosomal protein that controls and regulates ribosome biogenesis, the expression of which has been shown to be regulated by oxidative stress. [120] MMACHC/CblC is a recently identified molecular chaperone and decynase responsible for maintaining intracellular levels of cobalamin (vitamin B12) which in turn acts to protect against oxidative stress,[121-122] while ALDH16A1 encodes an aldehyde dehydrogenase which acts as an aldehyde scavenger in response to cellular lipid peroxidation [123]. Increased expression of the intermediate filament protein Nestin is known to confer protection against oxidative stress. [124] Given the central role of mitochondria in both producers of ROS and and targets of oxidative damage, up-regulation of a number of mitochondrial-specific ribosomal proteins is consistent with a requirement for newly synthesized mitochondrial proteins during periods of elevated autophagic flux. [125]

Significantly fewer proteins showed significant up-regulation or down-regulation when cells were exposed to **Ru(taptp)**. The reason for this is not yet clear although it may be a consequence of its reduced potency and the duration of cellular exposure, resulting in fewer significant changes in protein expression levels. Despite this, categories of proteins affected by **Ru(taptp)** were broadly similar to that of **Ru(tpphz)** and consistent changes in expression levels of several proteins (C1QTNF3, UCHL1 and HIST1H1D) were observed for both compounds. Of note, treatment of cells with **Ru(taptp)** resulted in altered expression of an additional protein (PPP2R4) associated with the DNA damage response. [126] Nevertheless, taken together it seems that both complexes provoke very similar cellular responses. Although the mode of DNA binding by each of the two complexes is different, they both appear to provoke a DNA damage response, as judged by proteomic analysis. This is not surprising as both intercalating agents such as doxorubicin, [127] as well as groove-binding compounds including Hoechst 33342, [128] are known to induce DNA damage by interfering with the progression of the replisome in S phase, as well as preventing efficient DNA repair. [127, 128]

A pictorial summary and comparison of the cellular processes affected by treatment with **Ru(tpphz)** and **Ru(taptp)** is shown in Figure 8C.

Conclusions

Despite being reported almost three decades ago, therapeutic leads based on the Ru^{II}(dppz) DNA light-switch complex that are intrinsically cytotoxic and nuclear targeting are almost non-existent.

Chao and co-workers have demonstrated that a cyclometallated mono-cationic derivative of the parent complex does localize within the nucleus and is potently cytotoxic through disruption of transcription [129]; however, this complex induces classical apoptosis and so will not be active in therapeutically resistant cancer in which apoptotic pathways have been abrogated. The MacDonnell group have demonstrated that dinuclear Ru^{II} complexes containing rigid, extended redox-active, bridging ligands are cytotoxic through a mechanism that involves the generation of reactive oxygen species, even in hypoxic conditions. [130] However these systems are not intrinsically luminescent and again only induce apoptosis. [131]

The induction of oncotic cell death by this group of Ru complexes is novel and unexpected. It is notable that the toxic stress induced by these complexes does not result in the destruction, via caspase-mediated proteolysis, of the enzyme PARP which plays a major role in the cellular response both to direct DNA damage in addition to that caused by the generation of reactive oxygen species. [132] PARP acts to modify target proteins via the synthesis and covalent attachment of polymeric adenosine ribose chains. Importantly significant PARP activation is known to deplete the cellular pool of the source of ADP ribose, NAD [133] which in turn results in oncotic cell death. [134] Future work will establish whether that is the mechanism of Ru complex-induced oncosis in this system.

The systems described herein offer a unique combination of nuclear targeting, a DNA light-switch effect, and the capability of inducing non-apoptotic cell death. Interestingly the effects induced by these systems are not solely dependent on their uptake concen-

trations, as they retain potency in a cell line known to possess an increased efficiency in drug efflux.

In particular, the mononuclear complexes, **Ru(tpphz)** and the newly reported **Ru(taptp)**, display a highly promising spectrum of properties. **Ru(tpphz)** potently blocks the proliferation of ovarian carcinoma cell lines with a potency similar to that of the cisplatin, which remains a frontline treatment for ovarian cancer. [135] Importantly, it does not show cross-resistance in cells that have developed resistance to cisplatin, and it induces cell death via a mechanism distinct from apoptosis, which may often be abrogated in tumors. It exhibits lower levels of toxicity than cisplatin in non-cancer model cell lines. Interestingly, it exhibits much lower potency against tested bladder and breast carcinoma cell lines. As both A2780 and MCF7 cells both retain wild-type p53, while T24 cells have a p53 mutant genotype, it is clear that p53 genotype cannot explain the differential potency of **Ru(tpphz).**

The activity profile of **Ru(taptp)** against MRC5 fibroblasts is no more cytotoxic than cisplatin and it is largely ineffective against HEK293 cells. As with **Ru(tpphz),** it shows no cross-resistance in platinum-resistant ovarian carcinoma. However, it is slightly more potent than cisplatin in breast carcinoma cells. Cisplatin plays an important role in adjuvant treatment of breast carcinoma after surgery, [136] in patients with inherited forms of breast/ovarian cancer syndrome arising from BRCA1 mutations [137] as well as following the onset of metastases. [138] It follows that **Ru(taptp)** may have promise in these settings following emergence of platinum resistance**.**

In the first proteomic study involving kinetically inert ruthenium complexes, we found that these mononuclear complexes induce protein expression changes that are indicative of both DNA damage and oxidative stress responses. This first observation is consistent with previous studies confirming that **Ru(tpphz)** binding directly stalls DNA replication forks, [51] however the up-regulation of proteins associated with oxidative stress suggest the complexes have a second mode of action. Although the complexes described by MacDonnell and coworkers are also cytotoxic through oxidative stress they contain ligands that – unlike tpphz [131]– are themselves redox active in biological conditions. Given this fact, and the different death mechanisms induced by the two classes of complexes, an involvement of a similar mechanism for **Ru(tpphz)** and **Ru(taptp)** can be discounted.

As therapeutic regimes displaying more than one mechanism of action offer great potential in the treatment of multigenic diseases such as cancer and are less susceptible to conventional resistance mechanisms, [139] **Ru(tpphz)** and **Ru(taptp)** offer great potential as metal based anticancer therapeutic leads. Further studies will focus on a more detailed analysis of the mode of action. For example, although it appears that DNA processing is disrupted by the complexes, details of any sequence or structurally specific binding targets - and the initial molecular events that lead to oxidative stress - still need to be delineated. A deeper understanding of these mechanisms will facilitate the synthesis of derivatives that enhance such effects and such studies will form the basis of future reports.

ASSOCIATED CONTENT

SUPPORTING INFORMATION

Experimental details, further ICP-MS details, additional microscope images, quantification of cell size changes and cell death times, further proteomic analyses (PDF).

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The manuscript was written through contributions of all authors.

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