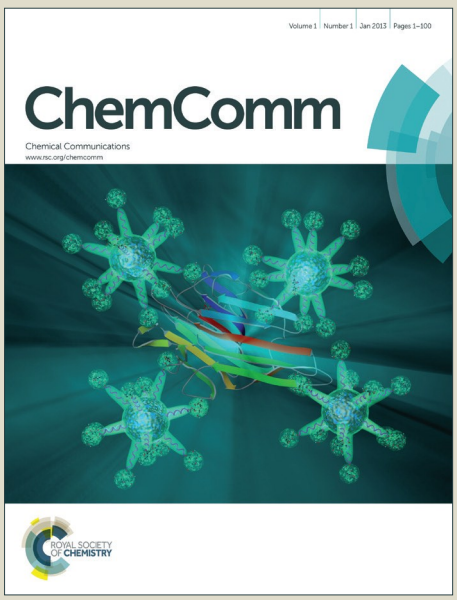
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

COMMUNICATION

Geometry matters: inverse cytotoxic relationship for *cis*/*trans*-Ru(II) polypyridyl complexes from *cis*/*trans*-$[PtCl_2(NH_3)_2]$

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

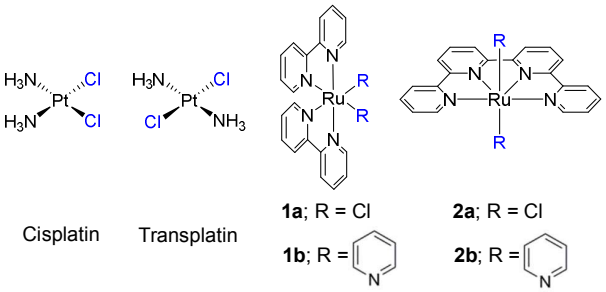
Erin Wachter,[a,†] Ana Zamora,[b,†] David K. Heidary,[a] José Ruiz,[b] and Edith C. Glazer[a,*]

Two thermally activated ruthenium(II) polypyridyl complexes, *cis*-$Ru(bpy)_2Cl_2$ and *trans*-$Ru(qpy)Cl_2$ were investigated to determine the impact of the geometric arrangement of the exchangable ligands on the potential of the compounds to act as chemotherapeutics. In contrast to the geometry requirements for cisplatin, *trans*-$Ru(qpy)Cl_2$ was 7.1–9.5x more cytotoxic than *cis*-$Ru(bpy)_2Cl_2$. This discovery could open up a new area of metal-based chemotherapeutic research.

Cisplatin, *cis*-$[PtCl_2(NH_3)_2]$ has received worldwide acceptance as a clinical drug for the treatment of various neoplastic diseases,[1] while its isomer transplatin, *trans*-$[PtCl_2(NH_3)_2]$ was found to be therapeutically inactive.[2] This observation was considered a paradigm for the structure-activity relationships (SAR) of Pt-based antitumor compounds, according to which the antitumor activity requires a neutral square-planar platinum center with two ammine ligands and two leaving groups in *cis*-geometry.[2] The lack of antitumor activity in transplatin has been associated with the formation of intrastrand cross-links between purine-pyrimidine[3] residues instead of purine-purine (major DNA adducts formed by cisplatin)[4] due to stereochemical constraints.

In addition to Pt based compounds, other metal complexes have been shown to have biological activity *in vitro*, including potency in cisplatin-resistant tumor cells.[5] Ruthenium has received particular attention in the present search for therapeutic agents, and ruthenium compounds exhibit antitumor effects as well as antibiotic, antiviral, and antimalarial activity.[5b] Two anionic Ru(III) coordination compounds, NAMI-A and KP1019, possess a strong ability to inhibit metastases of solid invasive cancers and successfully completed phase I clinical trials but ultimately failed in phase II clinical trials.[6] Generally, antitumor Ru(II) complexes can be divided in two primary families, *i.e.* the half-sandwich "piano-stool" and polypyridyl-types.[5b] The later family has been gaining attention due to their appealing physicochemical properties, which offer the possibility to use them in photodynamic therapy (PDT) and photoactivated chemotherapy (PACT).[7] However, all the Ru(II) compounds able to form covalent bonds to biomolecules exhibit a *cis* geometry, and no examples of *trans* polypyridyl Ru(II) isomers with biological activity are yet known, in spite of their interesting photophysical and catalytic properties.[8] But, does geometry matter in the design of anticancer Ru(II) complexes? Here we report that geometry appears to play a very important role, and moreover, a Ru(II) polypyridyl complex with exchangeable ligands with *trans* geometry exhibits *in vitro* anticancer activity significantly superior to the *cis* compound.

Chart 1. Structures of compounds included in this study. Exchangeable ligands are highlighted in blue



In order to determine the behavior of *trans* Ru(II) polypyridyl complexes, *trans*-$Ru(qpy)Cl_2$ (**2a**, qpy = 2,2':6',2'':6'',2'''-quaterpyridine) was synthesized and the Cl^- ligand exchange rate, DNA binding, cytotoxicity, and cellular uptake were investigated in comparison to *cis*-$Ru(bpy)_2Cl_2$ (**1a**, bpy = 2,2'-bipyridine) (Chart 1). The qpy ligand was chosen to generate a complex with exchangeable sites only in the *trans* arrangement; this was required as *trans*-$Ru(bpy)_2Cl_2$ is known

[a] Department of Chemistry, University of Kentucky, Lexington, KY 40506, United States. Tel: +1 859-257-2198, Fax: +1 859-323-1069, Email: ec.glazer@uky.edu.
[b] Departamento de Química Inorgánica and Regional Campus of International Excellence "Campus Mare Nostrum", Universidad de Murcia, and Institute for Bio-Health Research of Murcia (IMIB-Arrixaca), E-30771 Murcia, Spain.
† Authors contributed equally to this work.
Electronic Supplementary Information (ESI) available: experimental details, UV/Vis absorption profiles for thermal exchange and kinetic analysis. See DOI: 10.1039/x0xx00000x

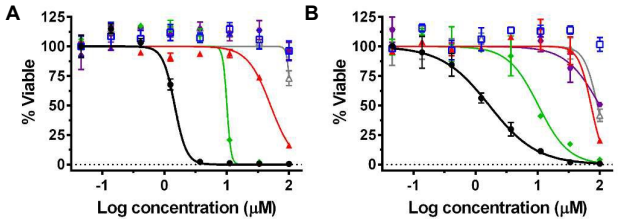


Figure 1. Cytotoxicity of cisplatin (black, ●), transplatin (blue, □), **1a** (red, ▲), **1b** (purple, ●), **2a** (green, ◆), and **2b** (grey, Δ) in (A) HL-60 and (B) A549.

to be highly insoluble.[9] Alternatively, attempting a comparison of the *cis*- and *trans*-$Ru(bpy)_2(H_2O)_2$ complexes is not viable as the two systems photoisomerize.[10] The qpy ligand was synthesized via an oxidative coupling reaction with 6-chloro-2,2′-bipyridine; coordination to $RuCl_3$ then yielded **2a**.[8d]

The qpy ligand and derivatives have been reported as a tetradentate ligand in mononuclear Ru(II) complexes,[8d, 8e, 11] but some derivatives may act as a bridging ligand in dinuclear Ru(II) complexes.[8a, 12] Thus, the mono-metallic structure of the complex **2a** was confirmed by the further synthesis and characterization of *trans*-$[Ru(qpy)(py)_2]^{2+}$ (**2b**, py = pyridine) from **2a**.[8d] As complex **2b** is not capable of ligand exchange, it also served as a control compound to assess if the addition of the qpy ligand itself to the Ru(II) center was responsible for the observed biological activity. Similarly, *cis*-$[Ru(bpy)_2(py)_2]^{2+}$ (**1b**) was used as a nonexchanging control for the *cis* geometry.

To determine the efficacy of **1a**, **1b**, **2a**, and **2b** in cancer cells, cytotoxicity studies were performed in HL-60 leukemia cells and A549 lung cancer cells and compared to cisplatin and transplatin (Figure 1 and Table 1). For square planar platinum compounds with exchangeable Cl^- ligands, the *cis* geometry is known to be more potent. Here, cisplatin was cytotoxic with IC_{50} values of 1.5 and 1.7 μM in HL-60 and A549 cells. As anticipated, transplatin had no effect on either cell line.

In marked contrast, for octahedral ruthenium complexes with exchangeable Cl^- ligands, the opposite relationship between geometry and cytotoxicity was observed. The

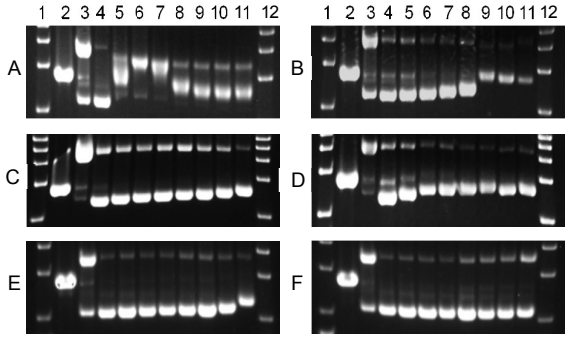


Figure 2. Agarose gel electrophoresis showing the dose response of (A) cisplatin, (B) transplatin, (C) **1a**, (D) **2a**, (E) **1b**, and (F) **2b** with 40 μg mL^{-1} pUC19 DNA incubated at 37 °C. Lanes 1 and 12: DNA ladder; Lane 2: EcoRI; Lane 3: $Cu(OP)_2$; Lane 4–11: 0, 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 μM compound. EcoRI and $Cu(OP)_2$ were used as controls to represent linear DNA and relaxed circle DNA, respectively.

Table 1. Cytotoxicity IC50 values for HL-60 and A549 cell lines.

Compound	HL-60 IC_{50} (μM)	A549 IC_{50} (μM)
Cisplatin	1.5 ± 0.1	1.7 ± 0.5
Transplatin	>100	>100
1a	96 ± 1	73 ± 1
1b	>100	98 ± 1
2a	10.1 ± 1.1	10.3 ± 1.2
2b	>100	93 ± 1

complex with exchangeable ligands in the *trans* geometry was 7.1–9.5x more cytotoxic than the *cis* geometry. For **1b** and **2b**, the control compounds that are incapable of ligand exchange, the cytotoxicity was eliminated in HL-60 cells, and only minimal toxicity was observed at 100 μM in A549 cells. Two hypotheses were proposed to rationalize the differences in cytotoxicity for the complexes: 1) the *trans* geometry interacts with different in vivo targets from the *cis* geometry; and 2) thermally exchangeable ligands are required for the cytotoxic effect to occur.

The DNA binding behavior for each compound was assessed using agarose gel electrophoresis to compare *cis* and *trans* Pt(II) versus Ru(II). Dose responses were performed with cisplatin, transplatin, **1a**, **1b**, **2a**, and **2b**, with pUC19 plasmid DNA after reaction at 37 °C for 12 hours (Figure 2). Cisplatin interacts with plasmid DNA at very low concentrations (15 μM) and effectively crosslinks the DNA. The adducts were visualized by the reduced mobility of the DNA at low concentrations of cisplatin, followed by increased mobility of the DNA at higher concentrations. In contrast, transplatin has minimal interaction with the DNA, even at high concentrations, where the migration of the DNA is only effected at ≥125 μM transplatin. Surprisingly, when incubated with plasmid DNA, **1a** and **2a** only showed minimal perturbation of DNA mobility, suggesting that either they do not interact strongly with plasmid DNA or the interaction does not cause significant changes to the supercoiled plasmid structure. Replacement of the Cl^- ligands with py ligands resulted in an even smaller effect, with only a slight decrease in mobility at the highest concentration of **1b** and **2b**.

Given that both **1a** and **2a** contain thermally labile chloride ligands, their ligand exchange rates were monitored using UV/Vis absorption spectroscopy. Compounds **1b** and **2b** were also studied, and were anticipated to be less susceptible to thermal exchange. Initially, the thermal exchange of **1a**, **1b**, **2a**, and **2b** was determined under different buffer and media conditions (Figures 3, S1–S4). Full spectrum absorbance was measured in 96 well plates incubated at 37 °C over the course of 15 hours. To determine the half-life ($t_{1/2}$) of ligand exchange, the change in absorbance was plotted as a function of time. While a $t_{1/2}$ could be determined for **1a**, compound **2a** underwent a very slow ligand exchange and the reaction never reached completion; therefore, the half-life could not be accurately determined. Not surprisingly, **1b** and **2b** show minimal changes in UV/Vis spectra following 15 hour incubation in aqueous solutions, confirming that they are essentially kinetically inert.

To compare the rates of ligand exchange under different conditions for **1a**, **1b**, **2a**, and **2b**, the spectral change at 500, 450, 345 and 325 nm, respectively, was determined. These wavelengths represent the maximal signal change for the majority of conditions tested. Striking differences were seen for **1a** and **2a**, where **1a** exhibited the fastest exchange in Opti-MEM supplemented with 1% FBS (fetal bovine serum, $t_{1/2}$ = 12.8 min; used as a control for the cell cytotoxicity experiments), and slowest in water ($t_{1/2}$ = 53 min, Figures 3 and S1, Table S2). On the other hand, **2a** had the largest spectral change in water (Δabs = 0.19) yet the smallest change in Opti-MEM with 1% FBS (Δabs = 0.011, Figure 3 and S3, Table S4). Compounds **1b** and **2b** were studied under the same conditions, but once more only showed minimal change (Δ_{abs} < 0.08) over the course of 15 hrs due to the thermally stable pyridine ligands.

The fast exchange for **1a** and minimal exchange for **2a** in Opti-MEM with 1% FBS may help to explain the drastic differences in cytotoxicity. The slow/minimal reaction of **2a** with cell culture media would potentially allow the complex to enter the cell without reaction with media components, whereas the fast reaction of **1a** could essentially deactivate the complex prior to entering the cell. In addition to aqueous media, the thermal exchange in the presence of duplex DNA and small molecules, used to mimic the side chains of amino acids, was tested.[13] The results from these studies revealed differences in the reactivity profiles for **1a** compared to **2a**, supporting that fast exchange reactions for **1a** prevents it from either entering the cell, or enables unintended side reactions with other biomolecules upon entering the cell.[13]

One of the causes for transplatin's inactivity is its high chemical reactivity, where it becomes deactivated through reactions with plasma and tissue proteins before entering a cell.[14] In a recent publication, transplatin was successfully internalized as the inactive molecule by encapsulation into nanocapsules, essentially preventing deactivation; following intracellular release it was able to induce a cytotoxic effect.[15] Likewise, we have previously reported Ru(II) complex prodrugs that are inactive, but when irradiated with light produce a *cis*-$Ru(bpy)_2L_2$ (L = H_2O or Cl^-) and are quite cytotoxic.[16] It appears that caging the "inactive" compound to allow uptake into cancer cells renders these compounds "active".

In order to test our deactivation hypothesis, cellular uptake of **1a** and **2a** was determined in HL-60 cells. The HL-60 cells were incubated in the presence of 20 μM **1a** or **2a** for 12 hours; following this time 90–95% of cells remained viable. The cells were then harvested and the ruthenium content was determined using graphite furnace atomic absorption spectrometry (GFAAS) for the media and cells separately. The total uptake for **2a** (475.8 ng) was 49x greater than the uptake for **1a** (9.8 ng); this represents 15% cellular uptake for **2a** (Figure S5). These results provide support for the deactivation hypothesis, where the fast reaction of **1a** in Opti-MEM renders the complex unable to accumulate in cells. On the other hand, the slow reaction of **2a** correlated to significant cellular accumulation, ultimately leading to the cytotoxic effect. Furthermore, flow cytometry confirmed the mechanism of cell death for **2a** occurs via apoptosis with no visible sign of necrosis.[13] Thus, the damage induced by compound **2a** triggers the programmed cell death pathway.

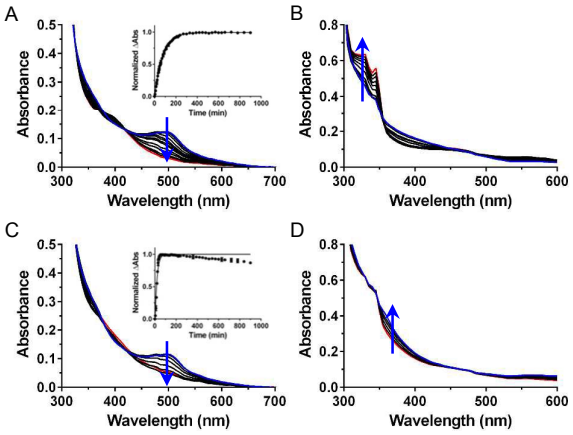


Figure 3. Thermal exchange studies of 40 μM **1a** and **2a** at 37 °C showing rapid exchange for **1a** and slow exchange for **2a**. (A) **1a** in water, (B) **2a** in water, (C) **1a** in Opti-MEM, 1% FBS, and (D) **2a** in Opti-MEM, 1% FBS. Insets show the change in absorbance fit to a one phase decay equation. Note: **2a** undergoes incomplete conversion over the course of 15 hours.

It is possible that the number of exchangeable ligands differed for the *cis* and *trans* complexes, and this also could contribute to the disparate biological activities. As both **1a** and **2a** react with imidazole, the complexes were incubated with this heterocycle until there was no further change in the absorption spectra, and then samples were analyzed by HPLC and mass spectrometry. Both complexes produced new species with longer retention times than the products that form in buffer alone. The reaction of complex **1a** with imidazole resulted in full conversion to *cis*-$[Ru(bpy)_2(imidazole)_2]^{2+}$ with the same retention time and absorption spectrum of the molecule produced by chemical synthesis (Figure S8). Mass spectrometry also confirmed that two imidazole ligands replaced the chloride ligands in both the *cis* complex **1a** (Figure S6) and the *trans* complex **2a** (Figure S7). Thus, both the *cis* and *trans* complexes can form biadducts, and should be capable of crosslinks, either between DNA bases, DNA and proteins, or within a protein.

SAR studies for cytotoxic metal compounds rarely address the impact of geometry. The history of research in platinum compounds and the inactivity of transplatin were interpreted as a demonstration that a particular geometric arrangement was required for efficacy. However, replacement of the NH_3 ligand in ineffective transplatin by planar N-heterocyclic amines produced *trans*-platinum complexes with significantly improved cytotoxicity due to enhanced rate of bifunctional interstrand adduct formation and altered sequence specificity.[17] Not only the geometry requirements for platinum species have been lifted; many compounds with "non-conventional" structures, including polynuclear,[18] monofunctional,[19] Pt(IV)[20] and organometallic[21] complexes have displayed anticancer potential. These findings highlighted that more chemical space is available for exploration among platinum compounds than previously thought. The same

appears to be true for ruthenium compounds, or, alternatively, different geometries should be investigated than are currently reflected in the literature.

In conclusion, we have synthesized two *trans* Ru(II) complexes and studied their binding interactions and cytotoxicities in comparison to their *cis* analogues. The *trans* complex containing exchangeable ligands is 7.1–9.5x more potent than the *cis* compound. In addition, the *trans* complex is accumulated in cells 49x more than the *cis* compound. We hypothesize that the slower rate of reactivity of **2a** is crucial for the cytotoxic activity. The fast and highly reactive *cis* complex **1a** can easily react with media and non-essential biomolecules, becoming inactivated before it is able to enter the cancer cell. On the other hand, the slow exchange of the *trans* complex **2a** allows it to avoid side reactions and reach a vulnerable target within the cell. Lastly, the ability of the Cl^- ligands of **2a** to exchange is crucial to the biological activity, as evidenced by the absence of activity for the complex incapable of ligand exchange. To the best of our knowledge, this is the first report of the cytotoxic behavior of a thermally activated *trans* polypyridyl Ru(II) complex. These results have the potential to open up a new area of research to develop a metal-based chemotherapeutic.

This work was supported by the National Institutes of Health (5R01GM107586). We would like to thank the University of Kentucky Environmental Research Training Laboratory (ERTL) for their assistance in some chemical analysis. Ana Zamora was supported by Fundación Séneca-CARM (Exp. 19020/FPI/13). The Spanish Ministerio de Economía y Competitividad and FEDER (Project CTQ2015-64319-R) is also acknowledged.

Notes and references

1. L. Kelland, *Nat. Rev. Cancer*, 2007, **7**, 573-584.
2. Farrell N., *Transition Metal Complexes as Drugs and Chemotherapeutic Agents*, Kluwer, Dordrecht, The Netherlands, 1989.
3. (a)V. Brabec and M. Leng, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 5345-5349; (b)V. Brabec, M. Síp and M. Leng, *Biochemistry*, 1993, **32**, 11676-11681; (c)A. Žákovská, O. Nováková, Z. Balcarová, U. Bierbach, N. Farrell and V. Brabec, *Eur. J. Biochem.*, 1998, **254**, 547-557.
4. (a)H. Huang, L. Zhu, B. R. Reid, G. P. Drobny and P. B. Hopkins, *Science*, 1995, **270**, 1842-1845; (b)A. M. Fichtinger-Schepman, J. L. van der Veer, J. H. J. den Hartog, P. H. M. Lohman and J. Reedijk, *Biochemistry*, 1985, **24**, 707-713.
5. (a)N. Cutillas, G. S. Yellol, C. de Haro, C. Vicente, V. Rodríguez and J. Ruiz, *Coord. Chem. Rev.*, 2013, **257**, 2784-2797; (b)S. Medici, M. Peana, V. M. Nurchi, J. I. Lachowicz, G. Crisponi and M. A. Zoroddu, *Coord. Chem. Rev.*, 2015, **284**, 329-350; (c)B. S. Murray, M. V. Babak, C. G. Hartinger and P. J. Dyson, *Coord. Chem. Rev.*, 2016, **306**, 86-114.
6. (a)A. Bergamo and G. Sava, *Chem Soc Rev*, 2015, **44**, 8818-8835; (b)S. Leijen, S. A. Burgers, P. Baas, D. Pluim, M. Tibben, E. van Werkhoven, E. Alessio, G. Sava, J. H. Beijnen and J. H. Schellens, *Invest. New Drugs*, 2015, **33**, 201-214.
7. C. Mari, V. Pierroz, S. Ferrari and G. Gasser, *Chem. Sci.*, 2015, **6**, 2660-2686.
8. (a)R. Zong and R. P. Thummel, *J. Am. Chem. Soc.*, 2004, **126**, 10800-10801; (b)G. Zhang, R. Zong, H. -W. Tseng and R. P. Thummel, *Inorg. Chem.*, 2008, **47**, 990-998; (c)R. Zong, B. Wang and R. P. Thummel, *Inorg. Chem.*, 2012, **51**, 3179-3185; (d)Y. Liu, S. -M. Ng, S. -M. Yiu, W. W. Y. Lam, X. -G. Wei, K. -C. Lau and T. -C. Lau, *Angew. Chem. Int. Ed. Engl.*, 2014, **53**, 14468-14471; (e)J. A. Rudd, M. K. Brennaman, K. E. Michaux, D. L. Ashford, R. W. Murray and T. J. Meyer, *J. Phys. Chem. A*, 2016, **120**, 1845-1852.
9. J. L. Walsh and B. Durham, *Inorg. Chem.*, 1982, **21**, 329-332.
10. B. Durham, S. R. Wilson, D. J. Hodgson and T. J. Meyer, *J. Am. Chem. Soc.*, 1980, **102**, 600-607.
11. (a)T. Renouard, R. -A. Fallahpour, M. K. Nazeeruddin, R. Humphry-Baker, S. I. Gorelsky, A. B. P. Lever and M. Grätzel, *Inorg. Chem.*, 2002, **41**, 367-378; (b)C. -W. Chan, T. -F. Lai and C. -M. Che, *J. Chem. Soc. Dalton Trans.*, 1994, 895-899.
12. E. C. Constable, C. J. Cathey, M. J. Hannon, D. A. Tocher, J. V. Walker and M. D. Ward, *Polyhedron*, 1999, **18**, 159-173.
13. E. Wachter, A. Zamora, L. A. Nease, D. K. Heidary and E. C. Glazer, *manuscript in preparation*.
14. (a)M. J. Cleare and J. D. Hoeschele, *Bioinorg. Chem.*, 1973, **2**, 187-210; (b)L. Trynda-Lemiesz, H. Kozlowski and B. K. Keppler, *J. Inorg. Biochem.*, 1999, **77**, 141-146.
15. O. Vrana, V. Novohradsky, Z. Medrikova, J. Burdikova, O. Stuchlikova, J. Kasparkova and V. Brabec, *Chem. Eur. J.*, 2016, **22**, 2728-2735.
16. B. S. Howerton, D. K. Heidary and E. C. Glazer, *J. Am. Chem. Soc.*, 2012, **134**, 8324-8327.
17. (a)U. Bierbach, Y. Qu, T. W. Hambley, J. Peroutka, H. L. Nguyen, M. Doedee and N. Farrell, *Inorg. Chem.*, 1999, **38**, 3535-3542; (b)J. M. Pérez, M. A. Fuertes, C. Alonso and C. Navarro-Ranninger, *Crit. Rev. Oncol. Hematol.*, 2000, **35**, 109-120; (c)M. Coluccia and G. Natile, *Anticancer Agents Med. Chem.*, 2007, **7**, 111-123; (d)S. M. Aris and N. P. Farrell, *Eur. J. Inorg. Chem.*, 2009, **2009**, 1293-1302; (e)J. Kasparkova and V. Brabec, *J. Inorg. Biochem.*, 2015, **153**, 206-210; (f)G. McGowan, S. Parsons and P. J. Sadler, *Inorg. Chem.*, 2005, **44**, 7459-7467.
18. (a)C. Billecke, S. Finniss, L. Tahash, C. Miller, T. Mikkelsen, N. P. Farrell and O. Bögler, *Neuro. Oncol.*, 2006, **8**, 215-226; (b)J. B. Mangrum and N. P. Farrell, *Chem. Commun.*, 2010, **46**, 6640-6650.
19. (a)K. S. Lovejoy, R. C. Todd, S. Zhang, M. S. McCormick, J. A. D'Aquino, J. T. Reardon, A. Sancar, K. M. Giacomini and S. J. Lippard, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 8902-8907; (b)G. Y. Park, J. J. Wilson, Y. Song and S. J. Lippard, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 11987-11992; (c)T. C. Johnstone, J. J. Wilson and S. J. Lippard, *Inorg. Chem.*, 2013, **52**, 12234-12249.
20. X. Han, J. Sun, Y. Wang and Z. He, *Med. Res. Rev.*, 2015, **35**, 1268-1299.
21. A. Zamora, S. A. Pérez, V. Rodríguez, C. Janiak, G. S. Yellol and J. Ruiz, *J. Med. Chem.*, 2015, **58**, 1320-1336.

Table of Contents Figure