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Discovery of a Strongly Apoptotic Ruthenium Complex Through Combinatorial Coordination Chemistry

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Discovery of a strongly apoptotic ruthenium complex through combinatorial coordination chemistry†

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A strategy for combinatorial parallel coordination chemistry is introduced that provides access to libraries of tris-heteroleptic ruthenium complexes in an economical fashion. Using this method, a library of 560 constitutionally unique, monocationic ruthenium complexes was synthesized, followed by a screening for anticancer activity and resulting in the identification of three hits with promising cytotoxic properties in HeLa cancer cells. A subsequent structure–activity relationship led to the discovery of the surprisingly simple anticancer complex $[Ru(t\mathrm{Bu}_2\mathrm{bpy})_2(\mathrm{phox})]PF_6$ (complex **1**), with $t\mathrm{Bu}_2\mathrm{bpy}$ = 4,4′-di-*tert*-buty-2,2′-bipyridine and Hphox = 2-(2′-hydroxyphenyl)oxazoline, displaying an LC_{50} value in HeLa cells of 1.3 μM and 0.3 μM after incubation for 24 and 72 h, respectively. Complex **1** also shows remarkable antiproliferative and apoptotic properties at submicromolar concentrations in more clinically relevant Burkitt-like lymphoma cells. A reduction of the mitochondrial membrane potential by **1** indicates the involvement of the intrinsic pathway of programmed cell death. Further investigations reveal that **1** requires caspase-3 for the induction of apoptosis but is insensitive to the proapoptotic and antiapoptotic proteins Smac and Bcl-2, respectively.

Introduction

Unique properties of metal complexes such as structural diversity, adjustable ligand exchange kinetics, fine-tuned redox activities, and distinct spectroscopic signatures, render them exciting scaffolds for the design of synthetic compounds with novel bioactivities.[1] In this light it is surprising that chemical biology and the pharmaceutical industry are dominated by organic chemistry, while metal-containing compounds play only a minor role.[2]

Nevertheless, a number of metal complexes are widely employed in clinical therapy and diagnosis, ranging from the important antitumor platinum complex cisplatin, *cis*-$Pt(NH_3)_2Cl_2$,[3] to silver(I) complexes with antibacterial properties,[4] to gadolinium(III) complexes[5] used as powerful magnetic resonance imaging (MRI) agents. The serendipitous discovery of the antiproliferative activity of cisplatin has spurred the development of novel anticancer complexes containing metals from all over the periodic table of elements,[6] such as reactive titanocene dichloride complexes,[7] redox-active ferrocene derivatives,[8] apoptotic organoiron nucleoside derivatives,[9] hypoxia-selective cobalt(III) complexes,[10] antiangiogenic cobalt-alkyne complexes,[11] apoptotic half-sandwich osmium(II) complexes,[12] gold-containing compounds,[13] gallium(III) complexes,[14] in addition to a rather large variety of anticancer ruthenium-containing compounds[15] with distinguished modes of actions. For example, Sadler and co-workers developed [η^6-arene)Ru(ethylenediamine)Cl]$^+$ complexes which exhibit high cytotoxicities against a variety of cancer cell lines and in tumors grafted on mice, most likely induced by a reaction of the ruthenium complexes with genomic DNA.[16] In contrast, the ruthenium(II)-arene-pta complexes (pta = 1,3,5-triaza-7-phosphaadamantane) developed by Dyson and co-workers display low cytotoxicities *in vitro* but show remarkable effects on metastasis *in vivo*.[17] Two octahedral antitumor ruthenium(III) complexes that are believed to become activated by reduction to ruthenium(II),[18] NAMI-A[17] and KP1019,[19] have successfully completed phase I clinical trials recently. Interestingly, even some completely unreactive ruthenium complexes have been reported to display anticancer activities. For example, Dwyer and coworkers already discovered more than 40 years ago that the inert hydrophobic ruthenium complex tris(3,4,7,8-tetramethyl-1,10-phenanthroline)ruthenium(II) dichloride inhibits the growth of Landschütz ascites tumor cells in mice,[20] whereas our group recently designed a class of unreactive ruthenium η^5-cyclopentadienyl pyridocarbazole complexes that bind reversibly to the ATP-binding site of protein kinases, with some complexes displaying strong apoptotic properties *in vitro*.[21] The ability of ruthenium to be tuned with respect to both reactivity and structure makes ruthenium one of the most versatile metals for the design of metal complexes with distinguished biological properties.

Technologies such as combinatorial chemistry and high throughput screening (HTS) offer the opportunity to significantly accelerate the process of discovering compounds with desired biological properties and have therefore been widely embraced by academia and the pharmaceutical industry for the identification of synthetic organic molecules with novel bioactivities. We were envisioning that the same discovery process should be highly applicable to octahedral metal complex scaffolds in which the synthesis of libraries would rely on combinatorial coordination

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chemistry.[22] Starting even from a limited number of reactive precursor complexes, combinatorial ligand exchange reactions should give access to a large number of metal complexes in a highly economical fashion. Importantly, since such compounds are built from a common structural center, the metal ion, it is the nature of the ligands in combination with their arrangement in the coordination sphere (relative and absolute stereochemistry) that determines the overall shape and functional group presentation of the derived complexes and therefore allows a large structural diversity if the ligands are chosen properly.[23]

We herein report a simple octahedral ruthenium complex **1** (Fig. 1) with striking apoptotic properties that was identified through such combinatorial chemistry, subsequent HTS and followed by a brief structure–activity relationship.

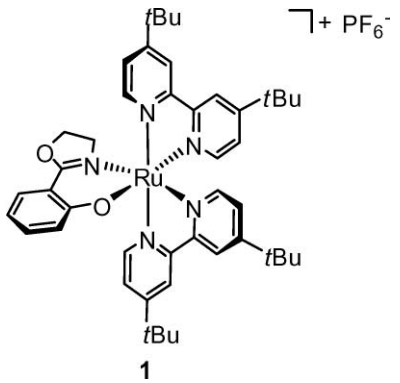

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Fig. 1 Anticancer complex **1** (racemate) was discovered through combinatorial coordination chemistry, followed by screening for cytotoxicity and a brief structure–activity-relationship. For clarity, only one enantiomer of **1** is shown.

Results and discussion

Synthesis of a ruthenium complex library

We started from the simple ruthenium complex precursor **2** in which ruthenium is coordinated to a bidentate ligand X–X in addition to four leaving groups (MeCN and chloride ligands) (Fig. 2).[24] It has been shown by others and us that in precursor complexes of this type, the labile monodentate ligands can be replaced readily in good yields by, for example, two bidentate ligands, thus leading to stable heteroleptic ruthenium complexes.[23,24] We expected that this reaction scheme would be highly suitable for combinatorial coordination chemistry because the reaction of precursor complex **2** successively with two different bidentate ligands would generate quickly very large ruthenium complex libraries. For example, the reaction of precursor **2** first with 100 bidentate ligands Y–Y, followed by another 100 bidentate ligands Z–Z would generate a library size of 10 000 constitutionally unique compounds without much effort.

After some rounds of optimization, we developed the reaction scheme shown in Fig. 2a. Accordingly, precursor complex **2** with X–X = 2,2′-bipyridine (bpy) was first reacted with a neutral bidentate ligand Y–Y in DMF at 75 °C for 3–4 h, followed by the addition of a monoanionic bidentate ligand Z–Z in ethylene glycol:water, with subsequent heating for another 2–3 h now at the elevated temperature of 90 °C. This optimized reaction scheme turned out to result only in minimal formation of side products and generated monocationic ruthenium complexes as the main products.

An initial library was synthesized starting from precursor complex **2** (X–X = bpy) plus a combination of 40 neutral bidentate ligands Y–Y and 17 monoanionic bidentate ligands Z–Z according to Fig. 2. The library was synthesized in sealed polypropylene

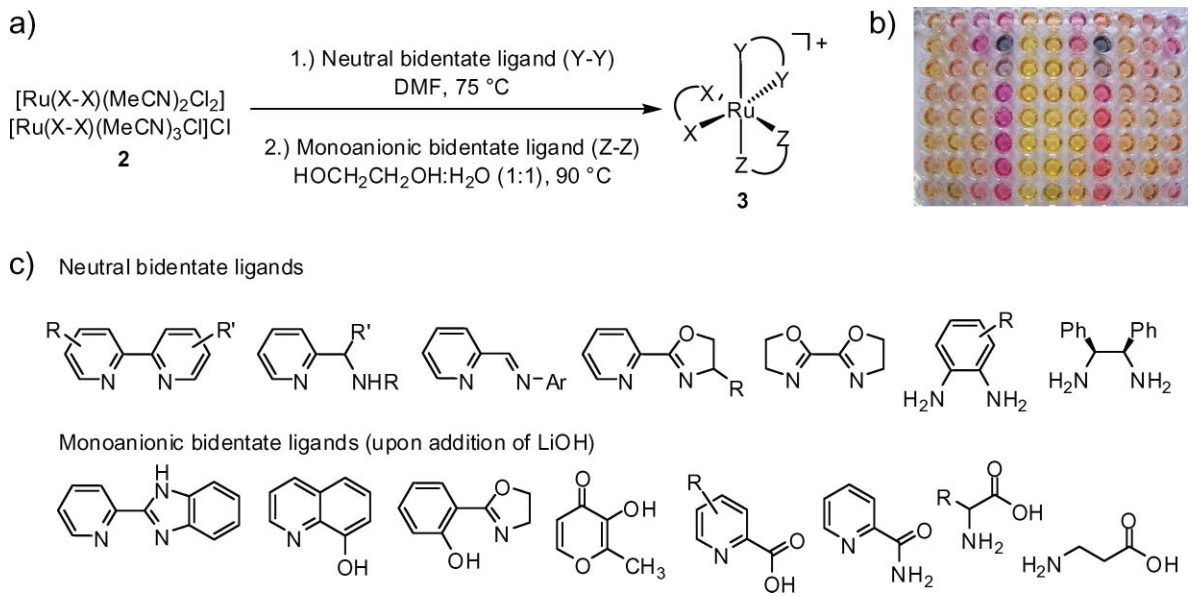

Fig. 2 Combinatorial parallel coordination chemistry for the synthesis of a library of monocationic tris-heteroleptic ruthenium complexes. (a) Optimized reaction scheme for the library synthesis. All compounds were formed as mixtures of enantiomers or diastereomers but for clarity only one metal-centered-isomer is shown for complex **3**. (b) Ruthenium complex library in 96-well plates. The image shows a library that was diluted to 100 μM after the synthesis. (c) Neutral and monoanionic bidentate ligands used for a more than 500-membered ruthenium complex library. See ESI† for more details.

Table 1 Cytotoxicity of ruthenium complexes **1**, **3a–i** in HeLa cells

Entry	Compound[a]	X–X[b]	Y–Y[b]	Z–Z[b]	LC_{50}[c]
1	**3a**	bpy	tBu$_2$bpy	phox	4
2	**3b**	bpy	non$_2$bpy	Br-picolinate	13
3	**3c**	bpy	non$_2$bpy	Cl-picolinate	13
4	**3d**	bpy	bpy	phox	>100
6	**1**	tBu$_2$bpy	tBu$_2$bpy	phox	1.3 (0.3)[d]
7	**3e**	tBu$_2$bpy	tBu$_2$bpy	8-HQ	2
8	**3f**	tBu$_2$bpy	tBu$_2$bpy	picolinate	40
9	**3g**	tBu$_2$bpy	tBu$_2$bpy	tBu$_2$bpy	20
10	**3h**	tBu$_2$bpy	tBu$_2$bpy	bpy	50
11	**3i**	tBu$_2$bpy	tBu$_2$bpy	dichloride	13

[a] Compounds **1** and **3a–i** were synthesized individually and purified. Charged complexes contain a PF_6 counterion. [b] bpy = 2,2′-bipyridine, tBu$_2$bpy = 4,4′-di-*tert*-butyl-2,2′-bipyridine, non$_2$bpy = 4,4′-dinonyl-2,2′-bipyridine, phox = deprotonated 2-(2′-hydroxyphenyl) oxazoline, Br-picolinate = 4-bromopicolinate, Cl-picolinate = 4-chloropicolinate, 8-HQ = deprotonated 8-hydroxyquinoline. [c] HeLa cells were incubated with different concentrations of complexes for 24 h at 37 °C after which point cell viability was determined with the MTT method. [d] 72 h incubation.

round-bottom 96-well plates with individual reaction volumes of 100 µL under nitrogen and with orbital shaking. The quality of the library was verified by analysis with LC/MS of a random selection of *ca.* 10% of the synthesized samples (see ESI† for more information). Some ligand combinations resulted in insoluble complexes which were eliminated from the library, thus resulting in an initial library of overall 560 soluble, constitutionally unique, monoanionic ruthenium complexes which were tested as mixtures of stereoisomers (see ESI† for more information).

Screening for cytotoxicity in HeLa cells

The crude reaction products were tested at an approximate concentration of 30 µM for their cytotoxicity in HeLa cells by using the MTT method.[25] Interestingly, out of the initial 560 library members (as mixtures of stereoisomers), most compounds did not show any significant activity in HeLa cells at these high concentrations for a 24 h incubation and only three compounds, **3a–c** (Table 1, entries 1–3), reduced cell survival below 5% (see ESI† for all compounds). These three compounds were resynthesized individually and the LC_{50} values (50% lethal concentration; concentration of compounds which cause the death of 50% of the cell population) of the purified compounds were determined to be 4, 13, and 13 µM for **3a**, **3b**, and **3c**, respectively. Complex **3a**, bearing a bpy, a 4,4′-di-*tert*-butyl-2,2′-bipyridine (tBu$_2$bpy), and a deprotonated 2-(2′-hydroxyphenyl)oxazoline (phox) ligand displayed the highest cytotoxicity and was selected as a lead structure for further studies.

Structure–activity relationship and discovery of complex 1

In order to evaluate which of the ligands in complex **3a** are required and responsible for its biological effect, we performed a brief structure–activity relationship (SAR) of individually synthesized and purified derivatives by exchanging single and multiple ligands of **3a** as shown in Table 1 and measuring the resulting cytotoxicities.

Interestingly, replacing the tBu$_2$bpy ligand of **3a** against bpy (**3d**, Table 1, entry 4) afforded an almost completely inactive complex (LC_{50} > 100 µM). However, a substitution of the bpy ligand in **3a** for another tBu$_2$bpy ligand afforded complex **1** which displayed an impressive LC_{50} value of 1.3 µM (24 h). This LC_{50} value decreased even further to 0.3 µM when the incubation time was elongated to 72 h. The cytotoxicities of **3a**, **3d**, and **1** correlate with their different hydrophobicities which directly affect the cellular uptake properties. On the other hand, replacing the phox ligand against any tested monoanionic (Table 1, entries 7 and 8) or neutral ligands (Table 1, entries 9 and 10) reduced the potency in all cases. Furthermore, it is also noteworthy that the reactive complex **3i** (Table 1, entry 11), containing in the coordination sphere two tBu$_2$bpy ligands and two chlorides, displayed a cytotoxicity in HeLa cells that was by an order of magnitude weaker compared to complex **1**. This brief SAR thus reveals that the combination out of the hydrophobic tBu$_2$bpy ligands and the monoanionic phox ligand is most beneficial for the cytotoxic behaviour in HeLa cells.

Antiproliferative and apoptotic properties of 1 in BJAB cells

In order to evaluate the anticancer properties of complex **1** in a more clinically relevant cancer, we tested the antiproliferative properties of **1** in Burkitt-like lymphoma (BJAB) cells. Surprisingly, even at a concentration as low as 100 nM, complex **1** was able to affect the viability of BJAB cells after a 24 h incubation in a dose-dependent fashion (Fig. 3a). Consistent with these results, a flow cytometrical analysis revealed a dose-dependent fragmentation of nuclear DNA, which is indicative of a highly efficient induction of apoptosis at submicromolar concentrations of complex **1** (Fig. 3b, 16% apoptotic cells at 100 nM **1**). More specifically, Fig. 3c shows that compound **1** strongly reduces the mitochondrial membrane potential which suggests the involvement of the intrinsic pathway of programmed cell death.[26] For example, after incubation of BJAB cells with 1 µM of ruthenium complex **1** for 48 h, already 69% of BJAB cells display a reduced membrane potential as determined by staining with the dye JC-1.[27]

Probing Smac-dependence in Jurkat cells

The release of proapoptotic factors from activated mitochondria into the cytosol are key events in the initiation of the final phase of apoptosis.[28] One of these death-promoting factors is the protein Smac which serves as a sensitizer for caspase activation.[29] We therefore tested the Smac-dependence of apoptosis induced by complex **1** in Jurkat T-lymphocyte cells that overexpress Smac and compared with regular Jurkat cells as shown in Fig. 4. Interestingly, no clear trend could be observed suggesting that the induction of apoptosis by **1** is independent of Smac and instead most likely relies on other proapoptotic factors instead.

Anticancer activity in drug resistant cancer cell lines

Drug-resistance poses a significant challenge for the successful clinical treatment of tumors.[30] We therefore tested complex **1** in vincristine (Vcr)- and doxorubicin (Dox)-resistant BJAB cells. The Vcr-resistance was achieved through an overexpression of the antiapoptotic protein Bcl-2,[31] whereas Dox-resistance was achieved due to the reduced levels of procaspase-3 expression. Interestingly, the results in Fig. 5 reveal that complex **1** cannot efficiently induce apoptosis in Dox-resistant cells but can overcome the Vcr-induced resistance, demonstrating that the induction of

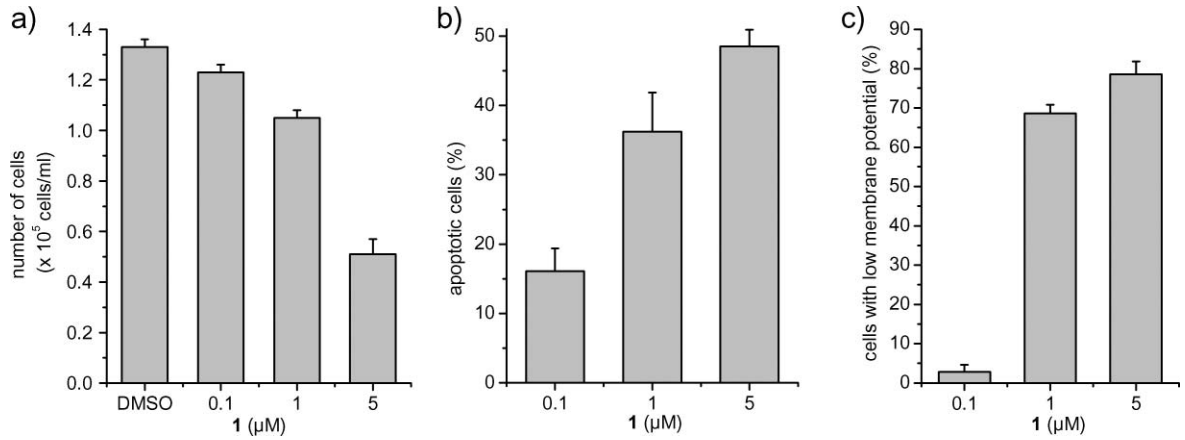

Fig. 3 Antiproliferative and apoptotic properties of complex **1** in BJAB cells. a) Inhibition of BJAB cell proliferation after treatment with **1** for 24 h as measured by a CASY cell counter. Bars indicate the number of cells after 24 h incubation. DMSO-treated cells served as the control. b.) Apoptosis induction of **1** in BJAB cells as measured by DNA fragmentation after treatment of BJAB cells with 1 for 72 h. Nuclear DNA fragmentation was quantified by flow cytometric determination of hypodiploid DNA. Data are given in percentage hypoploidy (sub-G1) ± SD ($n = 3$), which reflects the number of apoptotic cells. c.) Probing the induction of apoptosis by complex **1** *via* the intrinsic pathway by determining the influence of **1** on the membrane potential. The mitochondrial permeability transition was measured by flow cytometric analysis in BJAB cells after treatment with various concentrations of **1** for 48 h. Values of the mitochondrial permeability transition are given as the fraction of cells with decreased membrane potential in% ± SD ($n = 3$).

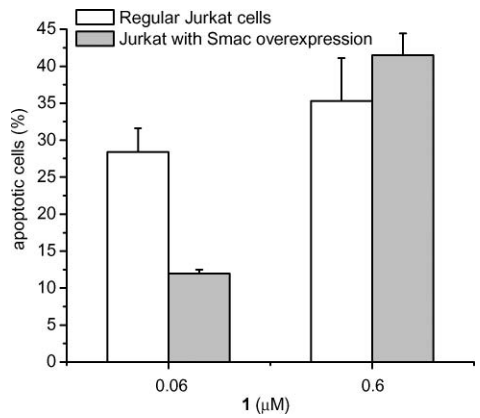

Fig. 4 Apoptotic properties of complex **1** as a function of Smac expression. Induction of apoptosis was measured by DNA fragmentation after treatment of regular and Smac overexpressing Jurkat cells with **1** for 72 h. Nuclear DNA fragmentation was quantified by flow cytometric determination of hypodiploid DNA. Data are given in percentage hypoploidy (sub-G1) ± SD ($n = 3$).

apoptosis by **1** is sensitive to caspase-3 but not Bcl-2. Thus, caspase-3 is necessary for ruthenium-complex **1** induced apoptosis.

Conclusions

In summary, we reported here a strategy for combinatorial coordination chemistry that gives access to libraries of ruthenium complexes in an economical fashion. Using this method, a library of more than five hundred monocationic ruthenium complexes was synthesized and screened for cytotoxic properties in HeLa cells, which resulted after a brief structure–activity study in the discovery of the anticancer complex **1**. The surprisingly simple ruthenium complex **1** displays potent antiproliferative and apoptotic properties in BJAB cells already at submicromolar concentrations and thus constitutes a promising anticancer drug candidate.

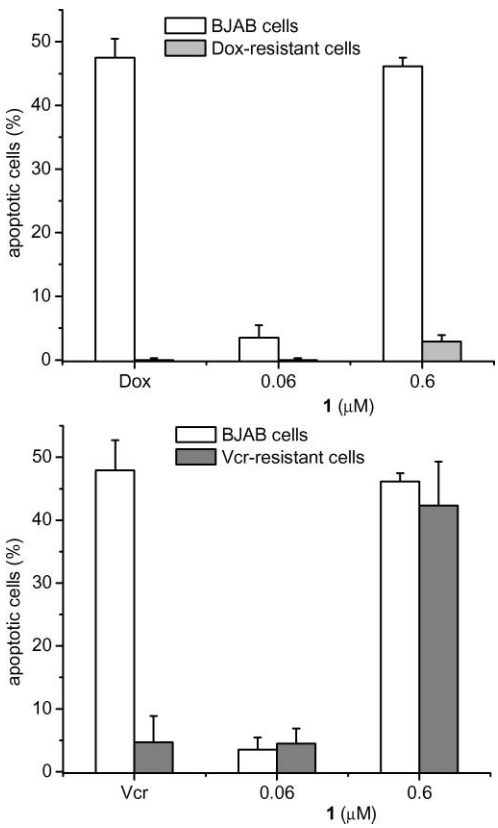

Fig. 5 Apoptotic properties of complex **1** in drug resistant BJAB cells. a) Doxorubicin (Dox)-resistant BJAB cells. Dox conc. = 170 nM. b.) Vincristine (Vcr)-resistant BJAB cells. Vcr conc. = 2 nM. Induction of apoptosis was measured by DNA fragmentation after treatment of regular and resistant BJAB cells with Dox, Vcr, or **1** for 72 h. Nuclear DNA fragmentation was quantified by flow cytometric determination of hypodiploid DNA. Data are given in percentage hypoploidy (sub-G1) ± SD ($n = 3$).

This work demonstrates that combinatorial coordination chemistry followed by screening is a suitable strategy for the discovery of metal complexes with novel biological properties and that this method should be applicable to larger library sizes, as well as the screening for other biological properties. Work along these lines is ongoing in our laboratory.

Experimetal

General methods

Preparative-scale reactions were performed in round-bottom flasks or test tubes equipped with rubber septa and a magnetic stir bar under argon or nitrogen atmosphere. Reactions performed in 96-well plates (250 μL well volume, round bottom) were heat-sealed with adhesive aluminium foil and shaken on a KEM-Lab Vortex Mixer from J-KEM Scientific equipped with a nitrogen balloon. Anhydrous DMF, ethylene glycol, and EtOH were obtained from commercial sources and further dried over molecular sieves (4 Å, 8–12 mesh). All other solvents were used as HPLC grade quality. The synthesis of complex **2** (X–X = bpy) has been reported.[24] See ESI† for the synthesis and analytical data of complexes **3a–i**. Cell viability experiments, DNA fragmentation, and the measurement of the mitochondrial permeability transition have been performed in analogy to a recently published protocol.[32]

Library synthesis

A stock solution containing 100 mM **2** (X–X = bpy) in DMF was purged with nitrogen thoroughly. Separate stock solutions containing 100 mM of each neutral bidentate ligand in DMF were also purged with nitrogen thoroughly. In a polypropylene round bottom 96-well plate, 30 μL of DMF was added to each well, followed by 10 μL of each neutral ligand in a predetermined order, and finally by 10 μL of the ruthenium precursor. The plate was sealed with heat sealing foil and then shaken from 55 °C to 75 °C over 1 h, and then held at 75 °C for 3 h. The plate was then cooled and centrifuged (4000 rpm, 5 min). The seal was removed and 40 μL of ethylene glycol was added, followed by 10 μL of a nitrogen-purged 100 mM stock solution of a monoanionic bidentate ligand as its lithium salt in ethylene glycol : water (1 : 1). The solution was mixed by pipetting, and then shaken from 70 °C to 90 °C over 1 h, and then held at 90 °C for another 2 h. The plate was then cooled, centrifuged at 2000 rpm for 1 min, and stored at 4 °C until used directly for screening without further work-up or purification. This procedure was repeated until all ligand combinations were synthesized. Ligand combinations that resulted in insoluble complexes were eliminated from the compound collection so that finally overall 560 constitutional unique compounds were obtained as mixtures of stereoisomers and subsequently tested without further purification. See ESI† for a quality evaluation of the library and the screening data.

Ruthenium complex 1

[Ru(*t*Bu$_2$bpy)$_2$Cl$_2$] (350 mg, 495 mmol) in 49.5 mL EtOH–H$_2$O (9 : 1) was treated with 2-(2′-hydroxyphenyl)oxazoline (81 mg, 495 mmol) and LiOH (21 mg, 495 mmol) and refluxed for 4 h. The resulting solution was concentrated *in vacuo* and purified by column chromatography on silica gel using mixtures of MeCN and 100 : 3 : 1 MeCN:H$_2$O:KNO$_3$ (sat. aq.) as eluent. The major colored fractions were collected and concentrated to dryness, then redissolved in 8 : 1 H$_2$O:EtOH and precipitated with saturated aqueous NH_4PF_6. The compound was then centrifuged and washed extensively with water. The purified product was redissolved in MeCN and tared to provide a purple solid (368 mg, 78%). ^{1}H NMR (MeCN-d_3) δ (ppm) 8.65 (m, 2H, Py*H*), 8.44 (m, 2H, Py*H*), 8.37 (d, J = 2.0 Hz, 1H, Py*H*), 8.32 (d, J = 2.0 Hz, 1H, Py*H*), 7.65 (d, J = 6.1 Hz, 1H, Py*H*), 7.61 (dd, J = 2.1, 6.1 Hz, 1H, Py*H*), 7.51–7.56 (m, 2H, Py*H*), 7.43 (d, J = 6.1 Hz, 1H, Py*H*), 7.18 (dd, J = 2.1, 6.0 Hz, 1H, Py*H*), 7.14 (dd, J = 2.1, 6.0 Hz, 1H, C$_6H_4$O), 7.01 (ddd, J = 1.9, 6.8, 8.9 Hz, 1H, C$_6H_4$O), 6.27–6.37 (m, 2H, C$_6H_4$O), 4.43 (dt, J = 1.2, 9.1 Hz, 1H, CH_2N), 4.23 (m, 1H, CH_2O), 3.48 (br m, 1H, CH_2N), 2.75 (br m, 1H, CH_2N), 1.49 (s, 9H, CCH_3), 1.47 (s, 9H, CCH_3), 1.37 (s, 9H, CCH_3), 1.34 (s, 9H, CCH_3). ^{13}C NMR (MeCN-d_3) δ (ppm) 161.5, 161.2, 160.9, 160.5, 160.4, 159.1, 158.7, 158.5, 153.7, 151.9, 151.7, 150.7, 133.2, 130.5, 124.9, 124.2, 124.1, 123.8, 121.5, 121.5, 121.2, 121.0, 67.6 (*C*H$_2$O), 55.0 (*C*H$_2$N), 36.2 (*C*CH$_3$), 36.1 (*C*CH$_3$), 36.0 (*C*CH$_3$), 36.0 (*C*CH$_3$), 30.8 (*C*H$_3$), 30.7 (*C*H$_3$), 30.6 (*C*H$_3$), 30.5 (*C*H$_3$). IR (thin film) ν/cm^{-1} 3441, 3066, 2963, 2911, 2874, 1685, 1615, 1540, 1469, 1412, 1339, 1240, 1026, 928, 850. UV (maxima in nm, 100 μM in 1% DMSO/MeCN) 360, 525. Mp > 300 °C. HRMS (ESI-pos) calcd for RuC$_{45}$H$_{56}$N$_5$O$_2$ (M-PF$_6$)$^+$ 800.3472, found 800.3490.

Cytotoxicity measurements in HeLa cells

HeLa cells were obtained from Sigma and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin at 37 °C under an atmosphere of 5% CO_2 and constant humidity. Cells grown to 70% confluency were first washed with phosphate-buffered saline (PBS) and then trypsinized for 5 min at 37 °C. The cells were then harvested, centrifuged for 5 min at 1000 rpm, resuspended in 5 mL fresh DMEM and counted using a hemacytometer. The cells were then plated into sterile 96-well plates at a concentration of 10 000 cells per well (100 μL total volume) and grown for 24 h (cells grown for 72 h had 2000 cells per well). Afterwards, 100 μL of a 2X stock solution containing 60 μM Ru complex in 0.6% DMF:ethylene glycol (1 : 1) in DMEM, prepared directly from 10 mM library solutions in 1 : 1 DMF:ethylene glycol, were added and the cells were incubated for 24 h. The final concentration of complexes was 30 μM in 0.3% DMF:ethylene glycol and each data point was measured in triplicate. As a control, the cells were treated with 0.3% DMF:ethylene glycol. To ensure proper growth of the cells, the edges of the plate contained only buffer. After the incubation period, the media was removed *via* pipet and replaced with 200 μL fresh media and 20 μL of a 5 mg mL^{-1} solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were incubated for 3 h, at which point 155 μL of this solution was removed. To the remaining solution was added 90 μL DMSO which solubilized the purple crystals. The plates were then incubated at 37 °C for 10 min and then an absorbance measurement of each well was taken at 535 nm. A background correction was made by subtracting the absorption containing DMEM media and MTT plus DMSO. Percent cell survival was

then calculated as a percentage of the maximum absorbance for cells treated with only 0.3% DMF:ethylene glycol.

Measurements of LC_{50} values in HeLa cells

The HeLa cells were harvested and plated as described above. Then, 100 µL of a 2X stock solution containing a dilution series of ruthenium complexes in 1% DMSO in DMEM, prepared from a 20 mM stock solution of pure complexes, was added and the cells were incubated for 24 (or 72) h. The final concentration of ruthenium complexes was 100 nM–100 µM in 0.5% DMSO and each data point was measured in triplicate. As a control, the cells were treated with 0.5% DMSO. After the incubation, the media was removed *via* pipette and the MTT assay was performed as described. Percent cell survival for each concentration was then calculated as a percentage of the maximum absorbance for cells treated with only 0.5% DMSO. LC_{50} values were determined from a semi-log plot of percent cell survival and $\log_{10}$ (concentration) for the average of three identical experiments.

Drug-resistant BJAB cells

To generate vincristine- or doxorubicin-resistant BJAB cells, BJAB cells were exposed to increasing concentrations of selected chemotherapeutic drugs until they tolerated high concentrations without loss of vitality. By this method we produced a vincristine-resistant BJAB cell line (BJAB/Vcr) and a doxorubicin-resistant BJAB cell line (BLAB-7CDA). Cell lines were maintained at 37 °C in RPMI 1640 medium (GIBCO, Invitrogen), supplemented with heat inactivated fetal calf serum (FCS, 10%, v/v), L-glutamine (0.56 g l^{-1}), penicillin (100 000 i.u.) and streptomycin (0.1 g l^{-1}). Cells were passaged 2–3 times per week by dilution to a concentration of 1×10^5 cells mL^{-1}. 24 h before the assay setup, cells were adjusted to 3×10^5 cells mL^{-1} to ascertain standardized growth conditions. For proliferation and apoptosis assays, cells were diluted to 1×10^5 cells mL^{-1} immediately before treatment with the respective agents.

Acknowledgements

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