

Anticancer Potential of Camalexin-Derived Ru(II) Metallacycles

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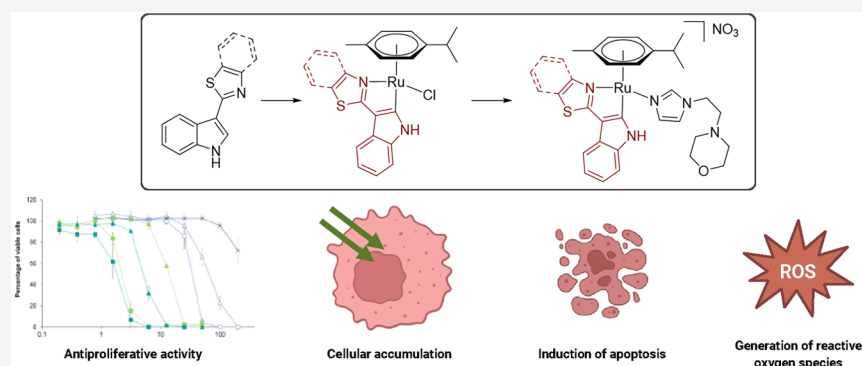
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ABSTRACT: Camalexin, a primary indole-analogous phytoalexin from *Arabidopsis thaliana* and other crucifers, inhibits the proliferation of various cancer cells. In this contribution, the synthesis and characterization of camalexin-derived Ru(II) half-sandwich complexes are described. Formation and sufficient purity of the complexes were confirmed by ^1H -, ^{13}C - and 2D-NMR techniques, X-ray or 3D electron diffractometry, high-resolution-mass-spectrometry (HRMS) and elemental analysis. Additionally, the stability in aqueous solution was studied under pseudophysiological conditions, revealing sufficient stability for further biological studies. Investigating the *in vitro* anticancer potency by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) and resazurin assays in four human cancer cell lines revealed IC_{50} values in the (mostly low) micromolar range. Tests for the capacity of generating reactive oxygen species (ROS) in the leukemic HL60 cell line identified one derivative with exceptionally high ROS induction via a 2',7'-dichlorofluorescein diacetate (DCFH-DA)-based approach. While increased ROS levels were not decisive for cell death induction, cytotoxic activity of the complexes was associated with mitochondrial membrane depolarization and functional perturbation. Lastly, the ability of the complexes to induce apoptosis was investigated, with the complexes showing remarkable effects, while the free ligands exhibited hardly any signs of apoptosis induction.

1. INTRODUCTION

Since the discovery of the DNA-binding agent cisplatin, the pursuit of new metal-based anticancer drugs has shifted toward the development of less toxic alternatives that maintain high efficacy and exhibit lower susceptibility to resistance.¹ In particular, certain ruthenium-based compounds have attracted interest due to their desirable pharmacological properties.^{2,3} Several Ru(III) compounds successfully entered clinical trials so far: NAMI-A and KP1019 displayed strong antimetastatic and anticancer activities *in vivo*, respectively, but studies were discontinued due to toxicity and solubility issues.² BOLD-100 (Figure 1), the more water-soluble successor of KP1019, is the most clinically advanced ruthenium-based therapeutic, currently being studied in phase II trials (NCT04421820). BOLD-100 induces reactive oxygen species and alters the unfolded protein response through GRP78 inhibition, leading to enhanced endoplasmic reticulum stress and apoptotic cell death.^{4–6} In-depth research into the mechanism of action of this type of Ru(III) complexes has revealed that they are

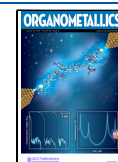
activated *in vivo* by reduction of inert Ru(III) to the more active Ru(II) oxidation state.^{7,8} Alongside BOLD-100, the Ru(II) complex TLD-1433 (Figure 1) is also in advanced clinical development, currently in a Phase II trial (NCT03945162) for photodynamic therapy of nonmuscle-invasive bladder cancer.^{9,10} Organometallic Ru(II) complexes enable a variety of structural modifications, which facilitates fine-tuning of the physicochemical and biological properties. The furthest developed representatives of this compound class are Sadler's RAED (e.g., RM175, Figure 1) and Dyson's RAPTA (e.g., RAPTA-T, Figure 1) complexes.^{11–13} To overcome the inherent instability commonly associated with

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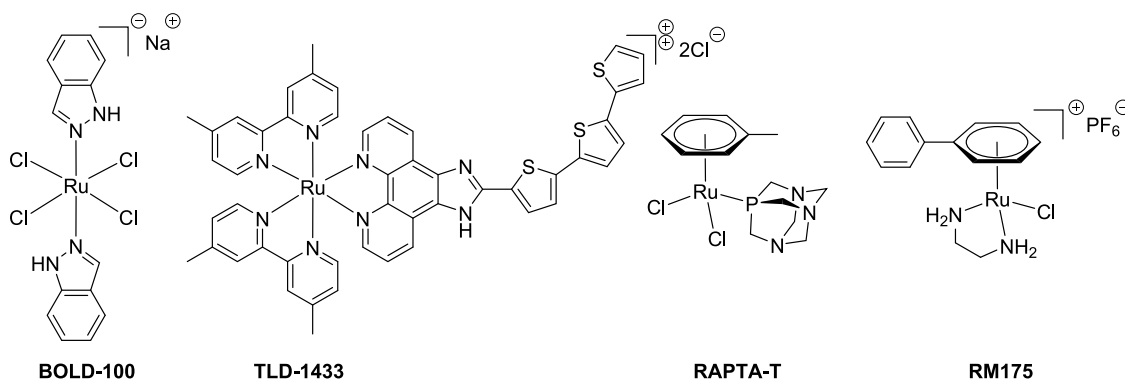
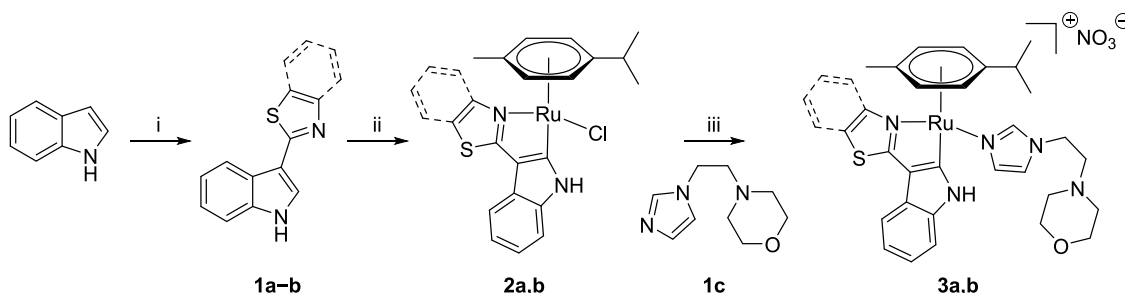


Figure 1. Chemical structures of Ru(III) compound BOLD-100, and Ru(II) compounds TLD-1433, RAPTA-T, and RM175.

Scheme 1. Synthetic Pathway towards Target Complexes^a



^ai: Mg, MeI, 2-bromothiazole or 2-bromobenzothiazole in Et₂O/toluene; ii: (Ru(*p*-cym)Cl₂)₂, NaOAc in DCM; iii: 4-(2-(1*H*-imidazol-1-yl)ethyl)morpholine, AgNO₃ in MeOH. camalexin = **a**; benzocamalexin = **b**.

conventional bidentate ligand structures in Ru(II) complexes, especially those with O,O, N,O, or S,O motifs,^{14,15} N,N or C,N ligand scaffolds have been employed with promising results. In particular, C,N-bidentate ligands have garnered considerable attention in recent years, due to their enhanced stability under physiological conditions together with notable antiproliferative activities.^{16–24} For example, Yellol et al. reported C,N-cyclometalated benzimidazole Ru(II) and Ir(III) complexes with remarkable antiproliferative and antiangiogenic properties, demonstrating their considerable potential as metal-based anticancer agents and establishing clear structure–activity relationships.¹⁶ Similarly, Martínez-Alonso et al. described Ru(II) arene complexes bearing 2-aryldiazole ligands that exhibited promising cytotoxic effects, revealing that subtle electronic and steric modifications of the ligands have a profound impact on their biological performance and providing further insights into the relationship between molecular structure and anticancer efficacy.²²

The coordination of bioactive ligands is a promising approach for the development of novel metallodrugs and can improve the anticancer potential, enable novel modes of action compared to the parental compounds, might help with overcoming resistances and improve drug stability.²⁵

Camalexin is a phytoalexin biosynthesized *de novo* by plants as a defense against fungal and bacterial pathogens.²⁶ After synthesizing a wide array of different (thiazolyl)indoles, Moody et al. initially discovered activity of camalexin against the human breast cancer cell line SK-BR-3.²⁷ Mezencev and co-workers hypothesized that the ability of camalexin to stimulate reactive oxygen species (ROS) production might play an important role in camalexin-induced apoptosis.²⁸ Further studies showed that fusion with a benzene ring on the

thiazole moiety increases the cytotoxicity and selectivity for cancer cells.²⁹

In this study, camalexin and benzocamalexin were employed as C,N-bidentate ligands for Ru(II)-*p*-cymene (*p*-cym) complexes, aiming for cumulative cytotoxic effects attributable to the inherent anticancer activity of both the ligands and the Ru(II)-arene-L_{CAN} scaffold. Due to the highly lipophilic nature of the resulting complexes, improving the aqueous solubility is desirable. Hence, we utilized the highly modular complex structure and introduced 4-(2-(1*H*-imidazol-1-yl)ethyl)morpholine as a leaving group, which can be cleaved off under acidic conditions present in the hypoxic tumor environment.^{30,31}

2. RESULTS AND DISCUSSION

2.1. Synthesis

The ligands, camalexin and benzocamalexin, were synthesized based on the procedure by Ayer et al.³² utilizing a Grignard reagent to deprotonate indole followed by a nucleophilic attack on 2-bromothiazole or 2-bromobenzothiazole (Scheme 1). Formation of the desired products was confirmed by ¹H NMR spectroscopy (Figures S1–S3).

Cyclometalation was accomplished via sp² C–H bond activation. The dimeric precursor, [Ru(*p*-cym)Cl₂]₂, was converted into the Ru(*p*-cym)(OAc)₂ species by treatment with an excess of sodium acetate. The resulting activated complex interacted with the ligand where, initially, the thiazole nitrogen coordinated to the metal center. Subsequently, deprotonation of the *ortho* hydrogen of the phenyl group by uncoordinated acetate, which simultaneously facilitated coordination to complete the cyclometalation process. Finally, the acetato complex, [Ru(*p*-cym)(L_{CAN})(OAc)], underwent li-

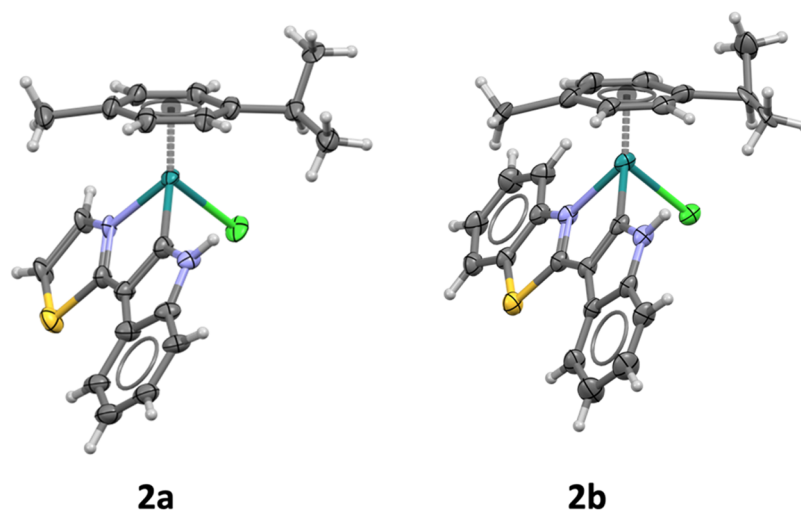


Figure 2. (a) ORTEP representation of the electron diffraction (ED) structure of complex **2a**, with thermal ellipsoids drawn at the 30% probability level. (b) X-ray diffraction structure of complex **2b**, with thermal ellipsoids shown at the 30% probability level; only one orientation of the disordered molecule is depicted.

gand exchange, yielding the desired chlorido complexes. Lastly, 4-(2-(1*H*-imidazol-1-yl)ethyl)morpholine was incorporated to increase the solubility of the parental complexes. Compounds **3a,b** were obtained after abstraction of the chlorido ligand with AgNO_3 , which facilitates the reaction with the *N*-donor molecule.

2.2. Characterization

The synthesized compounds were analyzed by standard analytical techniques, including ^1H -, ^{13}C -, and 2D-NMR spectroscopy, high-resolution electrospray ionization mass spectrometry (ESI-MS), 3D electron diffraction, X-ray crystallography and elemental analysis. 1D (Figures S4 (2a), S8 (2b), S12 (2c), S16 (2d)) and 2D NMR data (Figures S5–S7 (2a), S9–S11 (2b), S13–S15 (3a), S17–S19 (3b)) unambiguously confirmed the formation of the desired cyclometallates. Previous studies on related triazole and thiazole organometallics demonstrated that the chlorido leaving group is readily replaced by dimethyl sulfoxide (DMSO).²⁰ However, due to solubility issues of **2a** and **2b**, CDCl_3 and MeOD were also excluded as potential NMR solvents. Deuterated *N,N*-dimethylformamide (DMF) effectively addressed these challenges and was therefore selected as the preferred solvent for NMR measurements of the complexes.

Upon *C,N*-coordination, the aromatic *p*-cym protons were observed as four separate doublets in contrast to the dimeric precursor where only two doublets were found. These doublets were split farther apart in case of **2b** and **3b** due to the sterically more demanding benzocamalexin ligand (Figures S8 and S16). The coordination of morpholine-imidazole induces a downfield shift of the aromatic signals as can be seen in the ^1H NMR spectra (Figures S1–S7). All complexes showed hygroscopic behavior, which was confirmed by determination of the *O*-content via elemental analysis. In case of the chlorido complexes **2a,b**, dichloromethane (DCM) traces (0.20 equiv and 0.05 equiv, respectively) remained in the sample even after drying for several days at 50 °C *in vacuo* and the respective solvent signals were also found in the corresponding NMR spectra (Figures S4 and S8). These findings explained the obtained deviations from the theoretical values of the performed elemental analyses.

High-resolution mass spectrometry of the cyclometallates confirmed the presence of the chlorido-abstracted species $[\text{M}-\text{Cl}]^+$ along with acetonitrile (ACN) adducts $[\text{M}-\text{Cl} + \text{C}_2\text{H}_3\text{N}]^+$ in case of **2a** and **2b**. Additionally, the dimeric ACN adduct of **2b** $[2\text{M}-\text{Cl} + \text{C}_2\text{H}_3\text{N}]^+$ was observed (Figures S20 and S21). Morpholine-imidazole species **3a** and **3b** were observed as the free cation $[\text{M}-\text{NO}_3]^+$ (Figures S22 and S23).

Single crystals of compounds **2a** and **2b** were obtained by slow diffusion of a DCM complex solution into *n*-hexane. The crystals of **2a** were too small for conventional X-ray diffraction and were therefore analyzed using electron diffraction. Due to preferred orientation and the low symmetry of the $P2_1/c$ space group, data from five individual crystals were merged to achieve sufficient completeness for structure determination. In contrast, crystals of **2b**, which crystallized in the $P2_1/n$ space group, were of adequate size and quality for standard X-ray diffraction analysis. Detailed crystallographic data, including data collection parameters, structure refinement details, and CCDC deposition numbers, are provided in the SI (Table S1 and Figure S24). As shown in the crystal structures of **2a** and **2b** (Figure 2), both complexes adopt a characteristic “piano-stool” geometry, in which the π -bonded *p*-cymene ligand forms the “seat”, while the mono- and bidentate ligands constitute the three “legs” of the stool. The two structures are representative of monoclinic space groups $P2_1/c$ and $P2_1/n$, respectively. Within the pseudo-octahedral configuration of the compounds, a new type of disorder is observed in complex **2b**, originating from the *p*-cym ligand. The phenyl and thiazole rings are locked in a nearly coplanar arrangement, with torsion angles ranging from 5.08° to 4.45° along the thiazole–phenyl bond. The coordination bonds exhibit remarkable similarity between the two metal centers, with Ru–(*p*-cym) bond lengths of 1.65 Å in **2a** and 1.70 Å in **2b**, Ru(1)–N(2) distances of 2.134(16) Å in **2a** and 2.147(3) Å in **2b**, and Ru(1)–Cl(1) bond lengths of 2.482(7) Å in **2a** and 2.4119(11) Å in **2b**, respectively.

2.3. Stability in Aqueous Solution

Ensuring sufficient stability in aqueous solutions is crucial for anticancer therapeutics to monitor their cytotoxic potential.³³ Hence, the aqueous stability of the metallacycles was determined by UV–vis spectroscopy over 24 h at 20 °C

using 1% DMF as solubilizer in PBS (40 μ M compound concentration, pH 7.4, Figures S25–S28). DMF was used as an alternative to DMSO to avoid the formation of solvent adducts. Neutral organometallic chlorido complexes of this class are known to rapidly hydrolyze in aqueous solutions yielding the corresponding aqua species. However, due to their limited solubility, chlorido complexes precipitated significantly over time as there is an equilibrium between chlorido and aqua species, with **2b** precipitating much faster than **2a** (Figures S25 and S26). After the introduction of morpholino-imidazole, UV–vis spectra indicated fully stable complexes with no precipitation over 24 h, since no isosbestic points were observed (Figures S27 and S28). This behavior is in accordance with previous studies.³⁴

2.4. DNA Interaction Studies

Considering that our previous studies revealed that DNA can be a target of ruthenium piano-stool compounds,^{34,35} the DNA binding properties of the newly synthesized compounds **2a,b** and **3a,b** were evaluated using FRET melting assays on a panel of DNA sequences. These include the duplex control ds26 and five G-quadruplex (G4) forming oligonucleotides, here named htelo, kit1, httert, bcl2, and myc (Table S2 and Figures S29–S34). G4s are four-stranded DNA or RNA structures formed by stacks of guanine tetrads in G-rich genomic regions and they are a hot topic in cancer research because they regulate oncogene expression and telomere maintenance, making them attractive targets for selective anticancer therapies.³⁶ The duplex ds26 did not exhibit any significant change in melting temperature upon incubation with any of the tested compounds at the concentrations employed, indicating an absence of detectable stabilization and suggesting limited or no interaction with double-stranded DNA (Figure S29). In contrast, the G4 sequences bcl2 and myc showed a concentration-dependent thermal stabilization in the presence of compounds **2a** and **2b** (Figures S33 and S34). The observed ΔT_m values increased progressively with compound concentration, indicating a measurable affinity of **2a** and **2b** for these promoter-associated G4 structures. Compounds **3a** and **3b**, however, did not induce appreciable stabilization of any G4 tested under the same conditions. Additionally, the G-quadruplexes htelo, kit1, and httert displayed negligible or no thermal stabilization with any of the four compounds (Figures S30–S32). Overall, these results highlight a selective interaction of compounds **2a** and **2b** with the bcl2 and myc G4, while confirming their limited activity toward other G4 topologies and canonical B-DNA (Table 1). Of note, G4s forming in the promoter regions of genes *BCL2* and *MYC*, were found to modulate transcription by acting as regulatory switches. *BCL2* overexpression is implicated across multiple cancers, notably B-cell lymphomas/leukemias, as well as solid

tumors where it contributes to therapy resistance and poor prognosis, while *MYC* overexpression is implicated across many cancers, including breast, colon, cervix, small-cell lung cancer, osteosarcoma, glioblastoma, and myeloid leukemias.^{37,38} Stabilizing these G4s can regulate oncogenic expression, offering a promising anticancer strategy.

2.5. Antiproliferative Activity and Cellular Accumulation

The antiproliferative activity of the ligands **1a–c** and Ru(II) complexes **2a,b** and **3a,b** was assessed in human lung adenocarcinoma (A549), colon adenocarcinoma (SW480), ovarian teratocarcinoma (CH1/PA-1) cell lines by means of the colorimetric MTT assay and in promyelocytic leukemia (HL-60) cells by the fluorimetric resazurin assay (Figures S35 and S36). To assess antiproliferative activity in nonmalignant cells, human colon fibroblast were included in the testing panel. Cisplatin was used as positive cytotoxic control (Table 2 and Figures S37 and S38). Additionally, the cellular accumulation of all complexes was determined in SW480 cells to further elucidate the influence of ligand variation, especially the introduction of morpholine-imidazole **1c** as a leaving group.

The mild cytotoxicity of camalexin **1a** and its increase upon addition of a lipophilic benzene ring (**1b**) have been previously reported.^{27,29} Several sources suggest that camalexin effectively induces ROS in leukemic cell lines (Jurkat, NB4, HL-60)^{28,39} which inspired the investigation of the antiproliferative activity of camalexin and benzocamalexin bearing complexes **2a,b** and **3a,b** in the leukemia cell line HL-60. Unlike the free morpholine-imidazole ligand **1c**, whose IC_{50} value was not reached within the tested concentration range up to 200 μ M, the metallacycles **2a,b** and **3a,b** exhibited stronger cytotoxic effects than the free ligands, with IC_{50} values in the one- to two-digit micromolar range (Table 2 and Figures S35 and S36). Generally, the compounds (except inactive **1c**) were most active in the chemosensitive CH1/PA-1 and the leukemic HL-60 cell lines and exhibited the lowest activity in the multidrug-resistant A549 cell line. Previously published IC_{50} values of similar Ru(II) C,N half-sandwich complexes are in a comparable range.^{16–23,35} Within the tested compound series the benzocamalexin-bearing chlorido complex **2b** exhibits the highest cytotoxic effect followed by the morpholine-imidazole derivative **3b**. A marked decrease of antiproliferative activity was observed for the camalexin-bearing complex **2a** and its **1c**-bearing analog **3a**. Generally, the differences between IC_{50} values of the least and most potent complex indicate a moderate impact of the variable ligands **1a** and **1b**. Nevertheless, the antiproliferative activity seems to be directly proportional to the compounds' lipophilicity and therefore their cellular accumulation was determined. However, while complexes **2a,b** and **3a** feature high cellular accumulation values between 500 and 700 fg Ru/cell (see Table 2), compound **3b**'s cellular accumulation seems to be hampered (43 $\pm$ 15 fg Ru/cell). This behavior was rather surprising and does not match with the relatively linear trend in cytotoxicity. If those small amounts of **3b** give IC_{50} values comparable to those of the other compounds, its antiproliferative activity might arise from an altered mode of action. On the other hand, that both morpholine-imidazole-bearing complexes **3a** and **3b** (in contrast to the other two) yield higher IC_{50} values after 24 than 96 h (Table 2 and Figure S36) points to a slower onset of activity, consistent with a low cellular accumulation within the first 2 h for **3b** but not for **3a**, which could explain the observed

Table 1. ΔT_m Values for myc and bcl2 G4 Stabilization Measured in the Presence of Compounds **2a** and **3a** at the Three Different Concentration Ratios Indicated

[compound]/[oligo]		myc	bcl2
2a	5	5.8 $\pm$ 1.7	3.2 $\pm$ 1.5
	10	6.0 $\pm$ 1.6	6.6 $\pm$ 2.0
	20	9.0 $\pm$ 0.7	11.8 $\pm$ 1.2
3a	5	4.7 $\pm$ 0.9	3.1 $\pm$ 1.1
	10	6.2 $\pm$ 0.8	6.3 $\pm$ 1.5
	20	11.9 $\pm$ 0.7	16.4 $\pm$ 1.7

Table 2. Inhibition of Cancer Cell Growth in Four Human Cancer Cell Lines, Determined by the MTT Assay (A549, SW480, CH1/PA-1, CCD-18Co) and the Resazurin Assay (HL-60) (Exposure Time: 96 or 24 h, as Indicated)^a

Compound	A549 [μ M]	CH1/PA-1 [μ M]	HL-60 [μ M]	SW480 [μ M]	CCD-18Co [μ M]	SW480 [μ M]	fg Ru/SW480 cell
	96 h	96 h	96 h	96 h	96 h	24 h	
1a	135 $\pm$ 9	65 $\pm$ 7	77 $\pm$ 8	164 $\pm$ 25	>100	-	-
1b	36 $\pm$ 4	34 $\pm$ 2	35 $\pm$ 7	60 $\pm$ 7	-	-	-
1c	>200	>200	-	>200	-	-	-
2a	14 $\pm$ 2	3.1 $\pm$ 0.4	4.1 $\pm$ 0.2	6.9 $\pm$ 0.5	27 $\pm$ 1	4.0 $\pm$ 0.3	539 $\pm$ 40
2b	4.1 $\pm$ 0.1	2.2 $\pm$ 0.1	2.2 $\pm$ 0.1	2.1 $\pm$ 0.1	-	1.9 $\pm$ 0.3	646 $\pm$ 81
3a	53 $\pm$ 6	5.1 $\pm$ 0.3	7.4 $\pm$ 0.4	17 $\pm$ 3	71 $\pm$ 2	34 $\pm$ 7	561 $\pm$ 66
3b	11 $\pm$ 2	1.8 $\pm$ 0.3	2.5 $\pm$ 0.1	4.4 $\pm$ 0.5	-	7.1 $\pm$ 0.5	43 $\pm$ 15
Cisplatin	3.8 $\pm$ 1.0	0.073 $\pm$ 0.001	1.07 $\pm$ 0.01	1.8 $\pm$ 0.4	>20	-	-

^a50% Inhibitory concentrations (means $\pm$ standard deviations) from at least three independent experiments and cellular accumulation of compounds **2a,b** and **3a,b** (50 μ M, 0.5% DMF in MEM) in SW480 cancer cells (exposure time: 2 h).

discrepancy. Of note, human colon fibroblast CCD-18Co cells displayed significantly decreased sensitivity toward compound **3a**, while **2a** induced IC₅₀s comparable to that found in the colon cancer (SW480) cell model used in this study (Figure S38). This suggests superiority of compound **3a** over **2a** in terms of reduced potential side effects and enhanced drug tolerability. As expected, **1a** displayed no cytotoxicity up to a concentration of 100 μ M in the nonmalignant fibroblasts.

2.6. ROS

Leukemic HL-60 cells were tested for the impact on cellular reactive oxygen species (ROS) levels of ligands **1a,b** as well as complexes **2a,b** and **3a,b**. Our results, obtained by means of the DCFH-DA assay, are shown in Figure 3. Camalexin and benzocamalexin in the highest concentrations tested (200 μ M) showed different ROS-inducing capabilities. Surprisingly, camalexin (**1a**) did not seem to have any noteworthy impact on the cells, which contrasts with reported data in literature.³⁹ However, benzocamalexin (**1b**) elevated intracellular ROS levels by a factor of 2. This finding correlated well with their antiproliferative behavior, with **1b** being approximately twice as active after 96 h (Table 2). Compounds **2a** and **2b**, only varying in their bidentate ligands, decreased ROS levels concentration-dependently, with **2a** being more effective than **2b**. Both compounds showed solubility issues at their highest concentration in Opti-MEM, but the fact that the same trend of reducing ROS levels was also obvious at ten times lower concentration (20 μ M) lends support to the observation above. Remarkably, this trend was reversed when cells were treated with compounds **3a** and **3b**. The benzocamalexin- and morpholino-imidazole-bearing compound **3b** enhanced the ROS levels only slightly by 1.4-fold at the highest concentration tested, which was afflicted with solubility issues, too. At the concentration of 20 μ M, ROS levels increased only slightly with time and did not exceed that of the untreated control. For **3a**, though, a massive enhancement of ROS level was observable: At the intermediate concentration (20 μ M), an approximately 2.4-fold rise could be detected, and at the highest concentration (200 μ M) a 3.9-fold increase was observable. These results are remarkable, when compared to the positive control (200 μ M, *tert*-butylhydroperoxide), which only caused a 2.2-fold enhancement in ROS levels. However, cell death induced by **3a** was independent from enhanced ROS levels, since antioxidant *N*-acetyl-L-cysteine (NAC) could not rescue from cytotoxicity of the complex (Figure S39), while NAC clearly saved from cisplatin, a ROS-dependent cytotoxic agent.⁴⁰

2.7. Compound 3a Changes Inner Mitochondrial Membrane Potential

Prompted by enhanced ROS induction by compound **3a**, we investigated a possible change in mitochondrial membrane potential (MMP) using the cationic carbocyanine dye, JC-1 (Figure 4A,B).

In DMF solvent control samples, HL-60 cells showed high MMP reflected by J-aggregates accumulating in the mitochondrial matrix yielding intense red fluorescence intensity at 590 nm indicating intact mitochondrial membranes. Protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used as positive control for mitochondrial membrane depolarization inducing an expected shift toward green fluorescence. Treatment with **3a** decreased mitochondrial membrane potential dose-dependently reflected by weaker fluorescence at 590 nm and a shift toward green fluorescent monomers at 530 nm and significantly reduced red:green fluorescence intensity ratios (Figure 4B). Compound **2a** provoked the strongest shift toward green fluorescence while **1a**-treated cells resembled solvent controls. Contemporaneously, mitochondrial morphology was visualized using MitoTracker Red CMXRos (Figure 4C). As compared to ordered, elongated and homogeneously distributed mitochondria in solvent control-treated cells, mitochondria appeared bloated and shortened following **3a** treatment in a dose-dependent manner. Moreover, MitoTracker signal was diminished at higher dose of complex **3a** resembling CCCP-treated cells with depolarized mitochondria.

2.8. Induction of Apoptosis

After 24 h of treatment with the complexes and ligands, their capability to induce apoptosis (or necrosis) was tested in HL-60 cells via a double-staining experiment with annexin V-FITC and propidium iodide (PI). While annexin V selectively binds to phosphatidylserine on the outer leaflet of cell membranes, indicating the onset of apoptosis, the impermeable PI intercalates into DNA, indicating excessive membrane damage and is a measure for necrosis. Results are displayed in Figure 5 and Table S3. Ligands **1a** and **1b** showed hardly any signs of apoptosis induction in this cell line. Apoptotic cells quantified upon treatment with **1a** (2.6% to 4.7%) were in a range well comparable with that in the untreated control, while only the highest concentration of **1b** (50 μ M) yielded a slightly higher percentage (7.7% apoptotic cells). In contrast, complexes **2a,b** and **3a,b** displayed a marked concentration-dependent capability to induce apoptosis in the tested ranges. Benzocamalexin-bearing complexes (**2b** and **3b**) showed an even more pronounced induction of apoptotic cells (up to

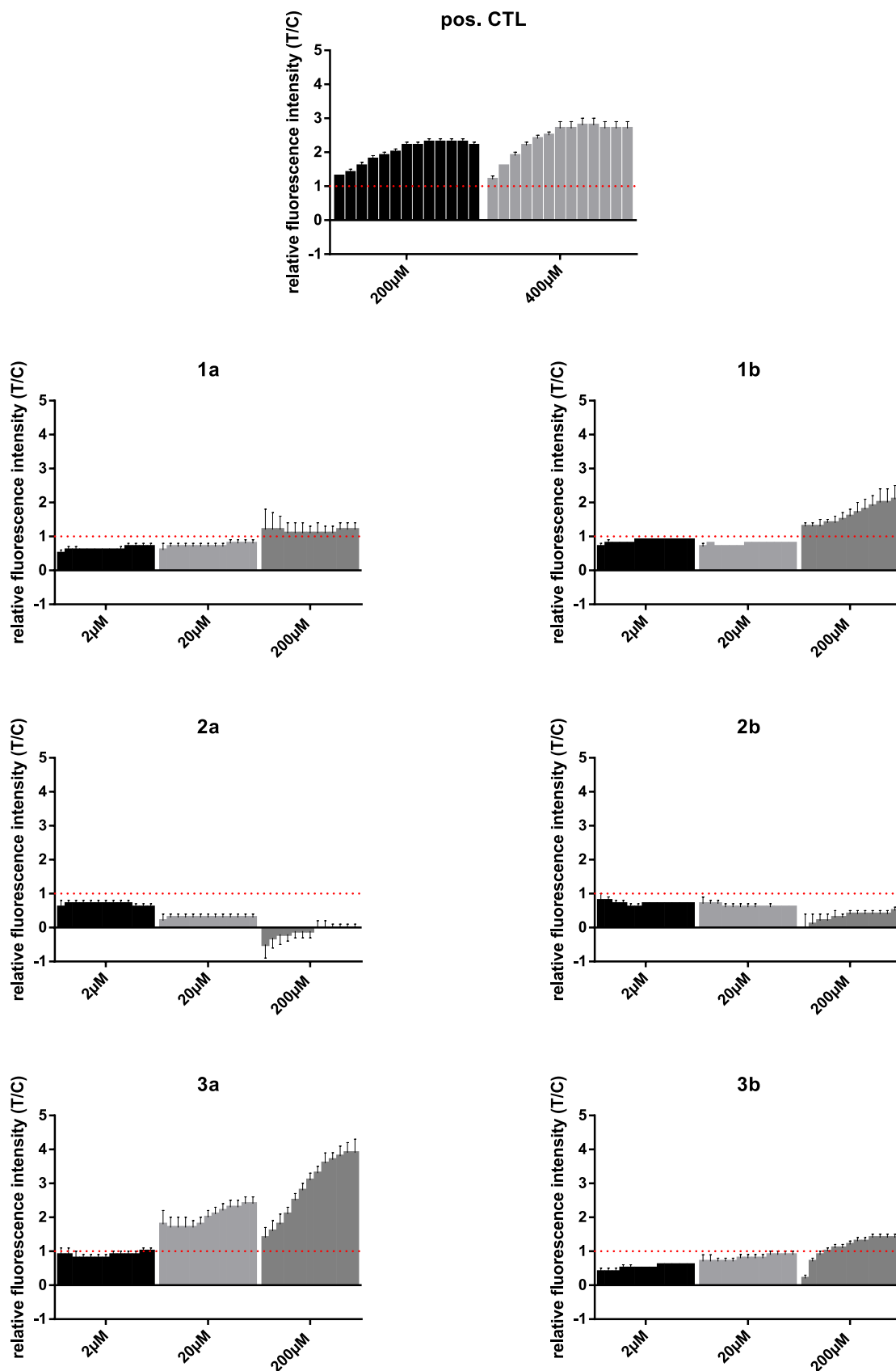


Figure 3. Induction of reactive oxygen species in HL-60 cells. Cells were stained with DCFH-DA and then treated with different concentrations of ligands **1a,b** and complexes **2a,b** and **3a,b**. *tert*-Butylhydroperoxide was used as positive control. All measured values were normalized to the negative control and the bars represent the respective standard deviations from at least three independent experiments.

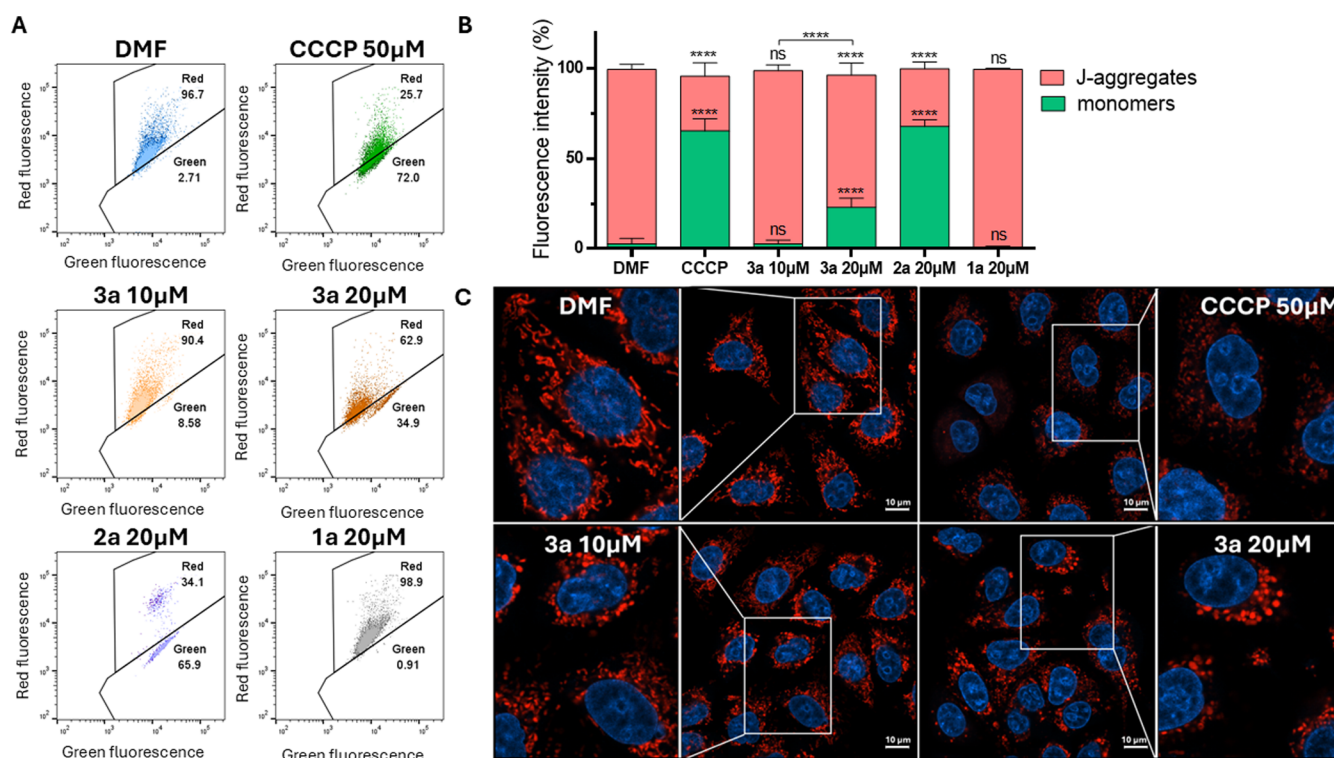


Figure 4. Complexes 3a and 2a impair mitochondrial function. (A) Flow cytometry detection of mitochondrial membrane potential (MMP) by JC-1 assay following 24 h of treatment of HL-60 cells at indicated drug concentrations, each in triplicates. Percentage of live cells with high and low MMP is given in upper and lower gates, respectively. CCCP: positive control, added 30 min prior measurement. One representative of three independent experiments is shown. (B) Percentage of JC-1 fluorescence intensities red:green (J-aggregate:monomer)-ratio quantified from (A). Values represent mean $\pm$ SD ($n = 3$), each in triplicates. **** $p < 0.001$ vs solvent DMF control. (C) Representative confocal microscopy images of MitoTracker (red; mitochondria) and HOECHST 33342 (blue; nuclei)-stained SW480 cells treated with solvent control or complex 3a for 24 h. CCCP: positive control, added 30 min prior imaging. 63 $\times$ magnification, scale bar: 10 μ m.

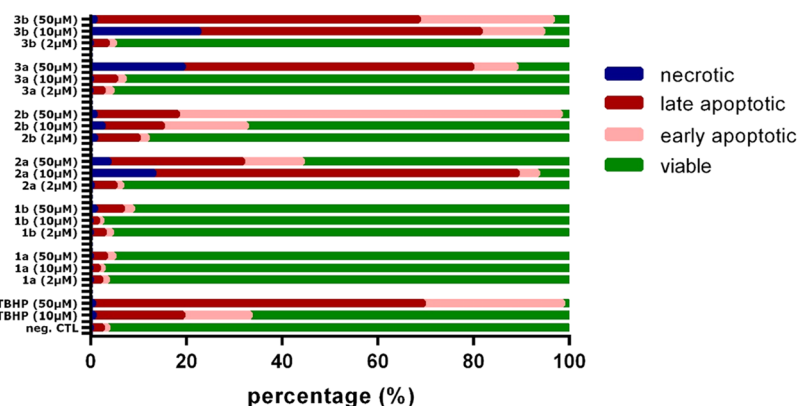


Figure 5. Apoptosis induction in HL-60 cells. Cells were double-stained with annexin V and propidium iodide after treatment for 24 h with different concentrations of ligands 1a,b and complexes 2a,b and 3a,b. *tert*-Butylhydroperoxide was used as a positive control. Stained HL-60 cells were measured with a flow cytometer and assigned to subpopulations of viable, early and late apoptotic as well as necrotic cells. Means were calculated from at least three independent experiments.

97.2% and 95.5%, respectively) than the camalexin-bearing complexes (2a and 3a: up to 80.1% and 69.5%). This trend correlates well with the IC_{50} values of the complexes, supporting the higher activity of benzocamalexin analogs (Table 2).

3. CONCLUSION

In this work, two sets of camalexin- and benzocamalexin-based complexes featuring different leaving groups were synthesized and characterized by means of standard analytical techniques

(NMR, HRMS, elemental analysis). The chlorido complexes exhibited poor aqueous solubility and, therefore, 4-(2-(1*H*-imidazol-1-yl)ethyl)morpholine was introduced as a solubility improving moiety. X-ray and electron diffraction confirmed the solid-state structures of the chlorido complexes. Stability studies in PBS confirmed the remarkable stability of the organometallics and no decomposition was observed over 24 h; however, chlorido complexes partially precipitated over time. The incorporation of the morpholine group resulted in significantly improved aqueous solubility, and these complexes

were also stable in aqueous solution. The used ligands and the four corresponding cyclometallates were investigated for their antiproliferative potency in four human cancer cell lines (A549, SW480, CH1/PA-1, and HL-60 cells), where the organometallics showed decreased IC_{50} values mostly in the low micromolar range compared to free ligand, while healthy human colon fibroblasts (CCD-18Co cells) were widely spared from cytotoxicity of **3a**. Additionally, the compounds' ability to induce ROS was investigated. **3a** exhibited a pronounced oxidative effect, even surpassing the positive control, while the **2a,b** slightly lowered the cellular ROS levels. To our surprise, the reported ROS generation of camalexin could not be confirmed in HL-60 cells. The complexes showed a remarkable capability to induce apoptosis in HL-60 cells, while the free ligands exhibited hardly any signs of apoptosis induction. While cell death induction by **3a** was not directly linked to increased ROS levels, cytotoxicity of **3a** was associated with mitochondrial membrane depolarization as well as morphological and functional disturbance of the cellular powerhouse. Overall, the coordination of camalexin derivatives to organometallic fragments gave rise to a series of highly cytotoxic compounds with remarkable stability under pseudophysiological conditions. In vivo studies are planned to clarify their potential as anticancer metallodrugs and further biological assays are necessary to reveal their mode of action which is obviously different to the free ligands.

4. EXPERIMENTAL PART

4.1. Materials and Methods

Methanol was distilled from Mg/I_2 and stored over molecular sieve (3 Å). Mg -chips (Sigma-Aldrich), Et_2O (>99.9%, Sigma-Aldrich), methyl iodide (99%, Sigma-Aldrich), toluene (ExtraDry, 99.9%, Acros Fisher), 2-bromothiazole (98%, Sigma-Aldrich), 2-bromobenzothiazole (95%, abcr), HCl (37%, Sigma-Aldrich), ethyl acetate (HPLC grade, Sigma-Aldrich), *n*-hexane (HPLC grade, Merck), dichloromethane (HPLC grade, Merck), sodium sulfate (99%, Alfa Aesar), silver nitrate (99.5%, Acros Fisher), 1*H*-imidazole (99.5%, Sigma-Aldrich), acetonitrile (HPLC grade, Merck), potassium hydroxide (85%, Alfa Aesar), 4-(2-chloroethyl)morpholine hydrochloride (95%, BLDPharm), tetra-*n*-butylammonium hydrogen sulfate (99%, Acros Fisher), molecular sieve (3 Å, beads, 4–8 mesh), phosphate buffered saline (pH 7.4, 10×, Gibco), α -terpinene (90%, Acros Fisher), sodium acetate anhydrous ($\geq 98.5\%$, Fluka), ruthenium(III) chloride hydrate (38–41% Ru, Johnson Matthey) were used without further purification. The dimeric metal precursor $[Ru(p\text{-cymene})Cl_2]_2$ was synthesized as described by Geisler et al.⁴¹

Purification via flash column chromatography was conducted with Biotage Isolera system and silica gel (VWR, mesh 40–63 μm).

1H -, ^{13}C - and 2D-NMR spectra of the complexes were recorded on a Bruker FT-NMR spectrometer Avance III HD 700.40 MHz or on a Bruker FT-NMR spectrometer Avance III 600.25 MHz, 1H NMR spectra of the ligands were recorded on a Bruker FT-NMR spectrometer AV NEO 500.10 MHz in d^6 -DMSO and referenced to the residual solvent signals.

High resolution ESI mass spectra of the metallacycles were recorded at the Mass Spectrometry Center of the University of Vienna (Faculty of Chemistry) on a Bruker maXis ESI-Qq-TOF Mass Spectrometer.

Electron diffraction data were collected as follows: A droplet of the *n*-hexane suspension of **2a** was placed onto a TEM grid featuring a lacey carbon film (copper, Ted Pella, 200-mesh). The evaporation of *n*-hexane was observed with a light microscope. The grid was then transferred to an ELSA698 tomography holder (Gatan INC) at 100 K and inserted into the JEM2100Plus TEM (200 keV LaB₆, JEOL), then warmed up to 163 K. The TEM was equipped with a 512 × 1024 pixel JUNGFRU detector, featuring a 320 μm -thick silicon sensor

and a pixel size of 75 μm . Diffraction patterns were recorded by illuminating crystalline grains of $\sim 1\text{--}2\ \mu m$ in length with the electron beam while rotating the sample stage from $\sim -60^\circ$ to 60° . Data were processed with XDS.⁴² Five data sets measured with different crystal grains were merged to improve completeness. The detector distance for each data set was refined during the CORRECT step of the XDS program to improve the accuracy of the unit cell parameters. The structures were solved by employing SHELXT⁴³ for ab initio phasing, followed by refinement and model building using SHELXL⁴⁴ and ShelXle,⁴⁵ respectively. The CIF file was deposited at the Cambridge Crystallographic Data Centre, under deposition number CCDC 2449653. Single crystal X-ray diffraction data for **2b** were collected with a Stadivari Diffractometer (STOE & Cie GmbH, Germany) equipped with an EIGER2 RS00 detector (Dectris Ltd., Switzerland). Data were processed and scaled with the STOE software suite X-AREA (STOE & Cie GmbH). Structures were solved with SHELXT⁴³ and refined with SHELXL⁴⁴ or Olex2.⁴⁶ Model building was done with Olex2 or ShelXle.⁴⁵ The CIF file was deposited at the Cambridge Crystallographic Data Centre, under deposition number CCDC 2449654. The structures were validated with CHECKCIF (<https://checkcif.iucr.org/>). Experimental data and CCDC-Codes (available online: <https://www.ccdc.cam.ac.uk/conts/retrieving.html>), as well as crystal data, data collection parameters, and structure refinement details can be found in Table S1.

Elemental analyses were performed by the Microanalytical Laboratory of the University of Vienna with a Eurovector EA 3000(2009) equipped with a high temperature pyrolysis furnace (HT, Hekatech, Germany, 2009). Elemental analyses samples were weighed on a Sartorius SEC 2 ultramicro balance with $\pm 0.1\ \mu g$ resolution. Sample weights from 1–3 mg were used. For calibration two NIST-certified reference materials were used: sulfanilamide ($C_6H_8N_2O_2S$) and BBOT (2,5-bis(5-*tert*-butyl-2-benzoxazol-2-yl)-thiophenone, $C_{26}H_{26}N_2O_2S$). The limit of quantification (LOQ) was 0.05 wt % for C, H, N and 0.02 wt % for S. The presented values are the average of determinations in triplicate. UV–vis data were recorded on a PerkinElmer Lambda 650 UV–vis Spectrophotometer with a Peltier element for temperature control.

4.2. Ligand Synthesis

4.2.1. Camalexin 1a and Benzocamalexin 1b. Mg -chips (194 mg, 8 mmol, 2 equiv) were suspended in dry Et_2O (16 mL) under argon atmosphere, and MeI (1646 mg, 11.6 mmol, 2.9 equiv) was added. After stirring at ambient temperature until Mg was dissolved (3 h), the solvent was removed *in vacuo*. The residue was taken up in dry toluene (10 mL) and indole (469 mg, 4 mmol, 1 equiv), dissolved in dry toluene (22 mL), was added to the reaction mixture dropwise. Subsequently, 2-bromobenzothiazole (1798 mg, 8.4 mmol, 2.1 equiv) or 2-bromothiazole (1377 mg, 8.4 mmol, 2.1 equiv) was added and the temperature was elevated to 95 °C for 20 h. After cooling to room temperature, the reaction was quenched with water and the pH was adjusted to 7 with 2 M HCl. The aqueous layer was extracted with $EtOAc$ (3×) and the combined organic layers were washed with brine (20 mL) before they were dried over sodium sulfate. After the solvent was removed under reduced pressure, the crude product was purified via chromatography on silica (50 g SiO_2), 30% $EtOAc$ in *n*-hexane (5% Et_3N). The purified product was dried *in vacuo* at 50 °C for 2 days to achieve elemental analysis purity (camalexin **1a**: 293 mg, 37%; benzocamalexin **1b**: 417 mg, 42%).

Camalexin **1a**: Elemental analysis found (calculated) for $C_{11}H_8N_2S \cdot 0.05H_2O \cdot 0.02C_6H_{14}$: C 65.75 (65.84), H 3.96 (4.17), N 13.76 (13.82), S 15.41 (15.78), O 0.30 (0.39). 1H NMR (500 MHz, d^6 -DMSO): δ = 11.71 (s, 1H), 8.21–8.15 (m, 1H), 8.07 (s, 1H), 7.81 (d, $^3J_{HH}$ = 3 Hz, 1H), 7.55 (d, $^3J_{HH}$ = 3 Hz, 1H), 7.51–7.45 (m, 1H), 7.25–7.15 (m, 2H) ppm.

Benzocamalexin **1b**: Elemental analysis found (calculated) for $C_{15}H_{10}N_2S \cdot 0.15H_2O$: C 71.04 (71.21), H 3.91 (4.11), N 11.0 (11.08), S 12.38 (12.65), O 0.56 (0.95). 1H NMR (500 MHz, d^6 -DMSO): δ = 11.95 (s, 1H), 8.41–8.35 (m, 1H), 8.27 (d, $^4J_{HH}$ = 3 Hz, 1H), 8.05 (d, $^3J_{HH}$ = 3 Hz, 1H), 7.96 (d, $^3J_{HH}$ = 8 Hz, 1H), 7.56–7.45 (m, 2H), 7.40–7.33 (m, 1H), 7.30–7.22 (m, 2H) ppm.

4.3. Complex Synthesis

4.3.1. [(Chlorido)(3-(1,3-thiazol-2-yl-(κ N))-1H-indolato-(κ C²))(η^6 -p-cymene)ruthenium(II)] (2a). (Ru(p-cym)Cl₂)₂ (200 mg, 327 μ mol, 1.0 equiv), anhydrous NaOAc (107 mg, 1.31 mmol, 4.0 equiv) and camalexin (131 mg, 653 μ mol, 2.0 equiv) were dissolved in dry MeOH (20 mL) and stirred for 24 h at room temperature (rt) under Ar atmosphere. The precipitated orange solid was collected via filtration, dissolved in dichloromethane, and filtered again. After removing the solvent under reduced pressure and drying at 50 °C *in vacuo* for 48 h, complex **2a** was obtained in elemental analysis purity (238 mg, 78%).

ESI-HR-MS⁺ *m/z* found (calculated): [M-Cl]⁺ 435.0461 (435.0469). Elemental analysis found (calculated) for C₂₁H₂₁ClN₂RuS·0.25H₂O·0.2CH₂Cl₂: C 51.62 (51.78), H 4.29 (4.49), N 5.78 (5.70), S 6.35 (6.51), O 1.04 (0.82). ¹H NMR (700 MHz, d⁷-DMF): δ = 12.01 (s, 1H, NH), 8.21 (d, ³J_{H,H} = 4 Hz, 1H, ArH_{Th-5}), 7.40–7.36 (m, 2H, ArH_{Ind-4+7}), 7.31 (d, ³J_{H,H} = 4 Hz, 1H, ArH_{Th-4}), 7.06–7.02 (m, 1H, ArH_{Ind-6}), 6.99–6.95 (m, 1H, ArH_{Ind-5}), 6.03 (d, ³J_{H,H} = 6 Hz, 1H, ArH_{Cym-h}), 6.00 (d, ³J_{H,H} = 6 Hz, 1H, ArH_{Cym-g}), 5.73 (d, ³J_{H,H} = 6 Hz, 1H, ArH_{Cym-f}), 5.40 (d, ³J_{H,H} = 6 Hz, 1H, ArH_{Cym-e}), 2.47 (hept, ³J_{H,H} = 7 Hz, 1H, CH₂_{Cym-c}), 2.06 (s, 3H, CH₃_{Cym-d}), 0.94 (d, ³J_{H,H} = 7 Hz, 3H, CH₃_{Cym-b}), 0.91 (d, ³J_{H,H} = 7 Hz, 3H, CH₃_{Cym-a}) ppm. ¹³C NMR (176 MHz, d⁷-DMF): δ = 192.4 (C_{Ind-2}), 169.6 (C_{Th-2}), 144.7 (C_{Th-5}), 142.3 (C_{Ind-7a}), 125.5 (C_{Ind-3a}), 120.7 (C_{Ind-6}), 119.9 (C_{Ind-5}), 117.1 (C_{Ind-3}), 116.7 (C_{Ind-7}), 112.0 (C_{Th-4}), 111.1 (C_{Ind-4}), 102.2, 102.2, 90.9 (C_{Cym-h}), 86.1 (C_{Cym-g}), 85.6 (C_{Cym-f}), 82.0 (C_{Cym-e}), 32.2 (C_{Cym-c}), 22.7 (C_{Cym-b}), 22.6 (C_{Cym-a}), 19.2 (C_{Cym-d}) ppm.

4.3.2. [(Chlorido)(2-(1H-indol-3-yl-(κ C²))-benzothiazolato-(κ N))(η^6 -p-cymene)ruthenium(II)] (2b). (Ru(p-cym)Cl₂)₂ (200 mg, 327 μ mol, 1.0 equiv), anhydrous NaOAc (107 mg, 1.31 mmol, 4.0 equiv) and benzocamalexin (164 mg, 653 μ mol, 2.0 equiv) were dissolved in dry MeOH (20 mL) and stirred for 24 h at rt under Ar atmosphere. The precipitated orange solid was collected via filtration, dissolved in dichloromethane, and filtered again. After removing the solvent under reduced pressure and drying at 50 °C *in vacuo* for 48 h, complex **2b** was obtained in elemental analysis purity (217 mg, 64%).

ESI-HR-MS⁺ *m/z* found (calculated): [M-Cl]⁺ 485.0622 (485.0625). Elemental analysis found (calculated) for C₂₅H₂₃ClN₂RuS·0.1H₂O·0.05CH₂Cl₂: C 57.07 (57.19), H 4.25 (4.46), N 5.39 (5.32), S 6.06 (6.10), O 0.32 (0.30). ¹H NMR (600 MHz, d⁷-DMF): δ = 12.35 (s, 1H, NH), 8.27 (d, ³J_{H,H} = 8 Hz, 1H, ArH_{BTh-4}), 8.05 (dd, ³J_{H,H} = 8 Hz, ⁴J_{H,H} = 1 Hz, 1H, ArH_{BTh-7}), 7.63–7.58 (m, 1H, ArH_{BTh-5}), 7.51 (d, ³J_{H,H} = 7 Hz, 1H, ArH_{Ind-4}), 7.45 (d, ³J_{H,H} = 8 Hz, 1H, ArH_{Ind-7}), 7.37–7.31 (m, 1H, ArH_{BTh-6}), 7.13–7.10 (m, 1H, 1H, ArH_{Ind-6}), 7.08–7.04 (m, 1H, ArH_{Ind-5}), 6.44 (dd, ³J_{H,H} = 6 Hz, 1H, ArH_{Cym-h}), 6.23 (dd, ³J_{H,H} = 6 Hz, ⁴J_{H,H} = 1 Hz, 1H, ArH_{Cym-g}), 5.95 (dd, ³J_{H,H} = 6 Hz, ⁴J_{H,H} = 1 Hz, 1H, ArH_{Cym-f}), 5.50 (dd, ³J_{H,H} = 6 Hz, ⁴J_{H,H} = 1 Hz, 1H, ArH_{Cym-e}), 2.29 (hept, ³J_{H,H} = 7 Hz, 1H, CH₂_{Cym-c}), 2.12 (s, 3H, CH₃_{Cym-d}), 0.89 (d, ³J_{H,H} = 7 Hz, 3H, CH₃_{Cym-b}), 0.80 (d, ³J_{H,H} = 7 Hz, 3H, CH₃_{Cym-a}) ppm. ¹³C NMR (151 MHz, d⁷-DMF): δ = 196.8 (C_{Ind-2}), 168.8 (C_{BTh-2}), 152.1 (C_{BTh-3a}), 142.9 (C_{Ind-3a}), 132.4 (C_{BTh-7a}), 127.9 (C_{BTh-5}), 126.0 (C_{Ind-7a}), 123.8 (C_{BTh-7}), 123.5 (C_{BTh-6}), 121.4 (C_{Ind-6}), 120.9 (C_{Ind-5}), 120.1 (C_{BTh-4}), 117.5 (C_{Ind-4}), 117.4 (C_{Ind-3}), 111.4 (C_{Ind-7}), 104.7 (C_{Cym-6}), 101.0 (C_{Cym-1}), 92.1 (C_{Cym-h}), 87.4 (C_{Cym-g}), 85.3 (C_{Cym-f}), 80.7 (C_{Cym-e}), 32.2 (C_{Cym-c}), 22.9 (C_{Cym-b}), 22.3 (C_{Cym-a}), 19.3 (C_{Cym-d}) ppm.

4.3.3. [(4-(2-(1H-imidazol-1-yl-(κ N³))ethyl)morpholine)3-(1,3-(thiazol-2-yl-(κ N))-1H-indolato-(κ C²))(η^6 -p-cymene)ruthenium(II)]nitrate (3a). Chlorido complex **2a** (200 mg, 426 μ mol, 1.0 equiv), 4-(2-(1H-imidazol-1-yl)ethyl)morpholine **4** (77 mg, 426 μ mol, 1.0 equiv) and silver nitrate (108 mg, 638 μ mol, 1.5 equiv) were stirred in MeOH (20 mL) under light protection for 3 h. Subsequently, methanol was removed under reduced pressure and the residue was purified using flash column chromatography on silica (25 g SiO₂, 4–7% MeOH in DCM). After drying at 50 °C *in vacuo* for 48 h, complex **3a** was obtained in elemental analysis purity (185 mg, 64%).

ESI-HR-MS⁺ *m/z* found (calculated): [M-NO₃]⁺ 616.1682 (616.1684). Elemental analysis found (calculated) for C₃₀H₃₆N₆O₄RuS · 0.5H₂O: C 52.73 (52.39), H 5.30 (5.43), N 11.94 (12.23), S 4.51 (4.65), O 10.62 (10.47). ¹H NMR (600 MHz, d⁷-DMF): δ = 12.44 (s, 1H, NH), 8.48 (d, ³J_{H,H} = 4 Hz, 1H, ArH_{Th-5}), 7.76–7.78 (m, 1H, ArH_{Imi-2}), 7.53–7.48 (m, 2H, ArH_{Th-4}, ArH_{Ind-4}), 7.38 (d, ³J_{H,H} = 7 Hz, 1H, ArH_{Ind-7}), 7.20–7.19 (m, 1H, ArH_{Imi-3}), 7.10–7.02 (m, 2H, ArH_{Ind-5+6}), 6.62–6.60 (m, 1H, ArH_{Imi-4}), 6.37 (dd, ³J_{H,H} = 6 Hz, ⁴J_{H,H} = 1 Hz, 1H, ArH_{Cym-h}), 6.33 (dd, ³J_{H,H} = 6 Hz, ⁴J_{H,H} = 1 Hz, 1H, ArH_{Cym-g}), 6.24 (dd, ³J_{H,H} = 6 Hz, ⁴J_{H,H} = 1 Hz, 1H, ArH_{Cym-f}), 5.83 (dd, ³J_{H,H} = 6 Hz, ⁴J_{H,H} = 1 Hz, 1H, ArH_{Cym-e}), 4.04 (t, ³J_{H,H} = 6 Hz, 2H, CH₂_{Ethyl-2}), 3.44 (t, ³J_{H,H} = 5 Hz, 4H, CH₂_{Morph-1}), 2.52–2.48 (m, 2H, CH₂_{Ethyl-1}), 2.34 (hept, ³J_{H,H} = 7 Hz, 1H, CH₂_{Cym-c}), 2.28–2.23 (m, 4H, CH₂_{Morph-2}), 1.79 (s, 3H, CH₃_{Cym-d}), 0.89 (d, ³J_{H,H} = 7 Hz, 3H, CH₃_{Cym-b}), 0.85 (d, ³J_{H,H} = 7 Hz, 3H, CH₃_{Cym-a}) ppm. ¹³C NMR (151 MHz, d⁷-DMF): δ = 189.7 (C_{Ind-2}), 170.7 (C_{Th-2}), 144.4 (C_{Th-5}), 142.4 (C_{Ind-7a}), 142.2 (C_{Imi-2}), 131.9 (C_{Imi-4}), 125.2 (C_{Ind-3a}), 122.3 (C_{Imi-3}), 121.3 (C_{Ind-6}), 120.8 (C_{Ind-5}), 117.6 (C_{Ind-3}), 117.1 (C_{Ind-7}), 113.9 (C_{Th-4}), 111.8 (C_{Ind-4}), 104.3 (C_{Cym-6}), 103.7 (C_{Cym-1}), 91.6 (C_{Cym-h}), 89.7 (C_{Cym-g}), 88.5 (C_{Cym-f}), 82.5 (C_{Cym-e}), 67.5 (C_{Morph-1}), 59.1 (C_{Ethyl-1}), 54.3 (C_{Morph-2}), 45.6 (C_{Ethyl-2}), 32.2 (C_{Cym-c}), 22.9 (C_{Cym-b}), 22.7 (C_{Cym-a}), 18.7 (C_{Cym-d}) ppm.

4.3.4. [(4-(2-(1H-imidazol-1-yl-(κ N³))ethyl)morpholine)(2-(1H-indol-3-yl-(κ C²))-benzothiazolato-(κ N))(η^6 -p-cymene)ruthenium(II)]nitrate (3b). Chlorido complex **2b** (200 mg, 385 μ mol, 1.0 equiv) and silver nitrate (97 mg, 577 μ mol, 1.5 equiv) were stirred in MeOH (20 mL) under light protection for 3.5 h. Subsequently, methanol was removed under reduced pressure and the residue was purified using flash column chromatography on silica (25 g SiO₂, 7% MeOH in DCM). After drying at 50 °C *in vacuo* for 48 h, complex **3b** was obtained in elemental analysis purity (206 mg, 74%).

ESI-HR-MS⁺ *m/z* found (calculated): [M-NO₃]⁺ 666.1840 (666.1841). Elemental analysis found (calculated) for C₃₄H₃₈N₆O₄RuS·0.75H₂O: C 54.91 (55.01), H 5.05 (5.37), N 11.47 (11.33), S 4.22 (4.31), O 9.96 (10.24). ¹H NMR (600 MHz, d⁷-DMF): δ = 12.80 (s, 1H, NH), 8.47 (d, ³J_{H,H} = 8 Hz, 1H, ArH_{BTh-4}), 8.15 (dd, ³J_{H,H} = 8 Hz, ⁴J_{H,H} = 1 Hz, 1H, ArH_{BTh-7}), 7.76–7.71 (m, 1H, ArH_{BTh-5}), 7.70–7.67 (m, 1H, ArH_{Imi-2}), 7.58 (dd, ³J_{H,H} = 7 Hz, ⁴J_{H,H} = 2 Hz, 1H, ArH_{Ind-4}), 7.52 (dd, ³J_{H,H} = 7 Hz, ⁴J_{H,H} = 2 Hz, 1H, ArH_{Ind-7}), 7.48–7.43 (m, 1H, ArH_{BTh-6}), 7.19–7.11 (m, 3H, ArH_{Imi-5}, ArH_{Ind-5}, ArH_{Ind-6}), 6.84 (dd, ³J_{H,H} = 6 Hz, ⁴J_{H,H} = 1 Hz, 1H, ArH_{Cym-h}), 6.60–6.55 (m, 2H, ArH_{Cym-g}, ArH_{Imi-4}), 6.47 (dd, ³J_{H,H} = 6 Hz, ⁴J_{H,H} = 1 Hz, 1H, ArH_{Cym-f}), 5.93 (dd, ³J_{H,H} = 6 Hz, ⁴J_{H,H} = 1 Hz, 1H, ArH_{Cym-e}), 3.99 (t, ³J_{H,H} = 6 Hz, 2H, CH₂_{Ethyl-2}), 3.37–3.28 (m, 4H, CH₂_{Morph-1}), 2.46–2.40 (m, 2H, CH₂_{Ethyl-1}), 2.24–2.14 (m, 5H, CH₂_{Morph-2}, CH₂_{Cym-c}), 1.80 (s, 3H, CH₃_{Cym-d}), 0.80 (d, ³J_{H,H} = 7 Hz, 3H, CH₃_{Cym-b}), 0.78 (d, ³J_{H,H} = 7 Hz, 3H, CH₃_{Cym-a}) ppm. ¹³C NMR (151 MHz, d⁷-DMF): δ = 193.9 (C_{Ind-2}), 170.4 (C_{BTh-2}), 151.4 (C_{BTh-3a}), 143.0 (C_{Ind-7a}), 142.1 (C_{Imi-2}), 132.4 (C_{BTh-7a}), 132.0 (C_{Imi-4}), 128.8 (C_{BTh-5}), 125.7 (C_{Ind-3a}), 124.5 (C_{BTh-7}), 124.3 (C_{BTh-6}), 122.6 (C_{Imi-5}), 122.0 (C_{Ind-6}), 121.7 (C_{Ind-5}), 119.6 (C_{BTh-4}), 118.0 (C_{Ind-3}), 117.8 (C_{Ind-7}), 112.1 (C_{Ind-4}), 106.2 (C_{Cym-6}), 102.5 (C_{Cym-1}), 92.2 (C_{Cym-h}), 90.6 (C_{Cym-g}), 89.9 (C_{Cym-f}), 81.0 (C_{Cym-e}), 67.4 (C_{Morph-1}), 59.0 (C_{Ethyl-1}), 54.2 (C_{Morph-2}), 45.5 (C_{Ethyl-2}), 32.1 (C_{Cym-c}), 22.8 (C_{Cym-b}), 22.7 (C_{Cym-a}), 18.7 (C_{Cym-d}) ppm.

4.4. Cell Lines and Culture Conditions

CH1/PA-1 ovarian teratocarcinoma cells (provided by L. R. Kelland, CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK; confirmed by STR profiling as PA-1 ovarian teratocarcinoma cells at Multiplexion, Heidelberg, Germany), SW480 colon carcinoma and A549 lung adenocarcinoma cells (both obtained from the ATCC, Manassas, VA, USA) as well as colon fibroblast CCD-18Co cells (a generous gift from Prof. G. Del-Favero, Department of Food Chemistry and Toxicology, University of Vienna) were grown as adherent cultures in 75 cm² culture flasks by using minimal essential medium (MEM) supplemented with 10%

heat-inactivated fetal bovine serum (FBS, Serana, Pessin, Germany), 1 mM sodium pyruvate, 4 mM L-glutamine and 1% (v/v) nonessential amino acids from 100-fold stock solution. HL-60 promyelocytic leukemia cells (from the ATCC) were grown as suspension cultures in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 4 mM L-glutamine in 75 cm² culture flasks. All cells were maintained under a humidified atmosphere containing 5% CO₂ in air at 37 °C. All culture media and supplements were purchased from Sigma-Aldrich (St. Louis, MO, USA) and all plasticware from Starlab (Hamburg, Germany) unless stated otherwise.

4.5. Cytotoxicity Assays and Cellular Accumulation

Cytotoxicity in adherent cell lines was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT, Acros Organics, Geel, Belgium) assay. Cells were harvested from culture flasks by trypsinization, seeded in 100 μ L aliquots into 96-well flat-bottom microculture plates in densities of 1×10^3 (CH1/PA-1), 2×10^3 (SW480, CCD-18Co) and 3×10^3 (A549) cells per well and incubated for 24 h. Stock solutions of test compounds were prepared in DMF, diluted in MEM (not to exceed a final content of 0.5% v/v of DMF in the test plates), and serial dilutions were added in triplicates of 100 μ L per well. After exposure for 96 h, the drug-containing medium was replaced with 100 μ L per well of staining medium, i.e., six parts of RPMI 1640 medium (supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine) and one part of MTT solution in phosphate buffered saline (5 mg/mL). After incubation for 4 h, the staining medium was removed, and the produced formazan crystals were dissolved in 150 μ L DMSO per well. Optical densities at 550 nm were measured spectrophotometrically with an ELx808 or Synergy HT microplate reader (BioTek, Winooski, VT, USA).

Cytotoxicity in HL-60 cells was tested by the fluorimetric resazurin assay. HL-60 cells were seeded into 96-well plates a density of 3000 cells/well 24 h prior to treatment with the test compounds, which was performed as described above. After exposure for 96 h, 20 μ L resazurin solution (110 μ g/mL resazurin sodium salt from Alfa Aesar in phosphate buffered saline) were added per well. Upon incubation for 4 h, fluorescence (ex/em 530/590 nm) was measured with a Synergy HT multimode reader (BioTek).

All 50% inhibitory concentrations (IC₅₀) were interpolated from concentration–effect curves from at least three independent experiments.

For assessment of the role of ROS on cytotoxic activity, cells were seeded for MTT assay as described above. Following overnight incubation, cells were pretreated with freshly prepared antioxidant N-acetyl-L-cystein (NAC, Sigma-Aldrich) for 20 min prior cotreatment with complexes or cisplatin for 96 h.

Cellular accumulation of ruthenium was determined as described in Cseh et al.⁴⁷ Briefly, 1 mL of supplemented MEM with 1.8×10^5 SW480 cells were seeded per well into tissue culture treated 12-well plates. After 24 h, compounds dissolved in DMF and immediately diluted in MEM were added to the cells to a final concentration of 50 μ M, and cells were incubated at 37 °C for 2 h. During incubation, the cell number was determined from three control wells of a plate seeded in parallel. After incubation, the medium was removed, all wells were washed with 3×1 mL PBS, cells were lysed by adding 400 μ L HNO₃ per well for 1 h at room temperature, and 300 μ L of each lysate were added to 7.7 mL Milli-Q water to obtain a total volume of 8 mL. Ruthenium content of the samples was determined with an Agilent 7800 ICP-MS instrument. Values were normalized to the cell number and results are given in fg [Ru]/cell from at least three independent experiments.

4.6. DNA Interaction Studies

FRET melting experiments were carried out on an Applied Biosystems QuantStudio 6 PCR cycler in a 96-well format using a FAM (6-carboxyfluorescein) detection filter. Oligonucleotides bearing 5'-FAM and 3'-TAMRA (6-carboxytetramethylrhodamine) labels were diluted to the desired concentrations in 60 mM potassium cacodylate buffer (pH 7.4). For myc sequence, the experiments were done in 10 mM potassium cacodylate buffer (pH 7.4), supplemented

with 50 mM LiCl. ODNs were folded into their respective B-DNA or G-quadruplex conformations by heating at 95 °C for 5 min, followed by slow cooling to room temperature overnight. The final oligonucleotide concentration was 0.2 μ M in a total volume of 30 μ L per well. Metal complexes were dissolved in DMF to obtain 2 mM stock solutions and subsequently diluted in buffer, ensuring a final DMF content below 1%. Fluorescence data were collected in duplicate over the 25–95 °C range, using a temperature ramp of 1 °C every 30 s. Emission values were normalized from 0 to 1 to enable comparison across experiments. The melting temperature ($T_{1/2}$) was defined as the temperature at which the normalized FAM emission reached 0.5. DNA concentrations are expressed as strand concentrations.

4.7. ROS Generation

HL-60 cells were centrifuged for 3 min at 1200 rpm (Thermo Scientific, Megafuge 1.0R). After aspiration of the supernatant, the cell pellet was resuspended in supplemented RPMI1640 medium. Afterward, HL-60 cells were seeded in 600 μ L aliquots in densities of 6.0×10^4 cells/well in 24-well plates (CytoOne, Starlab). Upon cultivation for 24 h at 37 °C, cells from each well were then harvested in single 1.5 mL Eppendorf tubes, centrifuged at 2100 rpm (Mikro 200R, Hettich) and washed with 500 μ L Hanks' balanced salt solution (HBSS; Sigma-Aldrich; supplemented with 1% heat-inactivated FBS). Following centrifugation, supernatant was removed and cells were incubated with 100 μ L of 25 μ M 2',7'-dichlorofluorescein diacetate (DCFH-DA; in supplemented HBSS) for 45 min at 37 °C.

Meanwhile, compounds were diluted in phenol-red-free Opti-MEM (Gibco, supplemented with 1% heat-inactivated FBS) from a 100% DMF stock solution. The positive control *tert*-butylhydroperoxide (TBHP) as well as different concentrations of the test compounds were added in triplicates to a flat-bottom 96-well plate (CytoOne, Starlab) and the plate was incubated at 37 °C until the end of the staining procedure.

After staining, HL-60 cells were centrifuged and washed with 500 μ L of supplemented HBSS. Cells were centrifuged and resuspended in 500 μ L phenol-red-free Opti-MEM (Gibco, supplemented with 1% heat-inactivated FBS). 100 μ L of the resulting cell suspension (approximately 12,000 HL-60 cells/well) were added to the proper concentration in the 96-well plate, resulting in treatment concentrations of 2, 20, and 200 μ M for each compound. For **2a**, **2b** and **3b**, measurements at the concentration of 200 μ M need to be interpreted with caution because of solubility issues.

Immediately after adding the cells, fluorescence (ex/em = 485/516 nm) was measured every 10 min for a total period of 2 h with a microplate reader (BioTek, Synergy HT). Blank-corrected values are given relative to negative controls (treated/untreated) from three independent experiments.

4.8. Assessment of Mitochondrial Membrane Potential and Morphology

For measurement of MMP, HL-60 cells were seeded at densities of 1.0×10^5 cells/well in 500 μ L of medium into 24-well plates. The following day, cells were treated with solvent control, respective ligands, or complexes for 24 h. Cells were treated with 50 μ M of CCCP, positive control for membrane depolarization, and 2 μ M of JC-1 (MedChemExpress, NJ, USA) for 30 min. After incubation, cells were washed with PBS (78.1 mM Na₂PO₄ $\times$ 2H₂O, 14.7 mM KH₂PO₄, 26.8 mM KCl, 1.37 M NaCl, pH 7.4) and measured immediately by flow cytometry (GuavaEasyCyte, Cytex) using bandwidth filters b530/30 (green, depolarized mitochondria) and 590 nm (red, polarized mitochondria). Data from three independent experiments were analyzed using FlowJo software (v10.10.0).

For visualization of mitochondrial morphology SW480 cells were seeded at a density of 2×10^4 in 8-well ibiTreat 1.5 polymer μ -slides (80 826, ibidi GmbH, Gräfelfing, Germany) in 300 μ L medium and incubated overnight. The next day, cells were treated with solvent control or complex **3a** at 10 and 20 μ M for 24 h. Prior assessment, positive control cells were treated with 50 μ M of CCCP for 30 min. After washing, mitochondria were stained with 0.05 μ M of

MitoTracker Red CMXRos (M7512, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min and cell nuclei were stained with 4 μ M Hoechst 33342 (H1399, Thermo Fisher Scientific, Waltham, MA, USA) for 20 min in the dark. Images were taken on a Zeiss LSM 800 Confocal Laser Scanning Microscope equipped with an Pln Apo 63 $\times$ /1.4 Oil DICII objective using Zeiss ZEN blue software (version 3.4.91.00000, Carl Zeiss Microscopy GmbH). Laser intensities and detector gains were maintained at the same level during all imaging sessions.

4.9. Induction of Apoptosis/Necrosis

HL-60 cells were seeded in densities of 5.0×10^4 cells/well in 600 μ L aliquots into 24-well plates. After 24 h, ligands and complexes, as well as a negative and positive controls, were added for another 24 h period. Afterward, cells (suspended in the supernatant) were collected and centrifuged for 3 min at 2100 rpm (Mikro 200R, Hettich). The supernatant was removed and cell pellets were resuspended in human recombinant annexin V/FITC protein (Invitrogen), diluted (1:150) in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl_2) and incubated for 15 min at 37 $^\circ\text{C}$. Then, propidium iodide (2 mg/mL suspension) was diluted (1:150) in binding buffer and cells were immediately measured with a flow cytometer (GuavaEasyCyte, Cytex). Data from three independent experiments were quantitatively analyzed with FlowJo software.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.organomet.6c00152>.

Synthesis of ligand **1c**, **1D** and 2D NMR spectra of the synthesized compounds, ESI-MS spectra of the complexes, X-ray and ED diffraction analysis data, stability in aqueous solution, MTT and Alamar Blue assays and apoptosis/necrosis assay (PDF)

Accession Codes

Deposition Numbers 2449653–2449654 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via the joint Cambridge Crystallographic Data Centre (CCDC) and Fachinformationszentrum Karlsruhe Access Structures service.

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Notes

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