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Ru(II) Metal Complexes: Synthesis, Interaction with Mitochondrial G-Quadruplex, and Biological Activity

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TABLE OF CONTENTS

Abbreviations.....	iv
Sommario	vi
Abstract	viii
1. Introduction	10
<i>1.1 DNA: From Molecular Architecture to Therapeutic Targeting</i>	<i>11</i>
1.1.1 DNA Structure and Function.....	11
1.1.2 DNA Alterations and Cancer Development	12
1.1.3 DNA-Targeted Anticancer Strategies	13
<i>1.2 G-Quadruplexes (G4s) as Non-Canonical DNA Structures</i>	<i>14</i>
1.2.1 Structural Features and Properties of G-Quadruplexes (G4s).....	15
1.2.2 Biological Roles of G4s and Therapeutic Potential	16
1.2.3 Small-Molecule Targeting of G4s	18
1.2.4 Methods: G-Quadruplex Structure & Small Molecule Binding.....	21
<i>1.3 Mitochondrial Function and Genome in Cancer.....</i>	<i>24</i>
1.3.1 Mitochondria: Overview of Structure and Functions.....	24
1.3.2 Structure and Functions of mtDNA	27
1.3.3 Role of Mitochondria in Cancer Cells.....	29
1.3.4 Mitochondrial Targeting in Cancer Treatment	31
1.3.5 Mitochondrial G4s: Evidence, Potential Functions, and Relevance in Cancer.....	33
<i>1.4 Metal-based Drugs and Biomedical Applications</i>	<i>34</i>
1.4.1 Therapeutic Applications of Metal Compounds in Oncology.....	35
1.4.2 Ruthenium Complexes: Properties and Anticancer Potential	36
1.4.3 Metal Complexes as G4 Binders.....	38
2. Results.....	41
<i>Chapter I: Ruthenium(II) Polypyridyl Complexes as Mitochondrial G-Quadruplex Binders: Synthesis,</i>	
<i>DNA binding and Biological Evaluation in Cancer Cells</i>	<i>42</i>
1. Introduction.....	42
2. Results and Discussion.....	43
3. Experimental	64

4. Conclusions	72
<i>Chapter II: Ruthenium(II) Arene Complexes with Mitochondrial Targeting Moieties for G-Quadruplex Binding.....</i>	
1. Introduction	74
2. Results and Discussion.....	74
3. Experimental	92
4. Conclusions	96
<i>Chapter III: Novel Ruthenium(II) Complexes with Polypyridyl and 1,2,4 Oxadiazole Ligands: From Synthesis to Biological Evaluation in Cancer Cells Lines.....</i>	
1. Introduction	97
2. Results and Discussion.....	97
3. Experimental	106
4. Conclusions	109
<i>Chapter IV: 4-phenylthiazole derived Ru(II) metalacycles and their interaction with G-Quadruplex</i>	
1. Introduction	111
2. Results and Discussion.....	112
3. Experimental	121
4. Conclusion	122
<i>Chapter V: Alloxazine-Based Ruthenium Complexes as G-Quadruplex Binders.....</i>	
1. Introduction	124
2. Results and Discussion.....	125
3. Experimental	127
4. Conclusions	128
3. Conclusions	129
Appendix A. NMR Spectra	131
Appendix B. Mass Spectra.....	135
Bibliography	139

Abbreviations

A2780 / A2780cis – Human ovarian carcinoma cell lines (cisplatin-sensitive / resistant)

ATP – Adenosine Triphosphate

AUC – Area under the curve

bpy – 2,2'-bipyridine

CD – Circular Dichroism

ct-DNA – Calf-Thymus DNA

DMF – Dimethylformamide

dppz – Dipyrido[3,2-a:2',3'-c]phenazine

dsDNA – Double-strand DNA

ECAR – Extracellular Acidification Rate

EDC·HCl – 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride

ESI-MS – Electrospray Ionization Mass Spectrometry

FAM – 6-carboxyfluorescein

FRET – Fluorescence Resonance Energy Transfer

G4 – G-Quadruplex

HCT116 – Human colorectal carcinoma cell line

HOBt – Hydroxybenzotriazole

IC₅₀ – Half-maximal inhibitory concentration

ICD – Induced Circular Dichroism

ICP-MS – Inductively Coupled Plasma Mass Spectrometry

mtDNA – Mitochondrial DNA

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NMR – Nuclear Magnetic Resonance

OCR – Oxygen Consumption Rate

ODN(s) – Oligodeoxyribonucleotide(s)

OXPHOS – Oxidative Phosphorylation

PBS – Phosphate Buffered Saline

PDB – Protein Data Bank

phen – 1,10-phenanthroline

PQS – Potential Quadruplex-Forming Sequence

SD – Standard Deviation

TAMRA – 6-carboxy-tetramethyl-rhodamine

T_m – Melting Temperature

TPP – Triphenylphosphonium

UV-Vis – Ultraviolet-Visible

Sommario

I composti a base di platino restano tra i pilastri della chemioterapia antitumorale, ma la loro efficacia è spesso limitata da elevata tossicità sistemica e dall'insorgenza di fenomeni di farmacoresistenza. Tali limiti rendono urgente lo sviluppo di nuovi metallo-farmaci alternativi. In questo contesto, i complessi di rutenio rappresentano una promettente classe di candidati, come dimostrano due molecole, BOLD-100 e TLD-1433, attualmente in fasi avanzate di sperimentazione clinica per il trattamento di diverse neoplasie. Tra i complessi di rutenio più studiati, gli *scaffold* polipiridilici di Ru(II) offrono ampie superfici π e geometrie ottaedriche, che favoriscono il riconoscimento degli acidi nucleici e manifestano il caratteristico effetto “*light-switch*” in seguito al legame con il DNA. Inoltre, numerosi complessi di Ru(II) hanno mostrato una notevole selettività verso strutture secondarie del DNA non canoniche, note come G-Quadruplex (G4).

Le strutture G4 sono particolarmente abbondanti nei telomeri e in regioni genomiche di rilevanza regolatoria, come i promotori di diversi oncogeni (ad esempio *MYC*, *KIT* e *BCL2*). Poiché i G4 sono in grado di modulare l'espressione di geni coinvolti nella tumorigenesi e di influenzare la progressione della replicazione cellulare, essi rappresentano bersagli terapeutici di grande interesse. Piccole molecole capaci di legarsi e stabilizzare le strutture G4 (*G4 binders*) possono alterare l'espressione di oncogeni, indurre stress replicativo e promuovere processi apoptotici.

Anche il DNA mitocondriale (mtDNA) contiene tratti ricchi in guanine che possono formare strutture G4 all'interno di geni e regioni regolatorie, suggerendo un ulteriore livello di regolazione genica a livello dell'organello. Questa evidenza apre nuove prospettive per lo sviluppo di composti capaci di legare selettivamente i G4 mitocondriali, combinando il riconoscimento strutturale con un'interferenza mirata nei processi bioenergetici mitocondriali e delineando così nuove opportunità per strategie terapeutiche di tipo mirato. Parallelamente, i mitocondri rappresentano un bersaglio anticancro di particolare interesse, data la loro funzione cruciale nel metabolismo, nella regolazione dell'apoptosi e nei meccanismi di resistenza farmacologica. Il *targeting* mitocondriale può essere ottenuto attraverso la coniugazione di gruppi trifenilfosfonio (TPP), che sfruttano il potenziale di membrana negativo del mitocondrio per favorire l'accumulo selettivo di molecole cationiche al suo interno.

L'obiettivo di questo progetto di dottorato è la sintesi di nuovi complessi di Ru(II), appositamente funzionalizzati per il *targeting* mitocondriale, con lo scopo di interagire con le strutture G4 presenti nel DNA mitocondriale e valutarne il potenziale effetto antitumorale. Questo progetto introduce i

primi complessi di rutenio specificatamente modificati per il *targeting* dei G4 mitocondriali. I composti sono stati caratterizzati mediante spettroscopia di risonanza magnetica nucleare, spettrometria di massa e analisi elementare. Successivamente, l'interazione con strutture G4 mitocondriali è stata indagata attraverso diverse tecniche biofisiche, tra cui la spettroscopia di assorbimento UV-Visibile (UV-Vis), il dicroismo circolare (CD), la spettroscopia di fluorescenza e misure di *melting* mediante la tecnica Förster Resonance Energy Transfer (FRET).

Lo studio dell'attività biologica dei complessi di Ru(II) è stato svolto durante un soggiorno di ricerca di otto mesi presso l'*Institute for Cancer Research* dell'Università di medicina di Vienna, in Austria. In tale contesto, sono stati eseguiti saggi di vitalità cellulare su colture 2D e 3D, anche in combinazione con modulatori metabolici (BAY-876 e metformina), analisi della funzionalità mitocondriale tramite Seahorse XF Analyzer, valutazioni dell'espressione genica mitocondriale mediante RT-qPCR e misure di *uptake* cellulare mediante ICP-MS.

La presente tesi è articolata in tre sezioni: (i) Introduzione, (ii) Risultati e (iii) Conclusioni.

La sezione dei Risultati è ulteriormente suddivisa in cinque capitoli. I primi due capitoli esplorano due diversi *scaffold* di complessi di rutenio, polipiridilici e arilici di tipo "*piano-stool*", funzionalizzati con gruppi TPP, analizzandone le proprietà di interazione con sequenze G4 mitocondriali e l'attività biologica su linee cellulari tumorali. Nel Capitolo III, l'attenzione si concentra su una nuova famiglia di complessi di Ru(II) contenenti leganti ossadiazolici, con l'obiettivo di ampliare la comprensione dei meccanismi di riconoscimento tra complessi di rutenio e strutture G4, questa volta di origine nucleare. Anche in questo caso, l'attività biologica dei complessi è stata valutata su diverse linee cellulari tumorali. Infine, nella parte conclusiva del lavoro (Capitoli IV e V), l'indagine si estende a ulteriori complessi di Ru(II), sviluppati e studiati nell'ambito di progetti collaborativi internazionali. Questi studi hanno contribuito ad approfondire la comprensione del riconoscimento dei G4 da parte di diversi *scaffold* di Ru(II).

Abstract

Platinum-based compounds remain among the cornerstones of anticancer chemotherapy; however, their clinical efficacy is often limited by severe systemic toxicity and the onset of drug resistance. These drawbacks highlight the urgent need to develop new classes of alternative metal-based drugs. In this context, ruthenium complexes have emerged as a promising family of candidates, as exemplified by BOLD-100 and TLD-1433, two compounds currently in advanced phases of clinical trials for the treatment of several malignancies. Among the most extensively studied ruthenium scaffolds, Ru(II) polypyridyl complexes offer extended π -surfaces and octahedral geometries that promote nucleic acid recognition and display the characteristic “light-switch” effect upon DNA binding. Moreover, many Ru(II) complexes have shown remarkable selectivity toward noncanonical DNA secondary structures known as G-quadruplexes (G4s).

G4 structures are particularly abundant in telomeric regions and in genomic sites of regulatory importance, such as oncogene promoters (e.g., *MYC*, *KIT*, and *BCL2*). Since G4s can modulate the expression of genes involved in tumorigenesis and affect the progression of DNA replication, they represent highly attractive therapeutic targets. Small molecules capable of binding and stabilizing G4 structures (G4 binders) can alter oncogene expression, induce replicative stress, and promote apoptotic processes.

Mitochondrial DNA (mtDNA) also contains guanine-rich regions capable of forming G4 structures within genes and regulatory sequences, suggesting an additional level of gene regulation at the organellar level. This evidence opens new perspectives for the development of compounds able to selectively bind mitochondrial G4s, combining structural recognition with targeted interference of mitochondrial bioenergetic processes, and thus offering new opportunities for precision therapeutic interventions. At the same time, mitochondria themselves are of particular interest as anticancer targets due to their central roles in metabolism, apoptosis regulation, and mechanisms of drug resistance. Mitochondrial targeting can be achieved through the conjugation of triphenylphosphonium (TPP) groups, which exploit the negative mitochondrial membrane potential to promote the selective accumulation of cationic molecules within the organelle.

The goal of this PhD project is the synthesis of novel Ru(II) complexes specifically functionalized for mitochondrial targeting, designed to interact with G4 structures within mtDNA and to assess their potential anticancer activity. This work introduces the first ruthenium complexes specifically engineered for the recognition of mitochondrial G4s. The compounds were characterized by nuclear

magnetic resonance spectroscopy, mass spectrometry, and elemental analysis. Their interaction with mitochondrial G4 structures was investigated using various biophysical techniques, including UV-visible absorption spectroscopy, circular dichroism, fluorescence spectroscopy, and Förster Resonance Energy Transfer (FRET) melting assays.

The biological activity of the Ru(II) complexes was investigated during an eight-month research stay at the Institute for Cancer Research, Medical University of Vienna (Austria). In this context, cell viability assays on 2D and 3D cultures were performed, both alone and in combination with metabolic modulators (BAY-876 and metformin). Mitochondrial function was analyzed using the Seahorse XF Analyzer, mitochondrial gene expression was evaluated by RT-qPCR, and cellular uptake was quantified by ICP-MS.

The present thesis is organized into three sections: (i) Introduction, (ii) Results, and (iii) Conclusions. The Results section is further divided into five chapters. The first two chapters explore two different Ru-based scaffolds, polypyridyl and arene “piano-stool” complexes, functionalized with TPP groups, focusing on their interaction with mitochondrial G4 sequences and their biological activity in cancer cell lines. Chapter III investigates a new family of Ru(II) complexes containing oxadiazole ligands, with the aim of broadening the understanding of recognition mechanisms between Ru(II) complexes and G4 structures, in this case of nuclear origin. Their biological activity was also evaluated in different tumor cell lines. Finally, in the concluding part of the work (Chapters IV and V), the study extends to additional Ru(II) complexes developed within international collaborative projects. These studies contributed to advancing the overall understanding of G4 recognition by diverse Ru(II) scaffold.

1. Introduction

1.1 DNA: From Molecular Architecture to Therapeutic Targeting

1.1.1 DNA Structure and Function

Deoxyribonucleic acid (DNA) is a fundamental polynucleotide that encodes the genetic instructions essential for cellular function and inheritance. The monomeric units of DNA are nucleotides (Figure 1a), each consisting of deoxyribose (a monosaccharide), one or more phosphate groups, and a nitrogenous base. In DNA, there are two purine bases, adenine (A) and guanine (G), and two pyrimidine bases, thymine (T) and cytosine (C). Phosphodiester bonds between the 3'-hydroxyl group of one deoxyribose and the 5'-phosphate of the next nucleotide form the sugar-phosphate backbone of the polymer.¹ The three-dimensional structure of DNA was first described in *Nature* in 1953 by Watson and Crick, based on critical experimental data from Rosalind Franklin's X-ray diffraction studies and Erwin Chargaff's base composition rules.² The canonical conformation of DNA, known as B-DNA, is a right-handed double helix in which two antiparallel strands interact via complementary hydrogen bonding (Figure 1b). Specifically, A pairs with T, while C pairs with G. The helix has a diameter of approximately 2 nm, with an axial distance between adjacent bases of 3.4 Å, and each base pair is rotated by 36°, resulting in one full helical turn every 10 base pairs (3.4 nm). Furthermore, the nonlinear alignment of the C1' atoms of opposing deoxyribose units gives rise to the major and minor grooves along the helix.¹

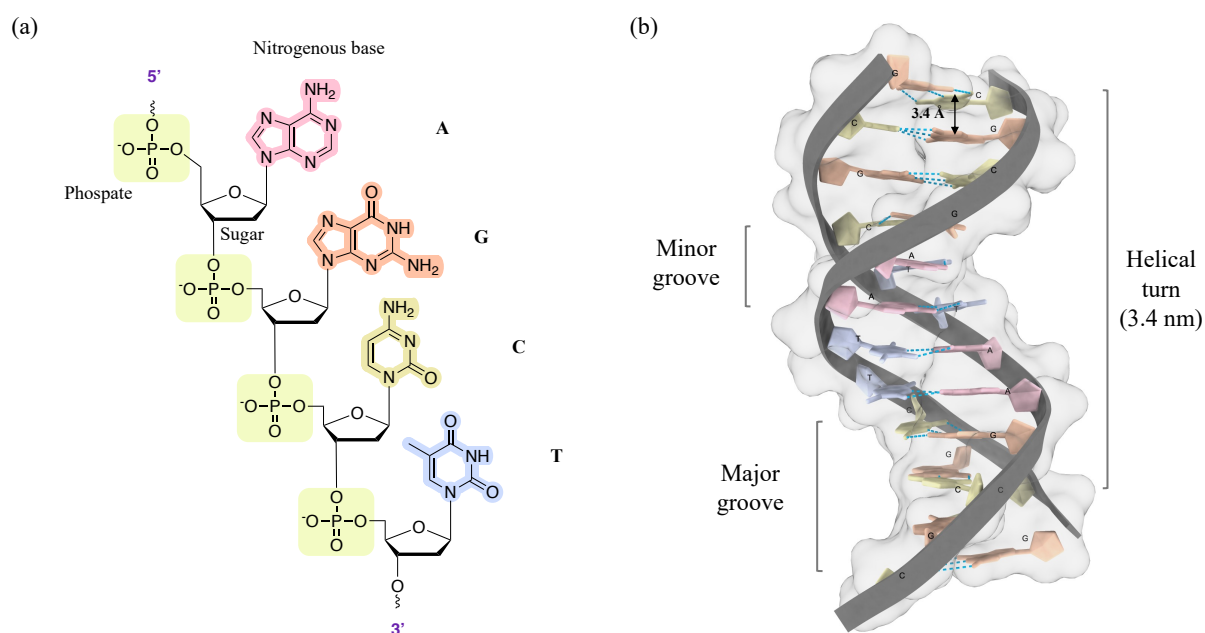


Figure 1. (a) Structure of DNA nucleotides, each composed of a phosphate group (green), a deoxyribose sugar, and one of the four nitrogenous bases: adenine (A), thymine (T), guanine (G), or cytosine (C). (b) Three-dimensional representation of the B-DNA double helix (PDB ID: 1BNA).

According to the central dogma of molecular biology, DNA encodes genetic information in its base sequence, which is transcribed into messenger RNA (mRNA). A gene is a DNA segment that specifies a functional product, most often a protein via an mRNA intermediate. In eukaryotes, non-coding introns are removed and exons spliced to form mature mRNA, which is then translated by ribosomes: transfer RNAs (tRNAs) deliver amino acids in order, while ribosomal RNAs (rRNAs) form the catalytic core that drives peptide-bond formation.³

To ensure faithful inheritance during cell division, DNA must be duplicated. Replication is a semi-conservative process in which the two parental strands separate and serve as templates for new complementary strands. It begins at specific origins, where helicases unwind the double helix, primase lays down RNA primers, and DNA polymerases extend new strands in the 5'→3' direction, continuously on the leading strand and discontinuously on the lagging strand, ultimately producing two identical DNA molecules.³

Remarkably, the three-dimensional conformation and dynamics of the double helix influence properties such as flexibility and bending, which in turn regulate the accessibility of the encoded information and affect protein recognition.⁴

Although the double helix provides a highly stable repository of genetic information, DNA remains vulnerable to damage and mutations that, if not properly repaired, can disrupt cellular function and ultimately drive diseases such as cancer.

1.1.2 DNA Alterations and Cancer Development

Cancer is defined as a heterogeneous group of diseases characterized by the uncontrolled proliferation of abnormal cells, their capacity to invade surrounding tissues, and their ability to disseminate to distant organs through metastasis.⁵ Carcinogenesis is a multistep evolutionary process driven by two fundamental processes. First, individual cells progressively accumulate DNA mutations, generating heritable variation within the population. Second, selective pressures in the tissue environment act on this diversity: deleterious mutations are typically eliminated, whereas advantageous alterations that promote growth or survival allow specific clones to expand and eventually dominate.⁶

DNA damage plays a critical role in cancer development by increasing the likelihood of mutation occurrence. Such damage can arise from endogenous sources, including replication stress or reactive oxygen species (ROS) generated during oxidative metabolism, and may also be influenced by hereditary predispositions. Exogenous agents such as toxins, ionizing radiation, ultraviolet (UV) light, and chemotherapeutic drugs can likewise induce structural alterations in DNA.⁷

Common forms of DNA damage include single-strand breaks (SSBs), double-strand breaks (DSBs), base modifications, and the formation of DNA adducts. Although such lesions occur physiologically

at a high frequency daily, cells rely on the DNA damage response (DDR), a complex signaling and repair network, to preserve genomic stability or, when damage is irreparable, to trigger apoptosis. However, dysregulation of DDR pathways promotes cancer initiation and progression. Indeed, genomic instability resulting from defective DNA damage responses is recognized as one of the hallmarks of cancer.^{8,9}

In addition to DNA repair genes, mutations in proto-oncogenes and tumor suppressor genes also increase cancer susceptibility. When activated, proto-oncogenes are converted into oncogenes, driving excessive production or activity of proteins that promote cell growth and survival. Conversely, tumor suppressor genes normally regulate cell cycle progression and apoptosis, thereby maintaining cellular homeostasis. Mutations affecting either class of genes disrupt this balance and can result in uncontrolled proliferation, ultimately leading to tumorigenesis.¹⁰

Given the central role of DNA alterations in driving tumorigenesis, it has long been considered a critical therapeutic target in the development of anticancer strategies.

1.1.3 DNA-Targeted Anticancer Strategies

Therapies directly or indirectly targeting DNA have long been established in clinical oncology and remain a cornerstone of anticancer treatment. Alkylating agents were among the first chemotherapeutic drugs to be discovered, and they are still widely employed in the treatment of various malignancies. By means of their electrophilic moieties, alkylating agents such as dacarbazine and cyclophosphamide form covalent adducts with nucleophilic sites in DNA, ultimately inducing diverse types of DNA damage.¹¹

Another important class of anticancer agents is represented by antimetabolites (e.g. methotrexate), structural analogs of folates, purines and pyrimidines that disrupt DNA and RNA synthesis either by inhibiting key enzymes in nucleotide metabolism or by being incorporated into nucleic acids. Furthermore, some compounds inhibit DNA-related proteins such as topoisomerases, which are essential for resolving torsional stress during DNA replication and transcription. Among these, it is worth mentioning camptothecin analogs (e.g. irinotecan) and antitumor antibiotics, such as anthracyclines, actinomycin D, and mitomycin C.¹² These agents can also act as DNA intercalators, using their planar aromatic moieties to insert between stacked base pairs of the double helix, thereby distorting the helical structure and impairing replication and transcription.¹³

Despite their efficacy, classical chemotherapeutic agents such as those mentioned are often associated with significant toxicity. In fact, since the toxic effect generally lacks tumor specificity, also rapidly proliferating healthy tissues, such as those of the bone marrow, gastrointestinal tract, and hair follicles, are damaged. Common adverse effects include myelosuppression, mucositis, nausea,

alopecia, and increased susceptibility to infections. These side effects not only compromise patient quality of life but also frequently limit dosing and long-term use, thereby motivating the search for more selective anticancer strategies.¹⁴

DNA continues to be a major focus of contemporary research, as illustrated by compounds such as lurbinectedin, a minor-groove binder recently approved (2020) for the treatment of small-cell lung cancer.¹⁵ A particularly successful modern approach exploits the concept of synthetic lethality through inhibition of poly(ADP-ribose) polymerase (PARP), a key enzyme in single-strand DNA break repair. Tumors harboring defects in homologous recombination repair (most notably BRCA1- or BRCA2-mutated cancers) are highly dependent on PARP-mediated pathways for survival. Pharmacological inhibition of PARP traps the enzyme and prevents the repair of SSBs, which eventually collapse into double-strand breaks during replication. In BRCA-deficient cells, the inability to repair these lesions leads to genomic instability and cell death. On this basis, several PARP inhibitors (e.g. olaparib) have been recently approved and are now used in the treatment of cancers carrying homologous recombination deficiencies.¹⁶ The chemical structures of some of the anticancer drugs discussed in this section are shown in Figure 2 above.

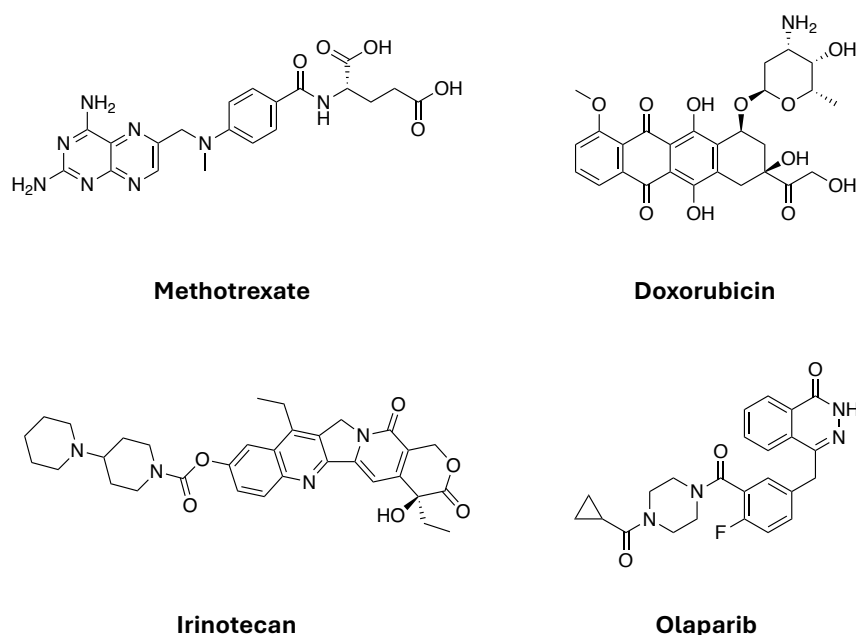


Figure 2. Chemical structures of selected anticancer drugs, cited in this section.

1.2 G-Quadruplexes (G4s) as Non-Canonical DNA Structures

The B-DNA conformation described above is the most prevalent form in cells, but it is not the only possible structure. Under certain conditions, DNA can deviate from Watson-Crick base pairing rules and adopt so-called non-canonical conformations. Factors such as nucleotide sequence, hydration

state, ionic environment, and interactions with small molecules (binders) influence the DNA structure and dynamics. As a result, DNA can fold into alternative topologies, including A-DNA, Z-DNA, hairpins, cruciforms, triplexes, G-quadruplex (G4), and i-motifs. These higher-order structures modulate cellular functions, thereby affecting key biological processes.¹⁷

Because of their widespread occurrence in key genomic regions and their emerging relevance as therapeutic targets, G4s constitute the central focus of this thesis. This section outlines their structural characteristics and biological functions, with particular emphasis on their involvement in cancer, as well as the molecules that target them and the experimental approaches used to investigate their structure and binding interactions.

1.2.1 Structural Features and Properties of G-Quadruplexes (G4s)

G4s are four-stranded secondary structures of nucleic acids that typically form within guanine-rich sequences. Four guanines arrange into a planar square tetrad known as G-quartet; within each quartet, every guanine participates in two Hoogsteen bonds (Figure 3a). Stacking of multiple G-quartets generates the characteristic G4 architecture, which is further stabilized by monovalent cations that interact with O6 carbonyls of guanine (Figure 3b,c). The stabilizing effect follows the order $K^+ > Na^+ > NH_4^+ > Li^+$, with K^+ being the most abundant cation in the intracellular compartment.^{18,19} Under physiologically relevant conditions, sequences following the motif 5'-G_{≥3}N₁₋₇G_{≥3}N₁₋₇G_{≥3}N₁₋₇G_{≥3}-3' will be able to form G4 structures.²⁰

G4s are highly polymorphic structures. They may form intramolecularly (unimolecular) within a single strand or intermolecularly (bi- or tetramolecular) from two or four strands. Furthermore, there are three principal G4 topologies, which differ according to strand orientation. In the parallel form, all four strands run in the same direction, whereas in the hybrid and antiparallel conformations, one or two strands are oriented oppositely, respectively. The glycosidic torsion angle of guanines is a key determinant of the topology. In parallel quadruplexes, all guanine residues adopt the *anti* conformation. By contrast, antiparallel structures contain a mixture of *syn* and *anti*, arranged in distinct patterns that depend on the specific topology and relative orientation of the four strands.²¹

The sequences connecting the guanine, the loops, can be classified into three types: propeller, lateral, and diagonal. Propeller loops connect adjacent parallel strands, lateral loops link adjacent antiparallel strands, and diagonal loops span across the G-quartet to connect opposite corners. Thus, parallel topologies contain three propeller loops, while hybrid ones contain two lateral loops and one propeller loop, and antiparallel structures contain either two lateral loops and one diagonal loop or three lateral loops.²²

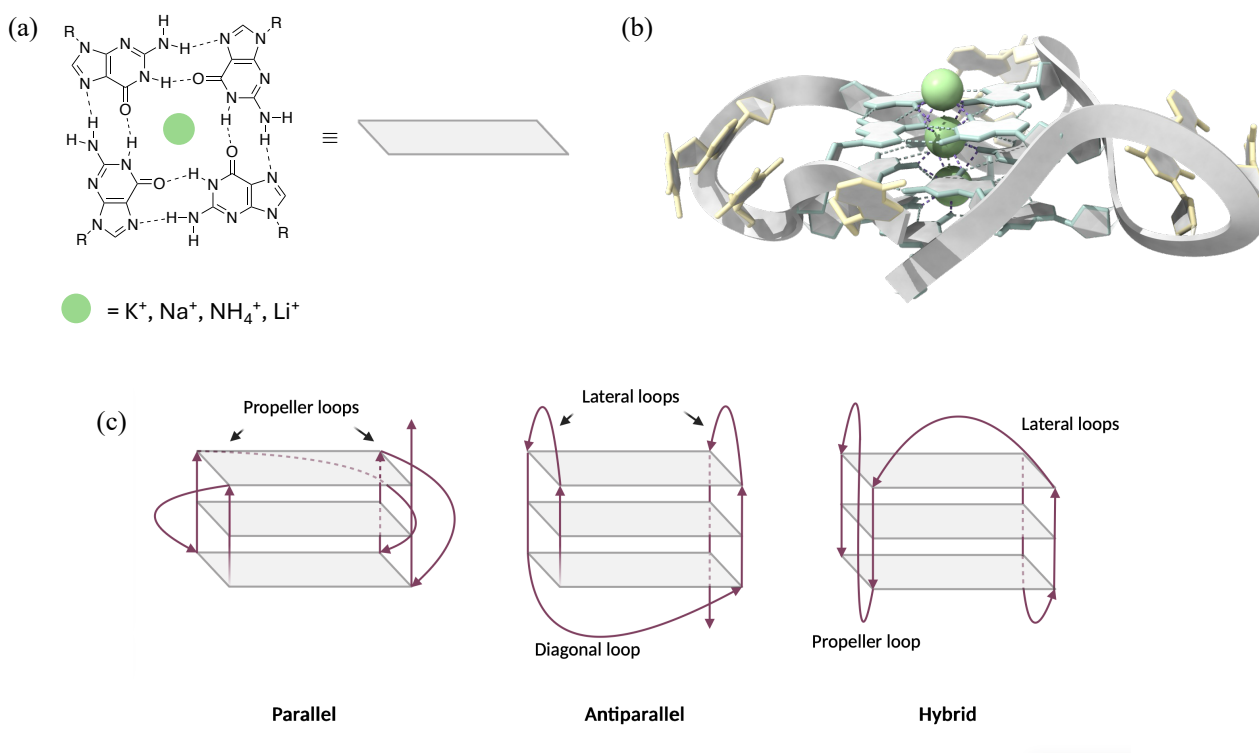


Figure 3. (a) Schematic representation of a G-quartet stabilized by a central monovalent cation (K^+ , Na^+ , NH_4^+ or Li^+). (b) Lateral view of one of the possible G4 structure adopted by the human telomere sequence 5'-AGGGTTAGGGTTAGGGTTAGGG-3'. The structure consists of a parallel G4 with three stacked G-quartets. PDB id: 1KF1. (c) The three main G4 topologies, parallel (left), antiparallel (center) and hybrid (right), with different types of connecting loops (propeller, lateral, and diagonal) are highlighted. Created with BioRender.com.

Topology and stability of G4s are determined by several factors, including the temperature, the molecular environment, the number of stacked G-quartets, the length and type of loops, the identity of the coordinating cation, and the sequence composition both within the G4 motif and in the flanking regions.²³

1.2.2 Biological Roles of G4s and Therapeutic Potential

To predict the genomic distribution of G4 structures, several computational algorithms have been developed, each with specific advantages and limitations. Firstly, Quadparser, described in the seminal work by Balasubramanian and co-workers, estimated that the human genome contains approximately 370,000 putative G4-forming sequences (PQS).²⁰ To address the false positives and negatives associated with this pattern-based approach, more advanced tools were later introduced, including G4Hunter,²⁴ which evaluates G-richness and G-skewness, and machine learning-based algorithms such as Quadron.²⁵ In 2015, the first experimental genome-wide G4 mapping was achieved using the G4-seq method, which detects polymerase-stalling sites under G4-stabilizing

conditions, revealing roughly 700,000 potential G4 structures in vitro.²⁶ Subsequently, the development of G4-specific antibodies, such as BG4, enabled chromatin immunoprecipitation coupled to high-throughput sequencing (ChIP-Seq) for G4 detection in cells. The first such protocol, reported by Balasubramanian's group, identified around 10,000 G4 structures in human cells.²⁷ Notably, these structures were more abundant in cancer cells than in normal ones and predominantly localized within regulatory, nucleosome-depleted regions, including telomeres, replication origins, gene promoters, somatic mutation hotspots, and untranslated regions (UTRs) of mRNAs.^{27,28}

Human telomeric G4s were the first to be recognized as biologically relevant. Telomeres are specialized nucleoprotein structures located at chromosome ends and composed of tandem repeats of the guanine-rich sequence d(TTAGGG). Their primary role is to safeguard genome integrity by protecting chromosome termini from degradation, recombination, and end-to-end fusions. Because replicative immortality is a hallmark of cancer, tumor cells must maintain telomere length, most commonly by reactivating telomerase (a reverse transcriptase normally silent in somatic cells but upregulated in ~80% of cancers), which elongates the 3' single-stranded telomeric overhang. This makes telomerase both a valuable cancer biomarker and a promising therapeutic target.^{29,30} Telomeric G4s most often adopt parallel or hybrid conformations, and these structural variations differentially can regulate telomerase activity: intramolecular antiparallel folds can act as barriers, whereas intermolecular parallel arrangements may still allow extension.^{23,28} Early G4 binders were therefore designed as telomerase inhibitors. However, it is now clear that telomeric G4s do not uniformly block telomerase. Instead, many binders primarily interfere with telomeric DNA processing rather than directly inhibiting telomerase extension.³¹

During DNA replication, helicases unwind the two DNA strands: one serves as the template for continuous leading-strand synthesis, while the lagging strand is replicated discontinuously. This transient single-stranded state of the lagging strand provides a favorable context for G4 formation, which acts as a natural barrier to replication. To maintain fork progression, cells depend on helicases such as BLM, WRN, and FANCI to resolve these structures. When control fails, unresolved G4s contribute to replication stress, chromosomal fragility, and genomic instability, especially in rapidly proliferating cancer cells. Consistently, mutations in these helicases underlie cancer predisposition syndromes such as Bloom syndrome, Werner syndrome, and Fanconi anemia.^{23,32}

Remarkably, one or more G4 motifs are located within 1,000 nucleotides upstream of the transcription start sites of nearly 50% of human genes, highlighting their potential role in transcriptional regulation.³³ Importantly, promoter G4s are particularly enriched in genes relevant to cancer biology, including *MYC*, *KIT*, *KRAS*, *BCL2*, *VEGF*, and *TERT*.³⁴ The functional outcome of G4 formation depends on strand orientation: G4s formed on the template strand can act as physical barriers to the

transcription machinery and repress gene expression, whereas G4s on the non-template strand may stabilize single-stranded regions, thereby facilitating transcription. In addition, proteins interacting with promoter G4s, such as transcriptional activators or repressors, further modulate their regulatory effects.³⁵ A noteworthy example is *MYC*, frequently overexpressed in ~70% cancers (e.g., breast, colorectal, cervical), making its downregulation an attractive strategy in cancer treatment. Directly targeting the MYC oncoprotein has proven extremely difficult because it lacks stable binding pockets and has a short half-life. Instead, attention has shifted to its promoter region where an intramolecular parallel G4 structure can function as a transcriptional silencer. Many G4s binders downregulate MYC transcription *in vitro* in cancer cell lines, leading to reduced proliferation, apoptosis, and tumor regression.³⁶

Finally, G4 motifs are enriched at sites of somatic mutations, where they may contribute to genomic instability,³⁷ and in mRNA, particularly within 5' untranslated regions (UTRs). UTRs are non-coding sequences that play a key role in regulating translation, and in this context, G4s can interfere with initiation by blocking ribosome scanning or promoting the use of upstream start codons. Accordingly, small molecules that stabilize RNA G4s within 5' UTRs have been proposed as potential inhibitors of oncogene translation.³⁸

1.2.3 Small-Molecule Targeting of G4s

As discussed above, G4s are enriched at key genomic regions and may influence cancer-related processes. Notably, G4 folding occurs more frequently in cancer cells than in normal cells, further underscoring their appeal as selective targets in anticancer therapy.¹⁸ This evidence has, in turn, driven the development of diverse classes of small molecules specifically designed to bind and stabilize G4s. The three-dimensional architecture of G4s provides multiple binding opportunities for small molecules (Figure 4). Most G4 binders contain extended π -planar aromatic systems that interact with the external G-quartets via end-stacking, and in some cases may intercalate between adjacent tetrads. However, intercalation is thought to be rare due to the inherent rigidity of the G4 core. Positively charged substituents can further enhance affinity by forming electrostatic interactions with the negatively charged phosphate backbone within the grooves. In addition, partial positive charges positioned to mimic the role of central cations (K^+ or Na^+) can further stabilize the quadruplex structure.^{39,40}

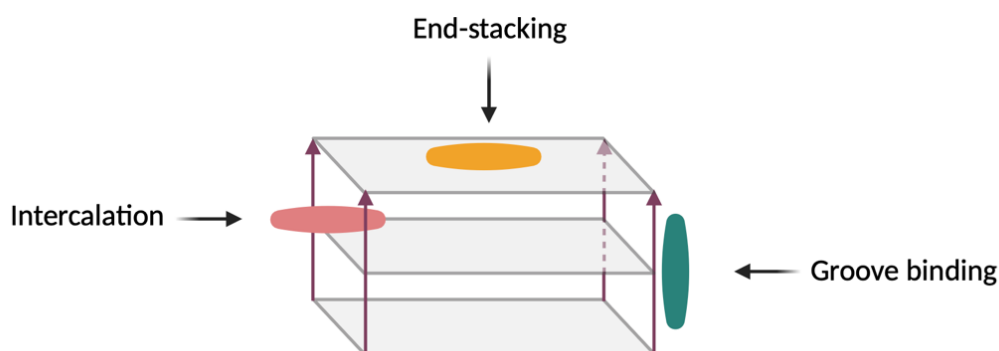


Figure 4. Schematic representation of possible binding modes of small molecules to G4 structures. Created with BioRender.com.

A crucial goal in a G4 binder design is achieving selectivity over duplex DNA. End-stacking provides a promising strategy, as the terminal G-quartets offer a significantly larger aromatic surface than duplex DNA. Accordingly, effective G4 binders typically require extended aromatic systems to maximize π - π overlap and enhance selectivity.⁴¹ The following subsections briefly survey representative classes of G4-targeting binders, highlighting the most relevant examples.

Acridines. **BRACO-19** was among the first molecule tailored to target telomeric G4s and inhibit telomerase; despite strong G4 affinity and preclinical antitumor activity, its poor pharmacokinetics curtailed development.^{42–44} In contrast, **RHPS4**, designed with an expanded aromatic core to enhance stacking, stabilizes telomeric G4s, inhibits telomerase, and induces telomere dysfunction with robust xenograft activity, but failed to progress clinically due to off-target cardiotoxicity.^{45,46}

Quinolones. **Quarfloxin**, the first G4 stabilizer to reach clinical trials, showed good tolerability but was ultimately limited by atypical pharmacokinetics and poor bioavailability.⁴⁷ In the same class of compounds, **CX-5461** was designed to improve drug-like properties and has emerged as a potent G4 stabilizer. It has shown favorable safety, tolerability, and pharmacokinetics and is still under active clinical evaluation.⁴⁸

Porphyrins. The benchmark compound in this class, **TMPyP4**, exhibits high affinity for G4s thanks to the extended aromatic surface but is limited by drawbacks such as auto-aggregation and poor selectivity among different G4 topologies.⁴⁰

Neutral macrocycles. **Telomestatin**, a natural macrocyclic compound isolated from *Streptomyces annulatus* in 2001,⁴⁹ strongly stabilizes G4 structures with remarkable selectivity over duplex DNA, acts as a potent telomerase inhibitor, and disrupts telomere maintenance by altering telomere

conformation and protein associations. However, synthetic complexity and scalability are major limitations.⁵⁰

Pyridostatin and derivatives. **Pyridostatin** and its derivatives constitute a promising class of selective DNA-damaging anticancer agents. Designed as G4-specific binders, they incorporate structural features that promote π - π stacking, and cation coordination. This class of molecules induces telomere dysfunction, displays strong selectivity for G4s over duplex DNA, and disrupts transcription at G4-rich oncogenes.⁵¹

Phenanthrolines. The phenanthroline scaffold provides an excellent platform for G4 recognition. The benchmark compound **Phen-DC3** shows strong selectivity over duplex DNA, and the ability to inhibit helicase-mediated G4 unwinding.⁵²

Metal complexes. The coordination of metal ions with organic ligands (such as phenanthrolines, porphyrins, and other heterocycles) provides versatile and powerful platforms for G4 recognition. Their binding properties benefit from tunable geometry, charge, and unique photophysical features conferred by the metal center.⁴¹ Because metal complexes represent a central focus of this thesis, a dedicated section will later provide a more detailed discussion of their structural features and therapeutic potential (see Section 1.4.3).

The chemical structure of the most relevant organic G4 binders, cited in this section, is reported below (Figure 5).

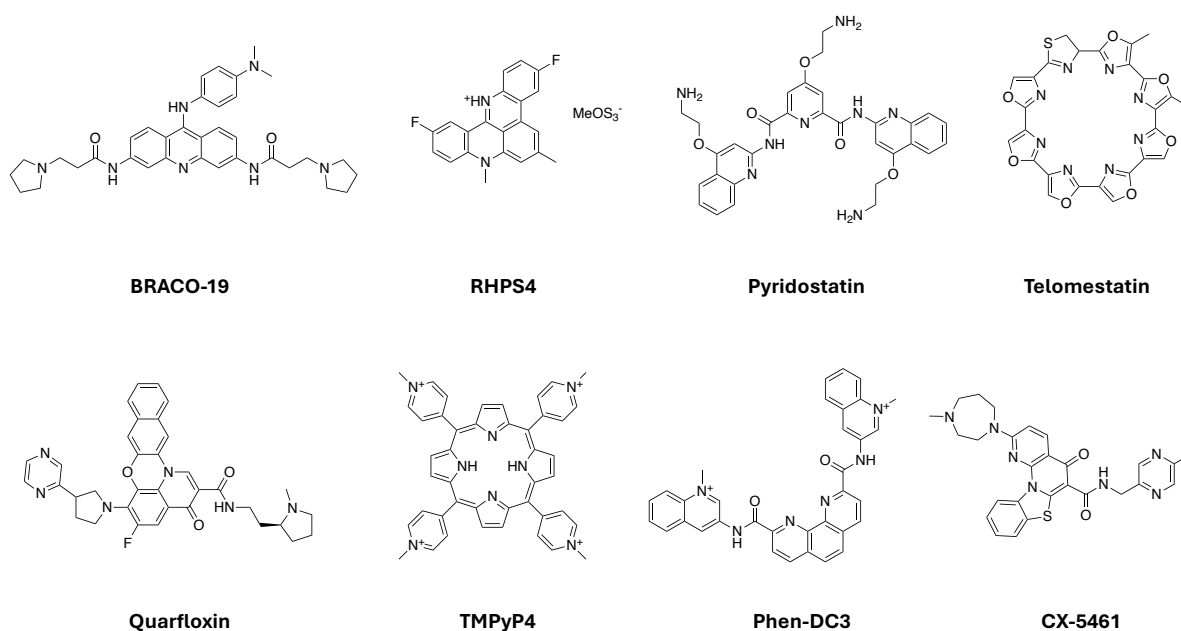


Figure 5. Chemical structure of the most common organic G4 binders.

1.2.4 Methods: G-Quadruplex Structure & Small Molecule Binding

A variety of biophysical methods are available for characterizing G4 structures and their interactions with binders. This section focuses on the most widely applied experimental approaches, which typically employ synthetic oligodeoxyribonucleotides (ODNs) of ~20 nucleotides. Before analysis, ODNs are usually pre-annealed in the appropriate buffer: slow cooling favors the formation of thermodynamically stable conformations, whereas rapid cooling promotes kinetically trapped structures.

Ultraviolet-visible (UV-Vis) spectroscopy. In G4s, far-UV absorbance (~190 nm) arises from the sugar-phosphate backbone, while the 200-300 nm region corresponds to $\pi \rightarrow \pi^*$ electronic transitions of the nitrogenous bases.⁵³ Guanosine shows peaks at 245 and 275 nm, and formation/dissociation of intramolecular G-quartets is sensitively monitored by absorbance changes at 295 nm.⁵⁴

An *isothermal differential spectrum* (IDS) is obtained by subtracting the spectrum of an ODN in the absence of stabilizing cations from that recorded in their presence, while a *thermal differential spectra* (TDS) results from subtracting spectra at high (unfolded) and low (folded) temperatures. With both methods G4s' spectra typically show a negative band near 295 nm, but since this feature can also appear in other structures, the overall spectral shape and ion dependence should be considered.

UV-melting experiments monitor absorbance changes upon heating, enabling determination of the melting temperature (T_m), defined as the temperature at which 50% of the structure is unfolded, commonly tracked at 295 nm for G4s. Because other nucleic acid structures can also produce transitions at this wavelength, complementary analyses are often required.⁵⁵

Beyond structure, UV-Vis spectroscopy also provides insight into the DNA-binding of small molecules. Shifts in a binder's absorption band provide qualitative evidence of interaction, while DNA-binder titrations with proper fitting yield stoichiometry and binding constants.³⁹

Circular dichroism (CD) spectroscopy. CD arises from the differential absorption of left- and right-handed circularly polarized light by a chiral molecule. When such light interacts with a chiral system, its left- and right-handed components are absorbed to different extents, reflecting the intrinsic molecular asymmetry. CD can be described in terms of the difference in molar extinction coefficients between the two polarization components ($\Delta\epsilon = \epsilon_L - \epsilon_R$ [$M^{-1} \text{ cm}^{-1}$]).^{56,57} Nucleic acids, including G4s, are chiral molecules because of their sugar-phosphate backbone and base stacking geometry. CD spectroscopy is a convenient tool to distinguish G4 conformations, as each topology exhibits characteristic spectral signatures. While parallel G4s typically display a positive maximum around 264 nm and a negative minimum near 245 nm, antiparallel G4s show a strong positive band near 295

nm accompanied by a negative one around 260 nm. Hybrid G4s exhibit mixed features, with positive peaks near both 295 nm and 260 nm, and a negative minimum around 245 nm. Similarly to UV-melting experiments, G4 thermal denaturation can also be monitored using CD spectroscopy.⁵⁸

In studying G4-binder interactions, CD is particularly useful for detecting binder-induced conformational switches, although such findings should be validated with complementary methods to avoid misinterpretation. An important feature is the induced CD signal (ICD), which arises when achiral binders interact with G4s, providing qualitative insights into binding mode. As an example, groove binders often yield strong ICD signals, while end-stackers show weak or no ICD. Compared to high-resolution techniques such as nuclear magnetic resonance (NMR) or X-ray crystallography, CD offers a faster, lower-cost, and solution-based approach that requires minimal sample amounts, making it particularly useful for routine studies.⁵⁹

NMR. By detecting the chemical shifts of imino protons involved in hydrogen bonding, NMR readily confirms G4 formation and distinguishes it from Watson–Crick base pairs. Characteristic signals in the 10-12 ppm region reveal G-quartets, offering insights into the number of stacked tetrads and the degree of structural polymorphism, though interpretation may be complicated by heterogeneity. Beyond simple confirmation, NMR provides detailed information on loop conformations, ion coordination, and small molecule binding modes, allowing the identification of end-stacking, groove binding, or intercalation events. While highly informative, this technique requires high sample concentrations and is not suited for high-throughput studies, but it remains the gold standard for in-depth structural characterization of G4s and G4-binder complexes.

Single-crystal X-ray crystallography. X-ray remains a key method for resolving the three-dimensional architecture of G4s, providing atomic-level insights into folding patterns, cation coordination, loop conformations, and binding modes. It has been particularly valuable in defining how small molecules interact with G-quartets or grooves, thereby supporting structure-based drug design. While crystallography captures static snapshots rather than the full dynamic nature of G4s, the accumulation of multiple crystal models builds a composite picture of their conformational diversity and binder interactions, making it an indispensable tool for validating binding modes and guiding binder optimization.^{59,60}

Numerous high-resolution G4 structures, both free and bound by small molecules, obtained either by NMR or X-ray, are available in the Protein Data Bank (PDB).^{61–66}

Fluorescence methods. Monchaud *et al.* developed the *G4-FID* assay, which monitors displacement of a fluorescent probe from DNA, offers a simple and cost-effective means to assess binder affinity and selectivity over duplex DNA. A decrease in fluorescence signals successful binding, though the method is limited to qualitative screening.⁶⁷

FRET (fluorescence resonance energy transfer) is a phenomenon in which energy is transferred from a donor to an acceptor fluorophore through dipole-dipole interactions. Because this transfer depends on the distance between the two fluorophores, changes in molecular conformation can be monitored as variations in FRET efficiency. The *FRET-melting assay* is a widely used, high-throughput method to evaluate G4 stabilization by binders. It uses oligonucleotides labeled with donor and acceptor fluorophores at opposite ends: when the G4 is folded, efficient FRET occurs, whereas melting separates the fluorophores, reducing FRET. Binder-induced shifts in melting temperature (ΔT_m) provide a measure of G4 stabilization or destabilization. The method is fast and cost-effective for comparing binder affinity and selectivity against duplex DNA, though it yields only indirect information.⁶⁸

Surface Plasmon Resonance (SPR) is a label-free, highly sensitive technique that combines optics and microfluidics to monitor G4-binder interactions in real time. By immobilizing a biotinylated G4 sequence on a sensor surface and flowing binders through a microfluidic system, changes in surface refractive index reveal kinetic and equilibrium parameters, yielding dissociation constants and binding curves. SPR requires only small sample amounts but is costly, technically demanding, and sensitive to immobilization conditions, which may perturb G4 conformation.⁶⁹

Isothermal Titration Calorimetry (ITC) and **Differential Scanning Calorimetry (DSC)** are calorimetric methods that provide a thermodynamic picture of G4-binder interactions, helping to distinguish enthalpy- versus entropy-driven binding processes. ITC directly measures the heat released or absorbed upon binding of small molecules to a G4. It provides quantitative thermodynamic parameters such as binding constants (K_a), stoichiometry, enthalpy (ΔH), and entropy (ΔS). ITC is label-free and does not require structural assumptions, making it powerful for studying the driving forces of binding.

DSC measures heat changes during the thermal denaturation of G4s in the presence or absence of binders. It yields information about stability, melting temperature (T_m), and the thermodynamics of unfolding.³⁹

Mass spectrometry (MS). Electrospray ionization MS (*ESI-MS*) is a powerful method to study G4-binder interactions because it preserves non-covalent complexes in the gas phase. It requires minimal sample and yields information on G4 formation, binding stoichiometry, and affinity. Monitoring potassium ion binding provides insights into the number of G-quartets and conformational changes upon small molecule binding. Furthermore, *MS-melting* experiments enable evaluation of thermal stability and thermodynamics of G4-binder complexes, offering a complementary approach to spectroscopic methods.⁷⁰

1.3 Mitochondrial Function and Genome in Cancer

Building on the previous section, G4s are increasingly recognized as regulatory elements in nuclear processes, yet they are not confined to nuclear DNA. G4 motifs have also been identified within mitochondrial DNA, linking these non-canonical structures to mitochondrial function. To place this in context, this section provides an overview of mitochondria and their genetic system, before focusing on mitochondrial G4s.

1.3.1 Mitochondria: Overview of Structure and Functions

Within a single eukaryotic cell, there are typically hundreds to thousands of mitochondria.⁷¹ Although often described as the ‘powerhouse of the cell,’ these intracellular organelles are now recognized as multifunctional and highly dynamic structures. Mitochondria continually remodel their morphology and abundance, and in addition to generating metabolic energy, they play essential roles in metabolic regulation and signal transduction.⁷²

Mitochondria are enclosed by two distinct membranes, the outer membrane and the inner membrane, which define two separate compartments: the intermembrane space and the matrix. The inner membrane folds into structures called cristae, which greatly increase its surface area (Figure 6). These electron carriers subsequently donate their electrons to the respiratory chain complexes embedded in the inner mitochondrial membrane. The electrons flow through this transport system to molecular oxygen, which acts as the final electron acceptor and is reduced to water. This transfer of electrons drives proton translocation across the inner membrane, generating an electrochemical gradient that powers oxidative phosphorylation (OXPHOS), the primary process responsible for ATP synthesis in eukaryotic cells. While the outer membrane is relatively permeable via porin channels, supporting metabolite exchange and lipid metabolism, the inner membrane is impermeable to ions and small molecules, preserving the proton gradient essential for ATP synthesis.³

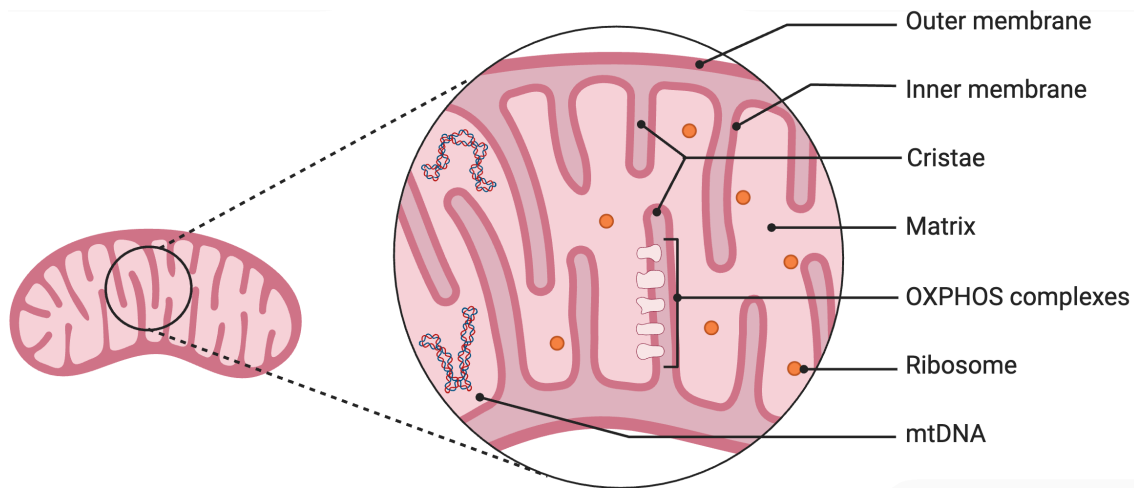


Figure 6. Schematic representation of mitochondrial structure. The mitochondrion is enclosed by a double membrane: the outer membrane and the extensively folded inner membrane, which forms cristae. Created with BioRender.com.

The OXPHOS system includes five different complexes, from Complex I to Complex V (Figure 7). Complex I (NADH:Ubiquinone Oxidoreductase) serves as the primary entry point for electrons derived from NADH, which in turn acquires them from cellular catabolic reactions. Electrons are transferred sequentially from NADH to flavin mononucleotide (FMN) and then through a series of seven iron–sulfur (Fe-S) clusters within Complex I, before reaching ubiquinone (CoQ), which is reduced to ubiquinol (CoQH₂). Ubiquinol then diffuses through the inner mitochondrial membrane to Complex III. During this process, conformational changes within Complex I facilitate the translocation of four protons from the matrix to the intermembrane space, contributing to the generation of the proton gradient. Complex II (Succinate Dehydrogenase) provides an alternative entry point for electrons by oxidizing succinate to fumarate in the TCA cycle. This enzyme transfers electrons from FADH₂ to ubiquinone, which is subsequently reduced to ubiquinol and delivers the electrons to Complex III. Unlike Complex I, however, Complex II does not pump protons and therefore does not directly contribute to the generation of the proton gradient. Complex III (cytochrome c reductase) facilitates the transfer of electrons from ubiquinol to cytochrome c through the sequential action of cytochromes b and c₁. This redox process is coupled to the translocation of two protons across the inner mitochondrial membrane per electron pair, contributing to the proton gradient essential for ATP synthesis. While ubiquinol donates two electrons, cytochrome c can accept only one at a time. To accommodate this mismatch, cytochrome c reductase functions as a dimer, allowing simultaneous interaction with two cytochrome c molecules. Complex IV (Cytochrome c Oxidase) accepts electrons from cytochrome c and uses them to reduce molecular oxygen (O₂) to water (H₂O). During this process, four additional protons are pumped into the intermembrane space, playing a significant role in maintaining the proton-motive force.⁷³

Electron transfer through the respiratory complexes is tightly coupled to proton translocation across the inner mitochondrial membrane, generating a substantial electrochemical potential ($\Delta\psi_m$) of approximately 150-200 mV. The latter provides the driving force for ATP synthase (Complex V), a rotary, turbine-like enzyme that catalyzes the synthesis of ATP from ADP and inorganic phosphate.⁷⁴

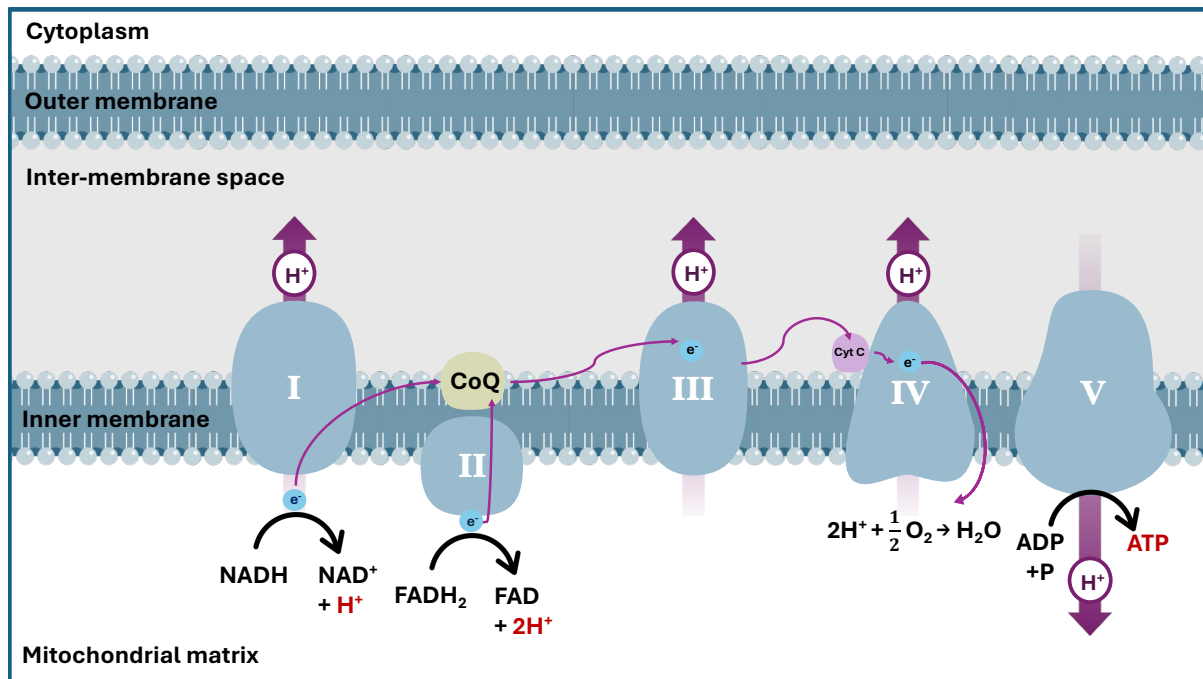


Figure 7. Representative diagram of the mitochondrial electron transport chain and ATP synthesis via oxidative phosphorylation. Complexes I, III, and IV translocate protons into the intermembrane space, generating a proton gradient used by ATP synthase (Complex V) to convert ADP and inorganic phosphate into ATP.

The mitochondrial membrane potential serves functions that extend well beyond ATP production. Indeed, it is essential for maintaining ion homeostasis, generating ROS, importing nuclear-encoded proteins, and enabling mitochondrial fusion. Owing to its central role in these processes, $\Delta\psi_m$ is a sensitive indicator of mitochondrial integrity and overall cellular health.⁷⁵

Calcium homeostasis in mitochondria is closely connected to metabolism and cell signaling, as calcium acts as a second messenger and its concentration directly affects ATP production. Additionally, calcium overload can trigger pro-apoptotic signals.⁷⁶

Within the cell, ROS are primarily generated in mitochondria during oxidative metabolism, where molecular oxygen undergoes a one-electron reduction to form superoxide ($O_2^{\cdot-}$). This radical is subsequently converted into hydrogen peroxide (H_2O_2) by superoxide dismutases located in both the mitochondrial matrix and the cytosol. Complexes I and II produce ROS exclusively within the matrix, whereas Complex III is capable of releasing ROS to both sides of the inner mitochondrial membrane, thereby facilitating their diffusion into the cytosol and enabling their involvement in redox signaling.

Indeed, ROS play crucial roles in the regulation of transcription factors, cell proliferation and differentiation, metabolic adaptation, immune and inflammatory signaling, cell death and survival, as well as the redox-based modulation of protein activity.⁷⁷

Apoptosis is a form of programmed cell death that enables the orderly disassembly of cellular components. Mammalian cells activate apoptosis through two main signaling pathways: the intrinsic and extrinsic pathways. Mitochondria have a central role in the regulation of the intrinsic pathway.⁷⁸ Various stimuli, such as elevated calcium levels, disruption of membrane potential or oxidative stress, can increase the permeability mitochondrial outer membrane, an event called “mitochondrial permeability transition” (MPT), which is regulated by Bcl-2 family proteins. Consequently, proteins like cytochrome c and apoptosis-inducing factors (AIF) are released from the intermembrane space to the cytosol. When cytochrome c is released in the cytosol, it binds to Apaf-1 to form the *apoptosome* protein complex. The latter eventually induces the caspase cascade, a sequence of enzymatic reactions that culminates in cell death.⁷⁹ AIF, on the other hand, can induce apoptosis independently of caspases by translocating to the nucleus, where it promotes chromatin condensation and DNA fragmentation.⁸⁰

Mitochondria constantly undergo dynamic changes in both number and morphology through the processes of fusion and fission. This phenomenon, known as mitochondrial dynamics, is essential for maintaining a healthy mitochondrial population within the cell and for ensuring optimal OXPHOS activity. Particularly, fission is necessary for proper mitochondrial distribution during cell division and for maintaining mitochondrial numbers, while fusion promotes mitochondrial functionality by facilitating the mixing of mitochondrial components.⁸¹ Remarkably, this dynamic behavior enables mitochondria to rapidly adapt to changing cellular demands and environmental stressors.⁸²

To accomplish their functions, mitochondria rely also on their own multi-copy genome (mtDNA), a small circular DNA molecule distinct from nuclear DNA. Its structure and functions, and their links to cancer, will be discussed in detail in the following sections.

1.3.2 Structure and Functions of mtDNA

In humans, mtDNA constitutes ~1% of total cellular DNA, as each cell contains about 1000 to 10,000 copies.⁸³ Human mtDNA was sequenced in 1981 and consists of a 16.5-kb circular, supercoiled, double-stranded DNA (dsDNA) without introns, located in the mitochondrial matrix. It encodes 13 essential subunits of the OXPHOS system, as well as 22 tRNAs and two rRNAs required for their translation (Figure 8). The remaining OXPHOS subunits (77 in humans), along with ~1,500 additional mitochondrial proteins, including those responsible for maintaining and expressing mtDNA, are encoded in the nucleus. Consequently, proper mitochondrial function depends on tight

coordination and communication between mitochondrial and nuclear gene expression to sustain oxidative ATP production and organelle homeostasis.

The two strands are distinguished as the heavy (H) strand, rich in purine bases and carrying most of the genetic information, and the light (L) strand, rich in pyrimidines.⁸⁴

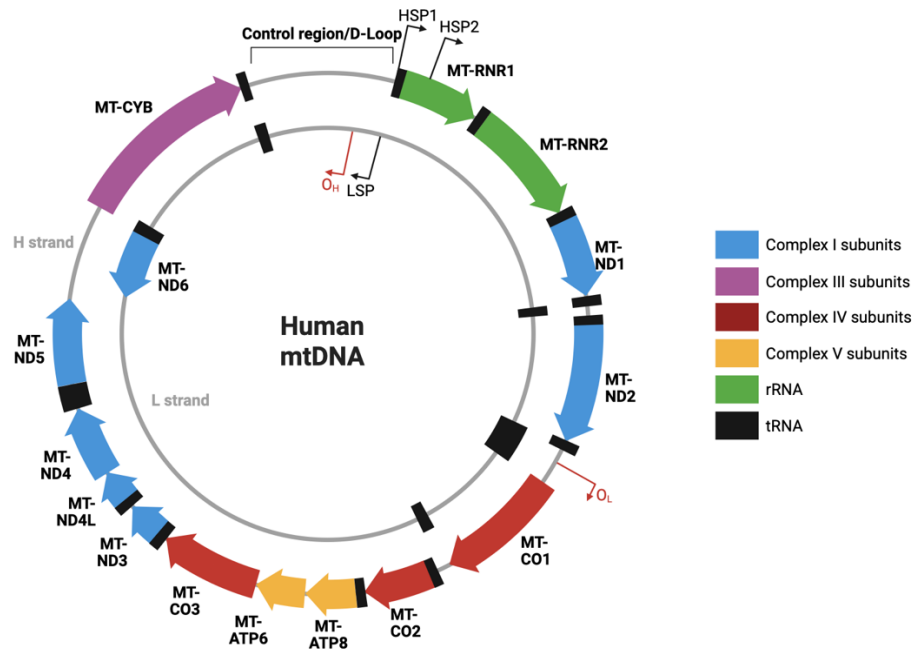


Figure 8. Map of the human mitochondrial genome showing the location of the 37 genes. The outer circle corresponds to the H-strand, while the inner circle represents the L-strand. Red bent arrows mark the origins of replication for the H-strand (O_H) and L-strand (O_L) and indicate the direction of DNA synthesis. Black bent arrows denote transcription initiation sites (HSP1, HSP2, and LSP) and the direction of RNA synthesis. Created with BioRender.com.

Unlike nuclear DNA, mtDNA is polycistronic: both the strands are transcribed as long continuous RNA molecules that contain multiple genes. These primary transcripts are then precisely processed to release individual rRNAs, tRNAs, and mRNAs. Both strands initiate in the D-loop, the major noncoding control region (HSP1/HSP2 on the H-strand; LSP on the L-strand), which also contains the H-strand replication origin and other elements regulating replication and transcription. LSP-derived transcripts also prime H-strand replication, so mitochondrial transcription simultaneously produces RNAs for gene expression and the primers/signals required for mtDNA maintenance. This compact, shared-control architecture coordinates expression but increases vulnerability to damage.⁸⁵ mtDNA continuously replicate across the cell cycle and is governed by an elaborate molecular machinery. According to the prevailing hypothesis, replication initiates at two strand-specific origins (O_H and O_L) and proceeds unidirectionally. Initiation occurs with RNA primers and extends new DNA strands using a dedicated mtDNA polymerase with proofreading activity. Helicases, topoisomerases, and single-stranded DNA-binding proteins assist in unwinding and stabilizing the template. Once

strand synthesis is complete, RNA primers are removed, ends are processed and ligated, and the two daughter molecules are separated.⁸⁶

Maintaining mtDNA integrity is essential for mitochondrial and respiratory chain function, relying on tightly regulated processes of replication, transmission, and repair. Unlike nuclear DNA, mtDNA lacks histone protection and lies close to the respiratory chain, where ROS can induce lesions such as oxidized bases and strand breaks. Although several repair enzymes encoded by nuclear genes have been identified, the mutation rate of the mitochondrial genome remains 10-20 times higher than that of nuclear DNA. mtDNA abnormalities account for ~20% of so-called ‘mitochondrial diseases’. Pathogenic variants include point mutations, deletions, insertions, and depletion, and often arise from defects in nuclear genes that regulate mtDNA maintenance.⁸⁷

1.3.3 Role of Mitochondria in Cancer Cells

Mitochondria contribute to cancer initiation, progression, and metastasis through diverse processes including mitochondrial biogenesis and turnover, mitochondrial dynamics, control of cell death, metabolism and bioenergetics, redox balance, signaling, and mtDNA homeostasis.

Biogenesis and turnover. Mitochondrial mass is controlled by biogenesis (new mitochondria production) and turnover (degradation, mainly via mitophagy). Both can act as positive or negative regulators of tumorigenesis depending on tumor type, stage, and microenvironment.⁸⁸

Dynamics. Fusion and fission are fundamental for maintaining mitochondrial function, and their dysregulation can drive cancer by disrupting mtDNA stability, energy metabolism, and apoptotic pathways.⁸⁹

Cell death control. Evasion of apoptosis is a hallmark of cancer, frequently achieved by suppressing the intrinsic mitochondrial pathway. Tumor cells may upregulate anti-apoptotic BCL-2 family proteins, inactivate or silence pro-apoptotic counterparts, or inhibit caspase activity downstream of mitochondrial permeabilization, thereby sustaining survival even under oncogenic or therapeutic stress.⁹⁰ In addition, dysregulation of ferroptosis, pyroptosis, and necroptosis, which are distinct regulated cell death pathways linked to mitochondria, can also contribute to malignant cell survival.⁹¹

Metabolic reprogramming. Another hallmark of cancer is the metabolic reprogramming, which supports macromolecule synthesis, meets bioenergetic demands, and promotes cell survival. The Warburg effect refers to cancer cells’ reliance on glycolysis over oxidative phosphorylation, even in the presence of oxygen. Although energetically less efficient, this reprogramming sustains rapid

proliferation by enhancing glucose uptake (e.g., via GLUT1) and channeling glycolytic intermediates into biosynthetic pathways.⁹² This initially led to the misconception that tumors rely on glycolysis due to defective respiration. However, many cancer cells retain functional mitochondria and can oxidize glucose through OXPHOS. Indeed, inhibition of glycolysis does not prevent tumorigenesis, and some tumors even enhance mitochondrial respiration when glycolysis is impaired. Overall, most cancers rely on a flexible combination of glycolysis and OXPHOS.⁹³ A key element of this flexibility comes from the TCA cycle, which serves as both an energy generator and a biosynthetic hub. In cancer, the TCA cycle is remodeled to support rapid proliferation, providing intermediates for nucleotide, amino acid, and lipid biosynthesis. Tumors often fuel the TCA cycle with alternative substrates like glutamine or fatty acids, ensuring sustained OXPHOS and metabolic plasticity under nutrient stress.⁹⁴

Redox balance. Mitochondria are a major source of ROS. While excessive ROS can damage DNA, proteins, and lipids, driving genomic instability, modest elevations act as signaling molecules that promote tumorigenesis. To survive elevated oxidative stress, cancer cells adapt by upregulating antioxidant defenses such as glutathione and thioredoxin. This creates a “ROS window” where levels are high enough to drive proliferation and signaling but buffered against cytotoxicity. Importantly, antioxidants have been shown to paradoxically enhance metastasis in melanoma models, underscoring the tumor-promoting role of finely tuned redox balance.⁹⁵

Cell signaling. A growing body of evidence indicates that classical oncogenes and tumor suppressors directly regulate mitochondrial biology, reshaping their functions to sustain tumor growth and survival. *MYC* promotes mitochondrial biogenesis and metabolism by upregulating nuclear-encoded mitochondrial genes,⁹⁶ while *RAS* rewires respiration and mitophagy to maintain proliferation under nutrient stress, ensuring metabolic flexibility within the tumor microenvironment.⁹⁷ The PI3K/Akt/mTOR pathway, frequently activated in cancers, enhances glucose uptake and glycolysis but also boosts mitochondrial oxidative metabolism, reinforcing both energy production and biosynthesis. Conversely, tumor suppressors also exert critical control over mitochondrial signaling.⁹⁸ *TP53* coordinates stress responses by regulating both nuclear and mitochondrial genes; it can enhance respiration by promoting the expression of factors required for respiratory chain assembly, but under stress it shifts toward pro-apoptotic functions by interacting with BCL-2 family proteins at the mitochondrial outer membrane.⁹⁹ Similarly, HIF-1 α , stabilized in hypoxia, reprograms mitochondrial metabolism by suppressing oxidative phosphorylation and favoring glycolysis, while modulating mitochondrial dynamics to adapt to fluctuating oxygen levels.¹⁰⁰

mtDNA homeostasis. The mitochondrial genome plays a multifaceted role in cancer, where both mtDNA mutations and copy number variations contribute to tumorigenesis. Somatic mtDNA mutations are common across many cancers and can enhance tumorigenic potential by altering ROS production, metabolism, and signaling. Some mutations directly promote cancer initiation, while others provide adaptive advantages that help tumors withstand stress. A recent large-scale analysis of mtDNA mutations revealed that in some cancers, mtDNA variation occurs mutually exclusively with nuclear driver mutations. In ovarian adenocarcinoma, for instance, ~40% of tumors carry only nuclear mutations, ~60% only mtDNA mutations, and very few harbor both. This suggests that tumors can fulfill distinct bioenergetic needs through either nuclear or mitochondrial DNA alterations, including changes in mtDNA copy number.¹⁰¹ Copy number changes also occur in a tumor- and context-dependent manner: decreases are observed in cancers such as bladder, breast, and liver, while increases are seen in lung adenocarcinoma and gliomas. These variations often correlate with cancer aggressiveness and patient survival, reflecting metabolic adaptation.

The balance between mtDNA damage and repair is central to cancer biology: while damage drives genomic instability and metabolic rewiring, tumors can adapt by upregulating repair or acquiring healthy mtDNA through mitochondrial transfer. This duality makes mtDNA both a driver of malignancy and a promising therapeutic target, as well as a potential biomarker for disease progression.^{102,103}

1.3.4 Mitochondrial Targeting in Cancer Treatment

As presented in the previous section, mitochondria orchestrate processes essential to tumorigenesis, making them compelling therapeutic targets. Moreover, growing evidence shows that directing drugs to specific organelles, including mitochondria, can substantially enhance anticancer efficacy.¹⁰⁴ Lipophilic cations are often used to deliver bioactive molecules into mitochondria, as exemplified by triphenylphosphonium (TPP). The plasma membrane maintains a potential of 30-60 mV relative to the extracellular space, leading to a 5-10-fold accumulation of TPP in the cytosol. By contrast, the mitochondrial membrane potential is much higher, around 150-180 mV, driving an additional 100-1000-fold enrichment of TPP in the matrix. Notably, in cancer cells, the mitochondrial membrane potential is typically ~60 mV more hyperpolarized than in normal cells, further enhancing the selective accumulation of lipophilic cations within tumor mitochondria (Figure 9).¹⁰⁵

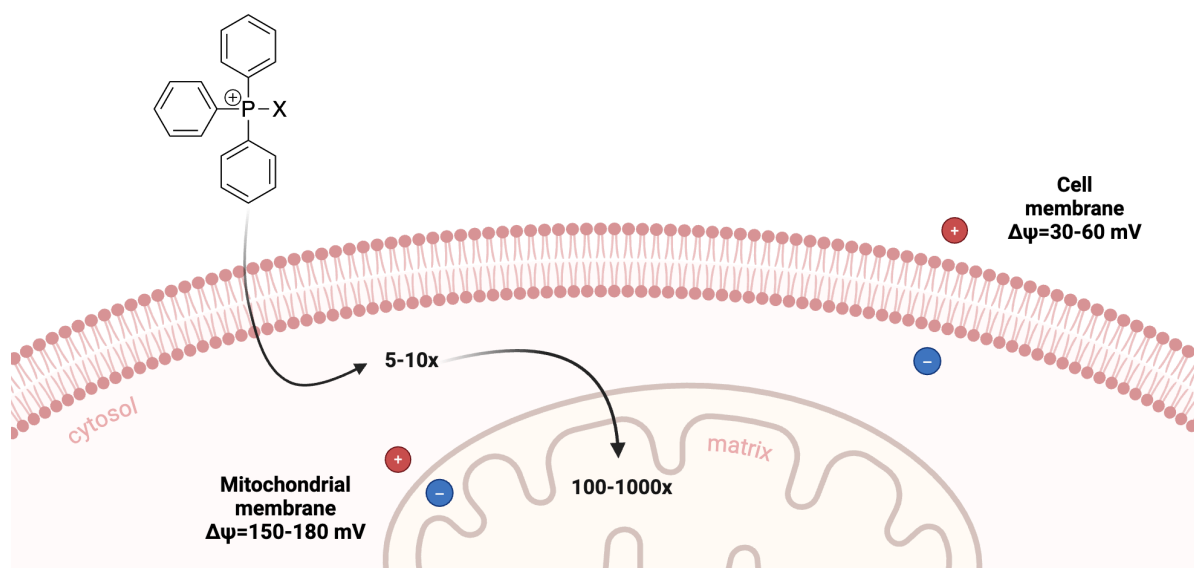


Figure 9. Schematic representation of cellular uptake of TPP-conjugated compounds, driven by plasma and mitochondrial membrane potentials. The high mitochondrial membrane potential enables rapid and preferential accumulation of TPP-containing molecules within mitochondria. Created with BioRender.com.

Various strategies can exploit the mitochondrial vulnerabilities in cancer. For instance, several cancers (e.g., breast cancer and classical Hodgkin lymphoma) display high OXPHOS activity, which contributes to therapy resistance and tumor repopulation. In such cases, OXPHOS inhibition has emerged as a promising strategy. Tumor regions frequently become hypoxic due to abnormal vasculature and high metabolic demand, driving aggressive progression and treatment resistance. By reducing oxygen consumption rate (OCR, the rate at which cells use oxygen to generate ATP), OXPHOS inhibition increases oxygen availability and promotes diffusion into previously hypoxic areas. Remarkably, even a modest OCR reduction (~30%) can alleviate severe hypoxia. This strategy is broadly applicable across tumor types, as it enhances intratumoral oxygen diffusion without requiring direct drug penetration into the hypoxic core. **Metformin**, commonly used for treating type 2 diabetes, is an OXPHOS inhibitor targeting Complex I. It reduces OCR in cancer cells and, in vivo, suppresses tumor growth while alleviating hypoxia and enhancing radiosensitivity. These effects have led to its evaluation in numerous clinical trials as a potential anticancer therapy.¹⁰⁶

Another strategy is to target mitochondrial apoptosis by altering membrane potential, triggering cytochrome c release, and modulating BCL-2 family proteins. A key advance is **venetoclax**, an FDA-approved BCL-2 inhibitor that has shown remarkable success in blood cancers driven by BCL-2 dependency. However, its effectiveness as a single agent in solid tumors is limited, partly due to resistance mechanisms. Current research is focused on combining **venetoclax** with other treatments to enhance its therapeutic benefit.¹⁰⁷

Mitochondrial metabolism represents a promising target in cancer therapy, often through disruption of pathways such as aerobic glycolysis. One example is **BAY-876**, a highly selective inhibitor of GLUT1, a glucose transporter frequently overexpressed in cancer cells to sustain elevated glucose uptake and support rapid proliferation. **BAY-876** has demonstrated tumor growth inhibition in preclinical models. However, the metabolic plasticity of cancer cells enables them to rewire pathways and develop resistance, highlighting the potential advantages of combination strategies or multi-target approaches over single-agent therapies.¹⁰⁸

Finally, recent evidence highlights mtDNA as a therapeutic vulnerability. Such approaches include pharmacological agents like nucleoside analogs (e.g. 2'3'-dideoxycytidine, alovudine) that block mtDNA replication, suppress cancer stem cells, and limit metastasis.^{109,110}

1.3.5 Mitochondrial G4s: Evidence, Potential Functions, and Relevance in Cancer

Since their discovery, G4 structures have been studied primarily in the nuclear genome. However, only over the past decade, an increasing body of evidence has demonstrated that they can also form in mtDNA.

Remarkably, a bioinformatic and experimental study identified ~200 potential PQS in mtDNA, and demonstrated that 71 of them can fold into stable G4 structures in vitro.²⁴ Due to the unique environment of the mitochondrial matrix, G4 structures are more likely to form in mtDNA than in the nuclear genome. The mitochondrial H-strand, for example, is enriched in guanine sequences.⁸⁴ Moreover, because mtDNA replication is relatively slow (~2 hours), the single-stranded G-rich H-strand remains exposed for extended periods, increasing the likelihood of G4 formation.¹¹¹ Finally, high potassium concentrations within mitochondria (~150 mM),¹¹² together with macromolecular crowding in the matrix, further promote G4 stability, as shown by both in vitro and cell-based studies.¹¹³

Although many questions remain, several hypotheses have been proposed regarding the potential roles of mitochondrial G4s. In 2021 Chen *et al.* demonstrated that mtDNA G4 folding occurs more frequently in cancer cells and is closely associated with enhanced glycolysis, highlighting mtDNA G4s as promising novel targets for anticancer therapy.¹¹⁴ Similar to nuclear G4s, mitochondrial G4 motifs have been associated with mtDNA deletion breakpoints. In cancer, such deletions occur across many tumor types, and their proximity to G4 motifs suggests a role in tumorigenesis and highlights their potential as biomarkers.¹¹⁵ Remarkably, recent studies have mapped mitochondrial G4s in cells using a mitochondrially targeted BG4 antibody, a well-established G4 probe, combined with chromatin immunoprecipitation sequencing (ChIP-seq). This approach showed that G4s form

transiently under normal conditions but become stabilized upon treatment with G4 binders (e.g., RHPS4). Their accumulation leads to rapid mtDNA loss, indicating that elevated G4 levels hinder replication fork progression and ultimately drive genomic instability.¹¹⁶

Another study showed that, at low concentrations, RHPS4 preferentially localizes to mitochondria, where it binds G4 structures and disrupts transcription, replication, and respiratory complex abundance, independently of nuclear DNA damage.¹¹⁷

Evidence suggests mitochondrial G4s also serve physiological roles. For example, at conserved sequence block II (CSBII), G4 formation regulates the transition from transcription to replication by mediating RNA priming, a process essential for proper mtDNA replication initiation.¹¹⁸ On the other hand, resolution of G4s is crucial for maintaining mitochondrial integrity. Helicases such as PIF1, DNA2, and other G4-resolving enzymes have been shown to mitigate G4 accumulation, and their dysfunction is associated with increased mtDNA deletions.¹¹⁹

Together, these findings present mitochondrial G4s as a double-edged sword: they may act as regulatory structures fine-tuning replication and transcription, but when unresolved or stabilized, they contribute to replication stress, deletions, and disease. This duality underscores their emerging relevance as both biomarkers of mitochondrial instability and potential therapeutic targets, with G4 binders or G4-resolving proteins offering new strategies to manipulate mitochondrial function in cancer and other pathologies.¹²⁰

1.4 Metal-based Drugs and Biomedical Applications

This section now turns to metal-based drugs, a cornerstone of oncology and a promising avenue for broadening therapeutic options. The use of metals in medicine has ancient roots, but the modern field emerged with the discovery of cisplatin in 1969, which revolutionized cancer therapy and highlighted the therapeutic potential of inorganic compounds. Since then, medicinal inorganic chemistry has advanced beyond platinum-based drugs to include contrast agents, radionuclides, and transition-metal complexes with distinctive catalytic and photodynamic activities. Today, metals in medicine span applications from oncology and infectious disease to imaging and nanomedicine, offering a broad and versatile platform for innovation.¹²¹

Coordination chemistry provides an unusually rich design space: metal centers enforce diverse geometries (e.g., linear, square-planar, octahedral), present multiple oxidation states, and bind a wide range of ligands. Tuning these features modulates thermodynamic and kinetic behavior, such as reactivity, stability, and photophysics, in biological environments. Many metallodrugs function as prodrugs, activated *in vivo* via ligand exchange, redox reactions, hydrolysis, or light-triggered

processes.¹²² This section examines metal complexes as anticancer drugs/drug candidates and their potential as G4 binders, with a particular focus on ruthenium complexes, a central theme of this thesis.

1.4.1 Therapeutic Applications of Metal Compounds in Oncology

As anticipated, the discovery of cis-diammine-dichloroplatinum(II), commonly known as cisplatin, marked a milestone for metal-based drugs in oncology. Cisplatin was the first metal-based chemotherapeutic to enter clinical use and, despite its significant toxicity, remains a first-line treatment for several malignancies. While its mechanism of action continues to be investigated, it is well established that DNA is a primary target. Once inside the cell, cisplatin undergoes aquation to form reactive species that bind DNA, producing intra- and interstrand crosslinks that distort the double helix. These lesions activate the DNA damage response, resulting in cell cycle arrest and, if unrepaired, induction of the mitochondrial apoptosis pathway.^{123,124}

To address cisplatin's limitations in toxicity and resistance, two derivatives have since been developed and FDA-approved: carboplatin and oxaliplatin (Figure 10). Carboplatin shares cisplatin's mechanism of forming DNA adducts but reacts more slowly, which reduces off-target toxicity and enables higher-dose chemotherapy. It is widely used in ovarian and lung cancers, though its main drawback is bone marrow suppression, and it often displays cross-resistance due to reliance on the same resistance mechanisms as cisplatin. In contrast, oxaliplatin contains a bulky 1,2-diaminocyclohexane ligand and oxalate leaving group, which modify its uptake and DNA binding profile. Although less reactive than cisplatin, oxaliplatin generates DNA distortions that escape mismatch repair recognition, rendering its cytotoxicity mismatch-repair independent. Clinically, it is a key treatment for colorectal cancer and retains efficacy in settings where cisplatin and carboplatin fail. Its major dose-limiting toxicity is peripheral sensory neuropathy, but overall, it offers a better safety profile and reduced cross-resistance compared to earlier platinum agents.¹²⁵

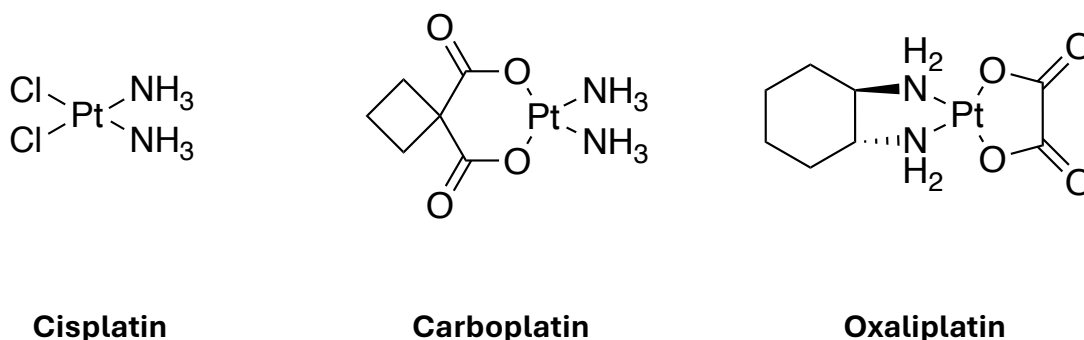


Figure 10. Chemical structure of cisplatin and its derivatives, carboplatin and oxaliplatin.

In recent years, increasing attention has shifted toward metal ions beyond platinum, including ruthenium, gold, palladium, copper, zinc, osmium and others, for their potential in cancer therapy and

diagnosis. Some of these compounds have already entered clinical use, while others show encouraging results in preclinical studies, offering novel mechanisms of action, the ability to overcome platinum resistance, and often a more favorable toxicity profile. Gold(III) complexes, for instance, inhibit thioredoxin reductase, thereby disrupting redox homeostasis and inducing apoptosis, with promising activity reported in liver cancer models. Copper(II) complexes combine DNA binding with the generation of ROS and have demonstrated efficacy even in platinum-resistant tumors, both in vivo and in early translational studies. Iridium-based photosensitizers are another noteworthy class: their cyclometalated complexes efficiently generate cytotoxic singlet oxygen upon light activation, enabling highly precise photodynamic cancer therapies. Organometallic iron compounds, particularly ferrocenyl derivatives of hormone therapies, exploit the redox-active ferrocene moiety to enhance cytotoxicity and expand structure-activity relationships. Finally, radiometals such as ^{177}Lu and ^{223}Ra are already in clinical use as radiotheranostics, while novel supramolecular radiotherapeutics are emerging to further harness coordination chemistry for targeted irradiation and imaging-guided therapy.^{126,127}

1.4.2 Ruthenium Complexes: Properties and Anticancer Potential

Among non-platinum metals, ruthenium complexes are among the most advanced class of metallodrugs, showing highly promising potential as anticancer agents.

The two main oxidation states of ruthenium complexes that are relevant under physiological conditions are Ru(II) and Ru(III). Ru(III) complexes are thermodynamically and kinetically stable and can act as prodrugs, becoming reduced to active Ru(II) species under tumor-associated conditions (hypoxia, acidic pH, and high glutathione levels). In contrast, Ru(II) complexes exert direct cytotoxic effects through multiple mechanisms and possess favorable photophysical and chemical properties, with ligand exchange flexibility. Altogether, ruthenium-based compounds represent valuable scaffolds for the design of next-generation, less toxic, non-platinum anticancer agents.^{128–130}

Ruthenium ions are typically hexacoordinated, adopting an octahedral geometry. When coordinated with π -bonded arenes, however, they form pseudo-octahedral structures known as ‘piano-stool’ complexes. The most widely explored ligand classes in this context include polypyridyl systems, arene ligands, and N-donor heteroaromatics.¹²² Emblematic examples of these scaffolds in the anticancer research are **TLD1433**, **RAPTA-C**, **NAMI-A** and **BOLD-100**, respectively (Figure 11).

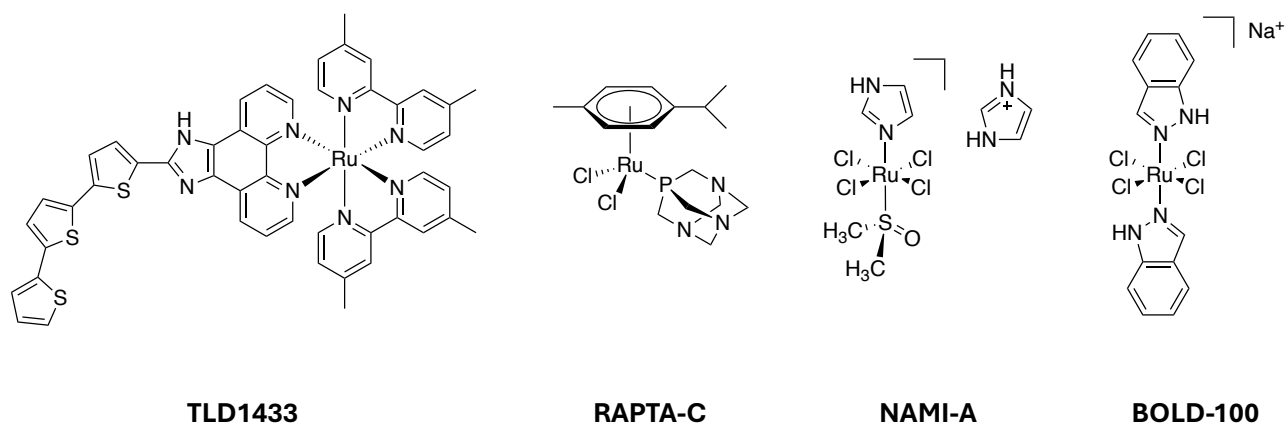


Figure 11. Chemical structure of the ruthenium complexes **TLD1433**, **RAPTA-C**, **NAMI-A** and **BOLD-100**.

Photodynamic therapy (PDT) offers a promising strategy for selectively targeting cancer cells, using light in presence of a photosensitizer and molecular oxygen. Ruthenium complexes are highly suitable for PDT because of their unique photophysical and photochemical properties. Unlike organic photosensitizers, they efficiently access long-lived triplet excited states through strong spin-orbit coupling, which enhances their ability to generate ROS upon light activation. These excited states also allow oxygen-independent pathways, providing cytotoxic activity even under hypoxic tumor conditions. Moreover, ruthenium complexes are synthetically versatile: their ligands can be tuned to adjust absorption wavelengths, excited-state lifetimes, and photoreactivity. A notable example is **TLD1433**, developed by McFarland and colleagues, the first Ru(II)-based photosensitizer to enter human clinical trials, where it has shown strong potential in advancing PDT applications.¹³¹

The RAPTA family of compounds, developed by Dyson's group, is characterized by a piano-stool geometry in which an η^6 -coordinated arene ligand stabilizes the Ru(II) oxidation state. One coordination site is occupied by the amphiphilic ligand PTA (1,3,5-triaza-7-phosphaadamantane), while the remaining two sites are typically filled by labile chlorido ligands. Among this family, the first to be reported was **RAPTA-C**, followed by many more derivatives. Unlike platinum drugs, they show low cytotoxicity in vitro but display strong antimetastatic and antiangiogenic effects in vivo. These effects appear to arise mainly from protein interactions (notably with histones and cytoskeletal proteins) rather than direct DNA damage, leading to altered adhesion, migration, and invasion of cancer cells. In animal models, RAPTA compounds significantly reduce metastasis formation and inhibit tumor vascularization while remaining well tolerated with low systemic toxicity.¹³²

NAMI-A, an imidazole–DMSO ruthenium(III) complex developed in 1992 by Alessio and co-workers, was the first Ru(III) compound tested in humans. In animal models, it displayed antimetastatic rather than cytotoxic activity, acting primarily by enhancing cell adhesion, reducing motility and invasiveness, and inhibiting angiogenesis. Although clinical trials were discontinued due

to limited efficacy, **NAMI-A** remains a landmark compound that significantly advanced the conceptual and mechanistic understanding of non-platinum anticancer metal drugs.^{133,134} Finally, the most prominent ruthenium complex in cancer research is **BOLD-100**, developed by Keppler and colleagues. It represents the first ruthenium-based drug to advance into late-stage clinical trials for cancer therapy. Its precursor, the indazole analogue **KP1019**, showed strong preclinical efficacy but was limited in clinical use by poor solubility.¹³⁴ This challenge was overcome with the development of the sodium salt **BOLD-100**, which offered markedly improved solubility while retaining Ru(III) redox stability in vivo.¹³⁵ **BOLD-100**'s mechanism of action is still under investigation, but evidence shows it binds to serum proteins like albumin, is reduced from Ru(III) to active Ru(II) in hypoxic tumors, and accumulates via the enhanced permeability and retention effect. It induces endoplasmic reticulum stress, promotes apoptosis, generates ROS, and can trigger immunogenic cell death. More recently, it was shown to inhibit glycolysis, reduce lactate release, and alter epigenetic regulation through histone acetylation. Clinically, **BOLD-100** has advanced to phase Ib/IIa trials with **FOLFOX** (a chemotherapy regimen that combines folinic acid, fluorouracil and oxaliplatin) for gastrointestinal cancers, showing good tolerability and early efficacy in heavily pretreated patients.¹³⁶

Importantly, many Ru(II) complexes, particularly those bearing polypyridyl ligands, preferentially accumulate in mitochondria driven by the highly negative membrane potential. Once localized, they can impair mitochondrial function, dissipate membrane potential, promote ROS generation, and activate intrinsic apoptotic pathways.¹³⁷

Given their therapeutic relevance, G4s were previously introduced as promising drug targets in cancer research. The following section revisits this concept with a focus on metal complexes, including ruthenium compounds, which exhibit strong G4-binding properties and thereby further expand their therapeutic potential.

1.4.3 Metal Complexes as G4 Binders

Metal complexes offer diverse structural and electronic features that make them highly versatile as G4 binders. For instance, metal centers can organize ligands into defined geometries, enabling optimal G4 binding and easier generation of compound libraries compared to organic molecules. Furthermore, the electron-withdrawing nature of metal centers enhances π -stacking with G-quartets, while the cationic charge can further stabilize the quadruplex by mimicking alkali metal binding.¹³⁸ Different metal scaffolds have been explored for their G4 affinity, including metalloporphyrins and derivatives, Schiff-base complexes, and polypyridyl complexes.

Metalloporphyrins and derivatives. Metalloporphyrins were the first metal complexes reported to stabilize G4 structures, particularly through insertion of metals such as Cu(II), Ni(II), or Mn(III) into the central cavity of **TMPyP4** (Figure 12a). These complexes remain among the most widely studied G4 binders, interacting primarily via π - π stacking with G-quartets. Their affinity and selectivity can be fine-tuned by altering either the central metal or the peripheral substituents: for example, Mn(III) porphyrins and derivatives with flexible cationic arms demonstrated significantly enhanced G4 selectivity and potent telomerase inhibition in vitro. Phthalocyanines, extended aromatic derivatives of porphyrins, also exhibit strong telomerase inhibition and have been applied in imaging studies.¹³⁹

Schiff-bases complexes. Schiff base metal complexes, particularly those derived from salen, salphen, and salnaphen ligands, have emerged as versatile G4 binders with promising anticancer potential (Figure 12b). Their square-planar geometry and tunable substituents allow strong π - π stacking with G-quartets, complemented by electrostatic interactions from positively charged side chains. Structural modifications, such as extended aromatic groups, cationic arms, or linkers, enhance affinity and selectivity for specific G4 topologies (e.g., telomeric or promoter G4s), while certain complexes also induce G4 topology switching, inhibit telomerase, or repress oncogene expression. Overall, Schiff base complexes are easily synthesized, highly tunable, and combine DNA stabilization with potential imaging or therapeutic functions, making them valuable candidates for G4-targeted anticancer strategies.¹⁴⁰

Polypyridyl complexes. Polypyridyl ligands include bipyridine, terpyridine, phenanthroline and their extended derivatives. Offering large π -surfaces that facilitate stacking interactions, they have emerged as important G4 binders. For instance, square-planar Pt(II)-phenanthroline complexes bearing ligands with extended aromatic surfaces, such as dipyrldophenazine (dppz) significantly enhance both affinity and selectivity for G4 over duplex DNA.¹⁴¹ On the other hand, octahedral metal complexes, particularly Ru(II), Ir(III), and Fe(III) polypyridyl systems, have been extensively investigated as G4 binders. Notably, Ru(II) complexes such as **[Ru(bpy)₂(dppz)]²⁺** and **[Ru(phen)₂(dppz)]²⁺** are classic DNA ‘light switches’: non-luminescent in aqueous media, they become strongly emissive upon binding to dsDNA (Figure 12c).¹⁴² Subsequent studies showed that they also interact with G4 DNA, though with relatively weak affinity and stabilization.^{143,144} However, ligand modifications, including imidazolone or flexible cyclic amine-based side chains in the dppz moiety, significantly enhanced their selectivity and stabilization of G4s, enabling strong telomerase inhibition and inducing antiproliferative effects comparable to cisplatin. These modified Ru(II) polypyridyl complexes also promoted apoptosis in cancer cells, often through mitochondrial dysfunction. Due to their octahedral

geometry, these metal complexes are unlikely to intercalate or end-stack directly onto G-quartets. Instead, it is the aromatic ligands with extended π -delocalized planar surfaces, such as dppz, that engage in end-stacking or intercalation with the G4 structure. Meanwhile, the overall positive charge imparted by the metal center enhances electrostatic interactions with the negatively charged DNA backbone, particularly within the grooves and loops.¹⁴⁵

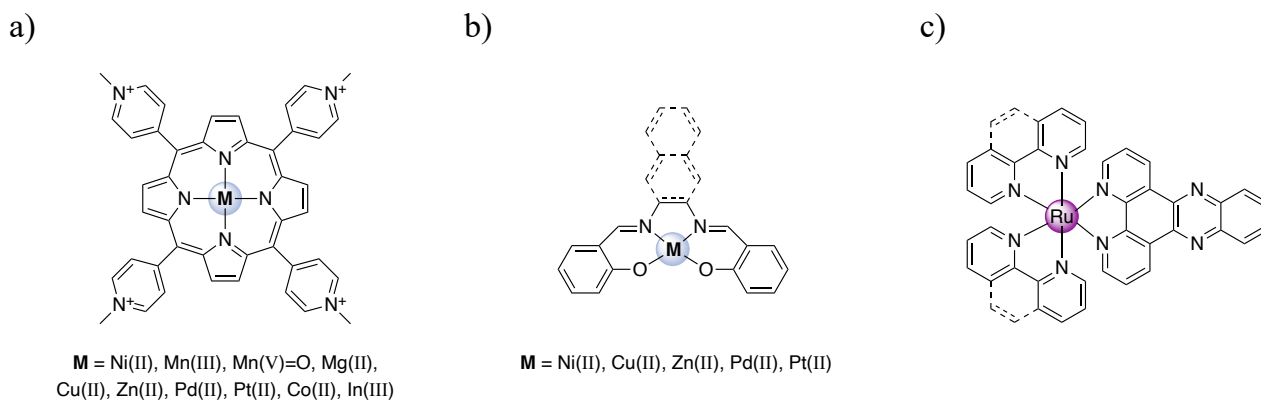


Figure 12. Representative chemical structures of metal complexes with strong G4-binding activity: (a) Metal–TMPyP4 derivatives with variable central metal ions. (b) General frameworks of salen, salphen, and salnaphen-like complexes. (c) Classic Ru(II) polypyridyl ‘light switch’ complexes $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ and $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$.

2. Results

Chapter I: Ruthenium(II) Polypyridyl Complexes as Mitochondrial G-Quadruplex Binders: Synthesis, DNA binding and Biological Evaluation in Cancer Cells

1. Introduction

As detailed in the introduction of this thesis (section 1.3.3), mitochondria are central to malignant metabolism, apoptosis regulation, and therapy resistance, making them attractive targets for precision chemotherapy. Guanine-rich sequences within mtDNA can adopt G4 structures that modulate several mitochondrial functions, including replication and transcription, creating exploitable structural vulnerabilities.¹²⁰ To reach the mitochondrial matrix, small molecules are often conjugated to triphenylphosphonium (TPP), a lipophilic cation that exploits the large mitochondrial membrane potential to achieve selective enrichment in cancer cells.¹⁰⁵

Ruthenium complexes are emerging as promising alternatives to platinum-based anticancer drugs.^{131,136} In particular, Ru(II) polypyridyl complexes combine tunable redox chemistry, reduced toxicity, and versatile ligand design.¹³⁷ Within this framework, these complexes stand out for their ability to bind DNA, with the archetypal “light-switch” complexes $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ and $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ becoming emissive upon intercalation into double-helical DNA. By exploiting these properties through targeted ligand modification, ligand engineering has shifted the focus from canonical duplex binding toward noncanonical G4 DNA structures, as the introduced substituents enable productive interactions with G4 topologies.¹⁴⁶ Importantly, many Ru(II) polypyridyl complexes also preferentially accumulate in mitochondria.¹³⁷

Despite the promise of mitochondrial G4s as anticancer targets, ligand discovery in this area remains limited compared to nuclear G4s, and studies on ruthenium complexes are especially scarce.^{147–149} This underscores the need for mitochondria-targeted Ru(II) systems that couple DNA recognition with organelle localization and are tested in functional cancer models. This chapter presents the first two TPP-conjugated Ru(II) polypyridyl complexes, designated to achieve selective mitochondrial accumulation and to interact with mitochondrial G4 structures. The rationale underlying this design was to couple the well-established DNA-binding properties of the dppz-containing ruthenium scaffold with a mitochondria-targeting vector, thereby creating hybrid molecules capable of perturbing mitochondrial genome regulation and energy metabolism.

2. Results and Discussion

Synthesis and characterization.

Two mitochondria-targeted Ru(II) polypyridyl complexes were synthesized, incorporating either bipyridine or phenanthroline ligands and a dipyrrophenazine (dppz) unit functionalized with a triphenylphosphonium (TPP) group. Starting from bipyridine or phenanthroline and ruthenium trichloride, the dichlorido precursors $[\text{Ru}(\text{bpy})_2\text{Cl}_2]$ and $[\text{Ru}(\text{phen})_2\text{Cl}_2]$ were obtained. The labile chlorido ligands were substituted by phenanthroline-5,6-dione, which was subsequently condensed with 3,4-diaminobenzoic acid to yield the intermediates $[\text{Ru}(\text{bpy})_2(\text{dppz-COOH})]^{2+}$ and $[\text{Ru}(\text{phen})_2(\text{dppz-COOH})]^{2+}$. In parallel, a TPP-bearing primary amine was obtained through nucleophilic substitution of the corresponding bromide. Amide coupling between the carboxylic acid of the ruthenium complexes and the TPP-amine furnished the desired conjugates in good yields (Figure 13). The resulting compounds, $[\text{Ru}(\text{phen})_2(\text{dppz-TPP})]^{3+}$ (**1**) and $[\text{Ru}(\text{bpy})_2(\text{dppz-TPP})]^{3+}$ (**2**), were isolated as hexafluorophosphate salts and fully characterized by ^1H NMR spectroscopy (Figures A.1-2, Appendix A), ESI-MS (Figures B.1-2, Appendix B), and elemental analysis, confirming the expected structures and purity.

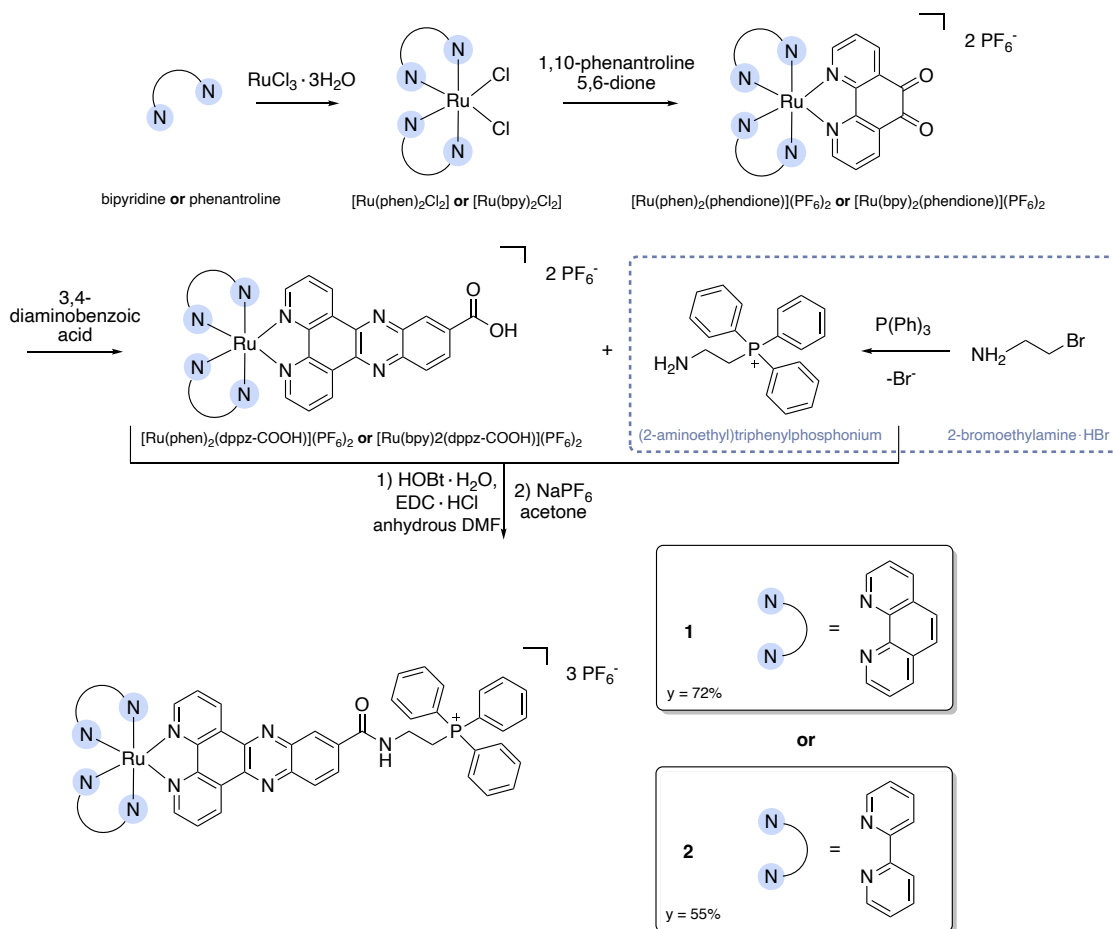


Figure 13. Synthetic route to compounds **1** and **2**, which differ only by the coordination of two phenanthroline or two bipyridine ligands, respectively.

In addition to the synthesis of the new derivatives, the well-known reference complexes $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ (**1a**) and $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ (**2a**) (Figure 14) were also re-prepared following established literature procedures, reported in the experimental section. These benchmark compounds were included in the study to provide a direct comparison with the new ones in the subsequent DNA-binding and biological evaluation experiments.

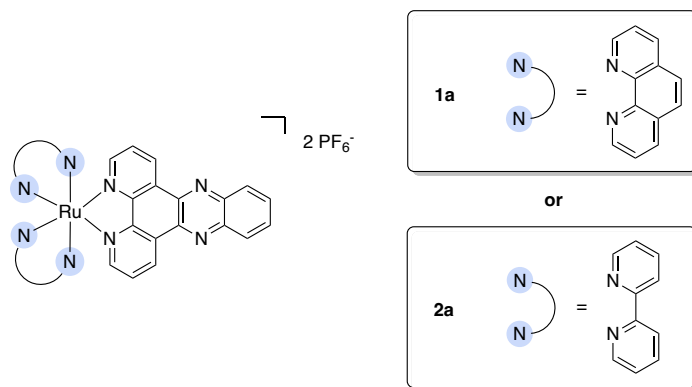


Figure 14. Structure of $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ (**1a**) and $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ (**2a**), used in this study as benchmarks. The synthetic procedure is reported in the experimental section of this chapter.

Stability in aqueous solutions.

UV-Vis spectroscopy was used to evaluate the stability of both compounds in aqueous media, revealing no appreciable spectral changes over 24 hours in Tris-KCl buffer (Figure 15). Notably, both absorption spectra display a band around 440 nm, characteristic of metal-to-ligand charge transfer (MLCT) transitions, as previously reported for related Ru-dppz complexes.¹⁵⁰

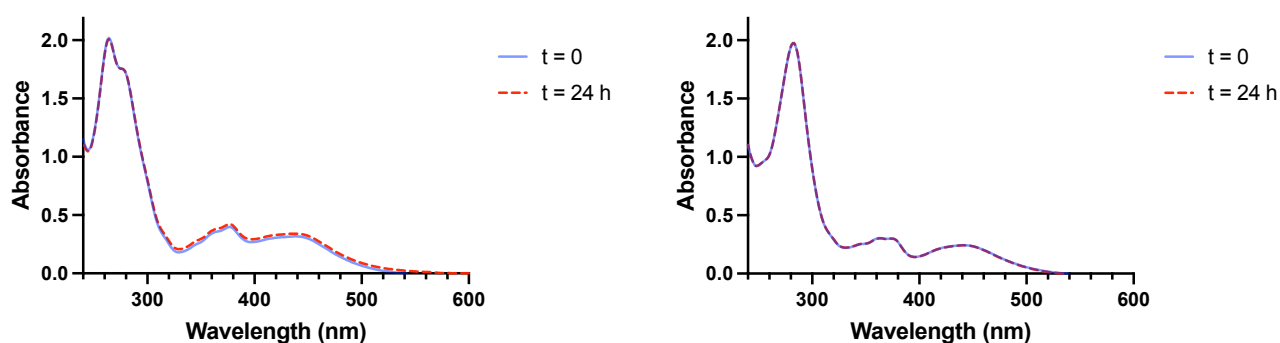


Figure 15. Compounds **1** (left) and **2** (right) in Tris-KCl buffer (50 mM Tris-HCl, 100 mM KCl, pH 7.4) at the two indicated time-points.

DNA interaction studies.

The interaction of compounds **1** and **2** with DNA was first evaluated by FRET-melting experiments using three mitochondrial G4-forming oligonucleotides (ODNs), namely atp6, nd2, and nd3, from the corresponding mitochondrial genes. These sequences were selected as representative models of

distinct G4 topologies: antiparallel (atp6), parallel (nd2), and mixed (nd3), as previously reported.²⁴ In addition, three nuclear G4-forming sequences (kit1 and kit2, from the promoter region of the *KIT* oncogene) and htelo (from human telomeric DNA),^{151,152} were included, together with a self-complementary duplex-forming sequence (dsDNA) used as a B-DNA control. The complexes **1** and **2** consistently induced higher stabilization (ΔT_m) of all G4 structures compared to their non-targeted analogues **1a** and **2a**, which lack the TPP group (Figure 16a). This effect was particularly pronounced for the G4 sequences, indicating that the introduction of the TPP unit enhances overall G4 affinity, likely due to the combined effect of additional positive charge that strengthens electrostatic interactions with the negatively charged nucleic acids and stacking interaction with the exposed tetrads. In contrast, no significant difference was observed between the ΔT_m values obtained for dsDNA upon treatment with **1/1a** or **2/2a**, indicating that the TPP functionalization does not increase the affinity for canonical duplex DNA. These findings demonstrate that the enhanced stabilization promoted by **1** and **2** is selective for G4 structures rather than unspecific DNA binding. Notably, compound **1** showed a preference for the antiparallel topology of atp6, with a T_m increase of 24 °C. Moreover, the stabilization induced by both **1** and **2** was found to be concentration-dependent, further confirming a direct interaction between the complexes and the G4-forming oligonucleotides (Figure 16b,c).

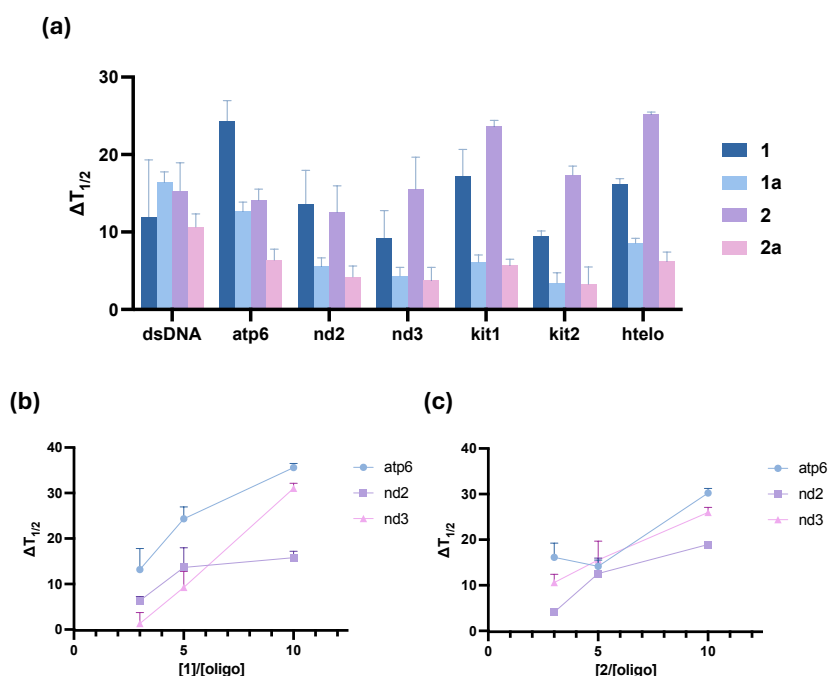


Figure 16. FRET-melting analysis of the interaction between the complexes and G4 structures. (a) ΔT_m values for mitochondrial (atp6, nd2, nd3) and nuclear G4 sequences (kit1, kit2 and htelo), and for a duplex control (dsDNA), upon incubation with **1**, **1a**, **2**, or **2a** (1 μ M complex, 0.2 μ M oligonucleotide). (b,c) Concentration-dependent stabilization of mitochondrial G4s by compounds **1** and **2**, respectively, expressed as ΔT_m increases. ODN concentrations are reported per strands.

To further investigate the structural impact of the complexes on DNA, CD titrations were performed with the mitochondrial G4s. For the B-DNA reference sequence ds26, compounds **1** and **2** induced dramatic alterations in the CD spectrum of B-DNA. The typical B-DNA positive band at around 275 nm suffers an inversion of the ellipticity after interaction with **1** or **2**. (Figure 17). It is known that 275 nm signal arises from π - π^* exciton coupling between adjacent bases and that its magnitude and sign are highly sensitive to base-pair step parameters such as twist, roll, tilt, slide, and the local electrostatic field.^{57,153} Intercalation of the dppz moiety between base pairs can re-stack bases and reorient transition dipoles so that the band inverts at $\sim$ 275 nm. However, given the steric hindrance imposed by the TPP group, such intercalation is unlikely. The observed effect may instead result from partial intercalation of the phenanthroline ligands in complex **1** or the bipyridine ligands in complex **2**. Despite the evident interaction of the dppz-TPP scaffold, most likely through groove binding, the absence of classical intercalation may actually be advantageous, as it contributes to the selectivity toward G4 structures, where the exposed tetrads enable π -stacking interactions with the ligand. In addition, the spectral change can also be interpreted as a local B-to-Z transition of the double helix. Indeed, left-handed Z-DNA is characterized by a negative CD band near 290 nm and a positive band around 260 nm, which are opposite in sign to those of right-handed B-DNA.⁵⁷ Finally, induced chirality of the complexes upon groove binding may contribute to the observed signal, potentially overlapping DNA's intrinsic near-UV CD band and influencing its sign around 275 nm. It is also worth noting that **1** and **2** are chiral compounds, but were used as racemic mixtures, which could further complicate the overall CD response.

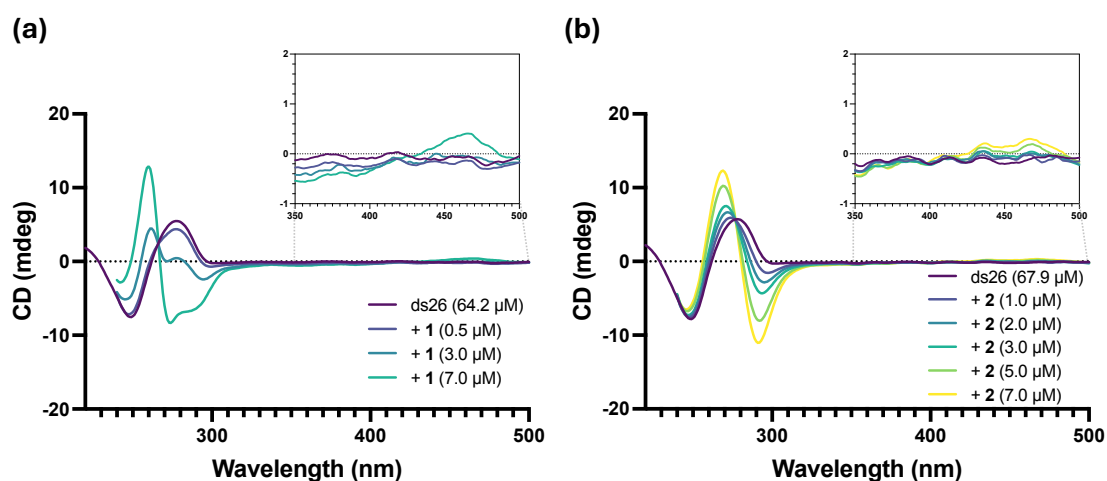


Figure 17. CD spectra of ds26, a B-DNA model, in presence of increasing amounts of **1** (a) and **2** (b). Inset: magnified view of the ICD region (350-500 nm). ODN concentrations are reported per bases.

The atp6, nd2, and nd3 sequences exhibit the characteristic CD signatures of antiparallel, parallel, and hybrid G-quadruplex topologies, respectively. Specifically, atp6 displays a positive band centered

at ~290 nm and a negative one at ~260 nm. In contrast, nd2 shows a positive band at ~260 nm accompanied by a negative band around 245 nm. Finally, nd3 presents the hallmark hybrid-type spectrum, with positive contributions near both 290 and 260 nm and a negative one around 245 nm. In the case of mitochondrial G4 sequences, both complexes induced changes in the characteristic G4 CD signatures, indicating modifications in the quadruplex topologies upon binding, or as seen for B-DNA, ICD bands contributions from the metal complexes themselves (Figure 18). Titration experiments revealed the emergence of ICD signals in the 370-500 nm region, which corresponds to the absorption bands of **1** or **2**, providing direct evidence of binding interactions between the complexes and the G4 structures. A particularly dramatic effect was noticed for atp6 spectrum upon incubation with compound **1**, consistent with its preference for the antiparallel topology, as also supported by the greater stabilization detected in the FRET-melting assay. Consistently, interaction of **1** with the parallel nd2 sequence resulted in the appearance of a shoulder around 290 nm, which may reflect an increased antiparallel contribution, indicative of a topology shift induced by **1**. For the hybrid nd3 sequence, compound **1** enhanced the parallel component relative to the antiparallel one. Complex **2** exhibited a comparable trend across the three G4 models, producing analogous spectral variations and ICD features. Nevertheless, it cannot be excluded that these spectral changes partly originate from ICD signals overlapping the intrinsic near-UV CD of DNA, or from a combination of ICD effects and topology rearrangements.

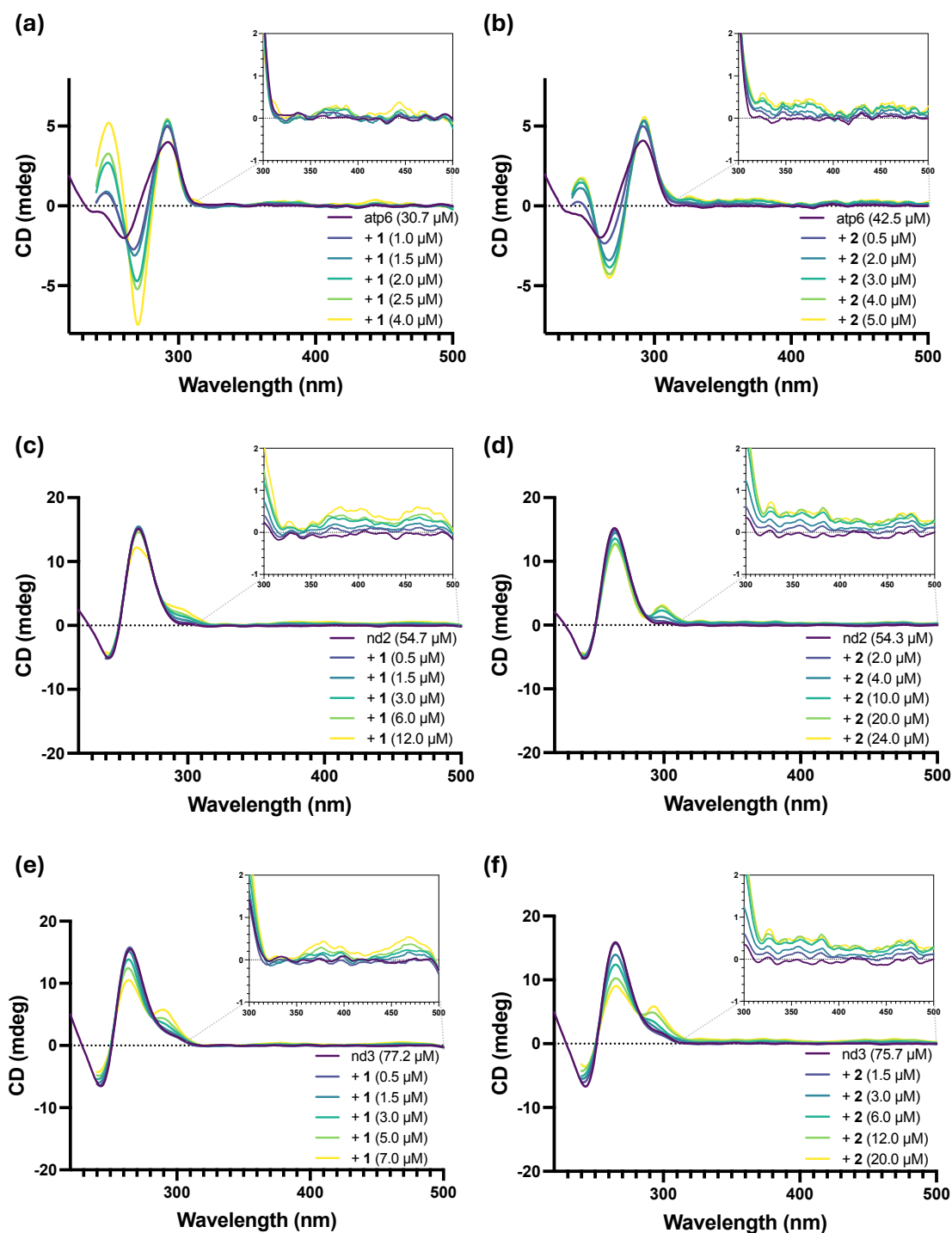


Figure 18. CD spectra of atp6, nd2 and nd3 G4s in presence of increasing amounts of **1** (a,c,e) and **2** (b,d,f). Inset: magnified view of the ICD region (300-500 nm). ODN concentrations are reported per bases.

The fluorescence properties of the new compounds were first evaluated in Tris-KCl buffer (Figure 19a,a'). To assess their ability to interact with B-DNA and to test for a potential light-switch effect similar to that reported for related Ru(II) polypyridyl complexes,^{142,154} ct-DNA was added into the solutions. Upon DNA addition, a measurable increase in emission intensity was observed, with **1** and **2** exhibiting an enhancement of approximately 7.7% and 19.7%, respectively (Figure 19b,b'). These

results confirmed that the compounds are capable of interacting with DNA and suggest partial protection of the emissive state from solvent quenching upon binding.

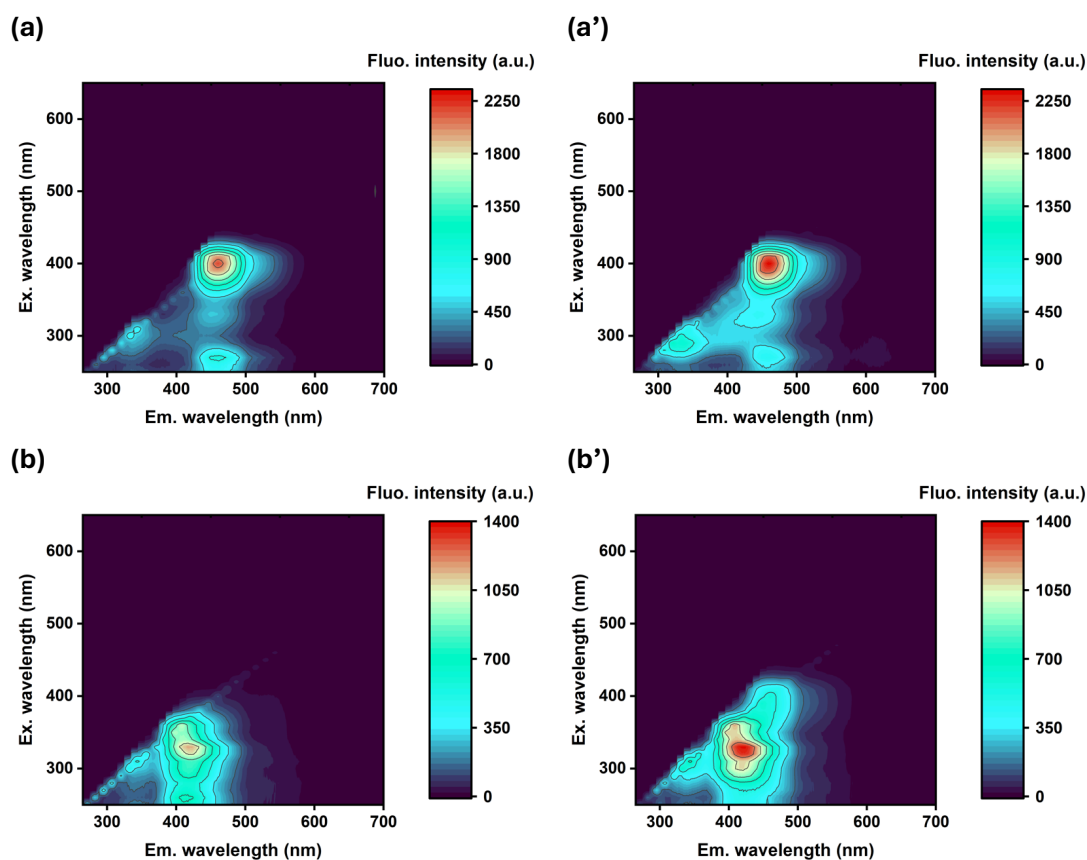


Figure 19. Emission contour plots of compounds **1** and **2** recorded in Tris–KCl buffer (50 mM Tris–HCl, 100 mM KCl, pH 7.4) before (a, b) and after addition of ct-DNA (a', b'). [**1**] or [**2**] = 5 μ M, [ct-DNA] = 100 μ M (expressed per strand).

Biological evaluation.

In the attempt to verify the potential biological activity mediated by mitochondrial targeting of the synthesized compounds, we first decided to assess the cellular uptake and cytotoxicity on cancer cell lines. The intracellular accumulation of ruthenium was quantified in HCT116 colorectal carcinoma cells for both complexes at three concentrations (10, 25, and 50 μ M) and two incubation times (5 and 24 h) (Table 1). Uptake of both compounds was concentration- and time-dependent. Overall, although comparable, compound **1** exhibited slightly higher cellular accumulation than **2**.

Compound	Concentration (μM)	Ru(ng)/ 10^6 cells	
		5 h	24 h
1	10	1.9 ± 0.2	3.7 ± 0.2
	25	3.3 ± 0.2	8.0 ± 0.3
	50	6.0 ± 0.4	<i>n.d.</i>
2	10	1.7 ± 0.2	3.1 ± 0.2
	25	2.6 ± 0.5	5.4 ± 0.1
	50	5.5 ± 0.2	<i>n.d.</i>

Table 1. Intracellular ruthenium uptake in HCT116 after incubation with compound **1** or **2** at the indicated concentration and times (*n.d.*, not determined). Values are reported as mean $\pm$ SD.

The cytotoxicity of the compounds **1** and **2**, measured by MTT assay, was evaluated in HCT116 cells within the 0-50 μM concentration range, where none of the complexes displayed significant toxicity (Figure 20a), and similar results were obtained for the non-targeted analogues **1a** and **2a** (*data not shown*). However, when the same treatments were analyzed by ATP quantification assays, a different trend emerged. Complexes **1** and **2** showed intrinsic cytotoxic activity as single agents, producing a concentration-dependent decrease in intracellular ATP levels relative to untreated controls, yielding IC_{50} values of approximately 15 μM for both compounds (Figure 20b). This discrepancy between the MTT and ATP assays may indicate an early impact of the compounds on mitochondrial bioenergetics, in line with their designed mitochondrial targeting properties, preceding the loss of overall cell viability.

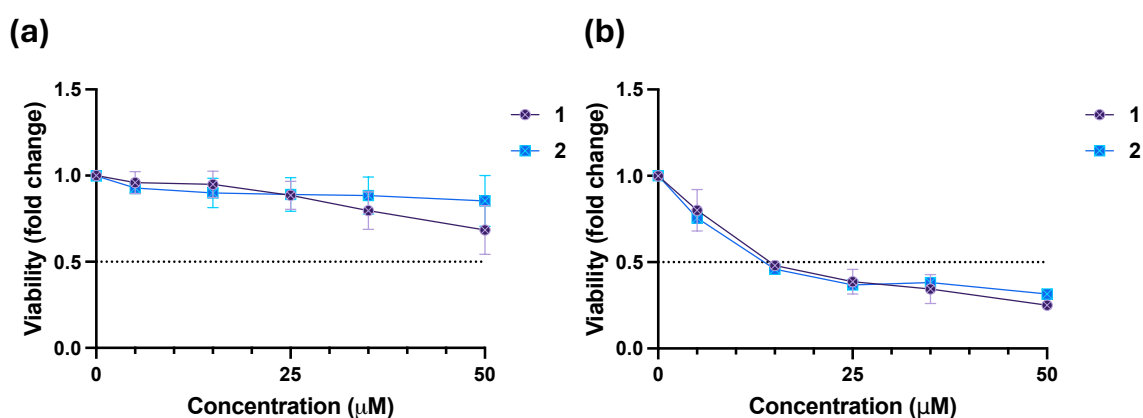


Figure 20. (a) MTT assay and (b) ATP assay measured after 72 hours incubation of HCT116 with either **1** or **2**. Data, normalized to the untreated control, are expressed as mean $\pm$ SD.

Having established both complexes' internalization and cytotoxicity, their effects on cell proliferation dynamics were next evaluated by monitoring whole-well confluency over time using the Incucyte®

live-cell imaging system. At 5 μM , compounds **1** and **2** showed no appreciable effect on cell confluency, whereas at 50 μM both induced a transient delay in the increase of confluency relative to the control. This behavior is consistent with a cytostatic response, which was more pronounced for compound **1**. The treated cells eventually reached confluency levels comparable to the control at the endpoint, attaining approximately 90% for compound **1** and full confluence for compound **2** (Figure 21a). Quantification of the proliferation over time, expressed as the area under the confluency curves (AUC), confirmed the more pronounced transient cytostatic effect of compound **1** (50 μM), with an AUC decrease of approximately 30% compared to the control (Figure 21b).

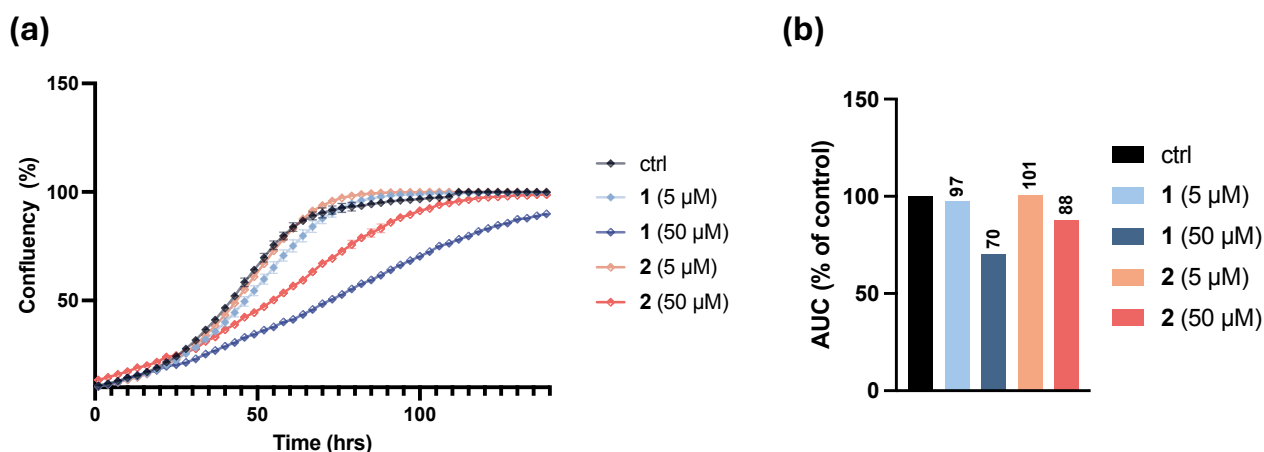


Figure 21. (a) Time-course of confluency (%) in HCT116 cells treated with **1** and **2** at the two indicated concentration, over 6 days; data are expressed as mean $\pm$ SD. (b) Quantification of proliferation expressed as the area under the confluency curves (AUC), normalized to the untreated control (%).

Encouraged by the interesting results, and to gain more information on the cytostatic activity of **1** and **2**, colony formation assay was performed. Both compounds exerted a pronounced cytostatic effect, leading to a significant reduction in colony number and overall staining density after one week of incubation. Among the two, complex **1** exhibited the strongest inhibitory effect, reducing colony density by approximately 45% compared to the control already at 10 μM , while complex **2** induced a reduction of about 32% under the same conditions (Figure 22). The decrease in colony-forming efficiency indicates that the complexes compromise the long-term proliferative potential of HCT116 cells, even in the absence of marked acute cytotoxicity.

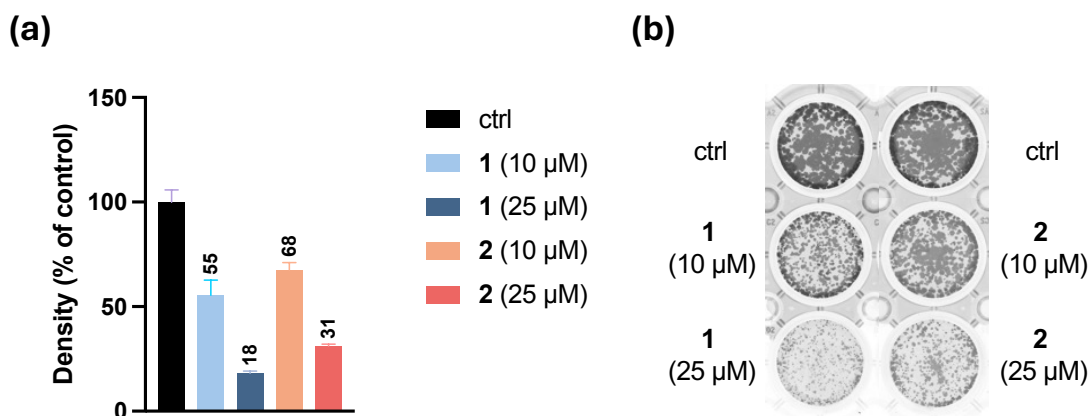


Figure 22. (a) Colony formation assay in HCT116 cells after treatment with compounds **1** or **2**. Data, normalized to the untreated control, are expressed as mean $\pm$ SD. (b) Representative stained wells of control and treated cells. Quantification of colony number and area was performed using ImageJ.

Since two-dimensional models may not fully capture the complexity of tumor organization, the compounds' effects were next investigated in three-dimensional spheroid cultures. Spheroid formation and subsequent replating assays were used to evaluate the impact of the treatments on tumor-like growth, recovery capacity, and clonogenic potential in a more physiologically relevant context. In the spheroid formation assay, cells were seeded and treated simultaneously to evaluate whether drug exposure interfered with spheroid development. Spheroids were monitored over a 4-day period using the Incucyte® live-cell imaging system. The resulting growth curves revealed a marked reduction in expansion rate upon treatment with compounds **1** or **2** (10 μ M) compared to the untreated control. The treated spheroids expanded more slowly and reached lower relative sizes over time, consistent with a cytostatic rather than cytotoxic effect (Figure 23a). To quantify these differences, the AUC was calculated. Among the two new complexes, compound **2** exhibited a lower AUC (72% vs 81% for compound **1**), indicating stronger inhibition of spheroid growth over time (Figure 23b). However, at the endpoint, spheroids treated with compound **1** were smaller relative to the control (59% vs 69% for compound **2**), suggesting a slightly greater final growth suppression (Figure 23c). Together, these results confirm that both **1** and **2** exert a sustained inhibitory effect on cancer cell proliferation in 3D culture, whereas the non-targeted analogues **1a** and **2a** displayed only minor effects.

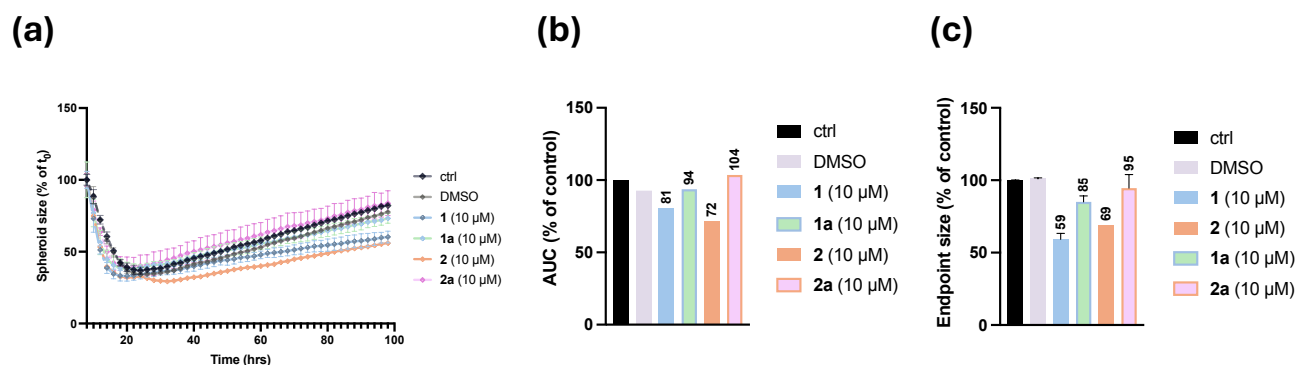


Figure 23. Spheroid formation assay with HCT116. (a) Representative spheroid growth curves normalized to the first measurable time point (8 h, set to 100); data are expressed as mean $\pm$ SD. (b) AUC values calculated from the normalized curves in (a) and expressed as percentage of the untreated control. (c) Endpoint spheroid size at the final time point, expressed as percentage of the control.

To further assess the invasive behavior of the cells following treatment, spheroids were replated onto adherent well plates to monitor the outgrowth of migrating cells from the spheroid core over around 4 days. The invasion kinetics were markedly reduced upon treatment with compound **1**, whereas compound **2** produced a milder effect compared to the untreated control, (Figure 24a) as also reflected by the corresponding AUC values (Figure 24b). At the endpoint, the invading area in spheroids treated with compound **1** was reduced by approximately 46% relative to the control (Figure 24c), demonstrating a pronounced inhibition of the outgrowth process. Conversely, compound **2** and the non-targeted analogues **1a** and **2a** exerted only limited effects.

The results obtained from the 3D culture models indicate that compound **1** not only restricts spheroid growth but also markedly impairs the invasive potential of cancer cells, showing a stronger overall effect than compound **2**.

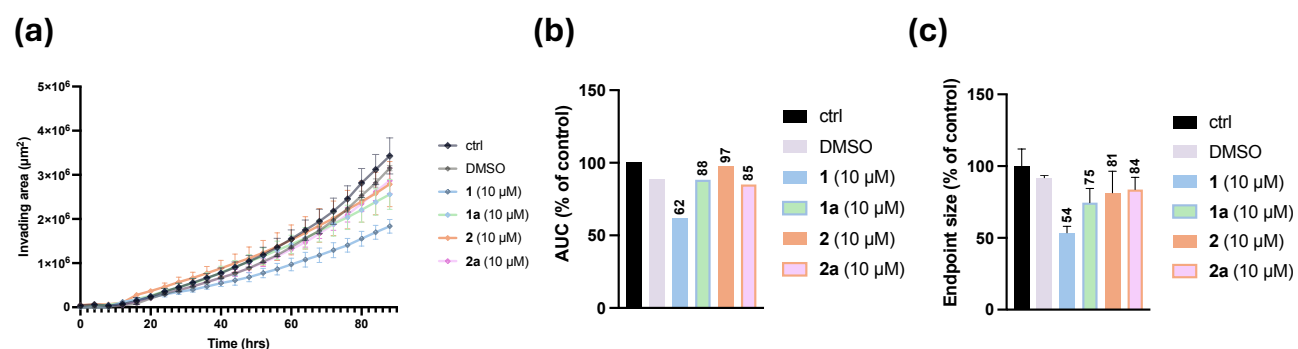


Figure 24. Spheroid replating assay with HCT116. (a) Raw invasion kinetics showing the expansion of the invading cell area over time; data are expressed as mean $\pm$ SD. (b) Quantification of the cumulative invasion, AUC from panel (a) and normalized to the untreated control. (c) Endpoint invading area at the final time point, expressed as percentage of the control.

The effect of the complexes on cell migration was further examined using scratch-wound assays, in which the rate of wound closure was quantified as a measure of cell migratory capacity under treatment conditions. Both compounds **1** and **2** markedly delayed wound closure to a similar extent relative to the untreated control, indicating a pronounced reduction in the migratory and proliferative potential of HCT116 cells. In contrast, the non-targeted analogues **1a** and **2a** showed minor effects on wound healing (Figure 25). These results further support the conclusion that mitochondrial-targeted complexes **1** and **2** effectively impair both cell proliferation and migratory behavior

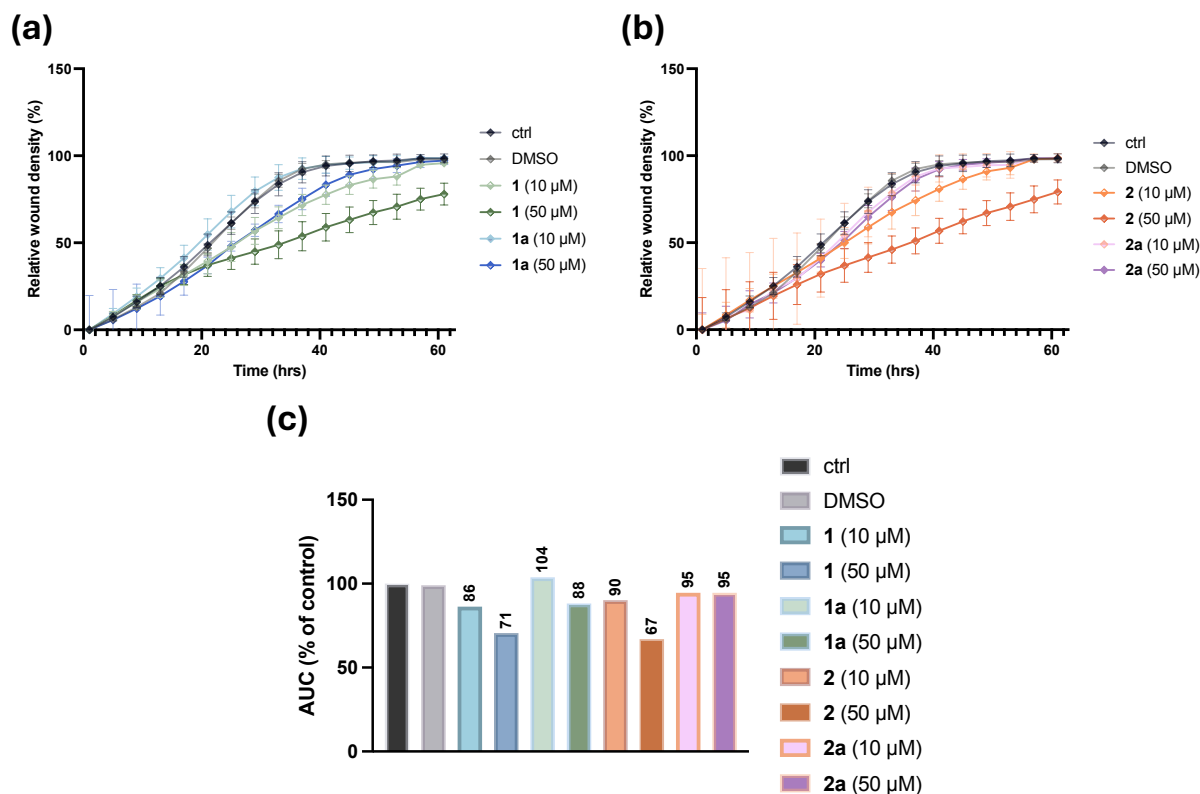


Figure 25. Scratch (migration) assay performed with HCT116 cells after treatment with (a) **1** or **1a** and (b) **2** or **2a**. Time-course of wound closure represented is represented as relative wound density, automatically normalized to cell confluence outside the wound. Additionally, relative wound density was normalized to the initial time point to correct for minor differences in scratch width. Data are expressed as mean $\pm$ SD. (c) AUC values from (a) and (b), expressed as percentage of the control.

In line with the mitochondrial-targeting rationale underlying the design of compounds **1** and **2**, mitochondrial respiration and glycolytic activity were evaluated in HCT116 cells using the Seahorse XF Mito Stress Test. As shown in Figures 26a–d, all compounds produced alterations in both oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) profiles, with marked effects observed at 50 μ M and milder responses at 5 μ M. Treatment with the complexes led to a reduction in both basal and maximal respiration, indicating impaired mitochondrial oxidative capacity. In particular, compounds **1** and **2** induced stronger inhibitory effects compared to their non-targeted

analogues **1a** and **2a**. Among all tested compounds, complex **1** produced the most pronounced decrease in both OCR and ECAR, suggesting a broad suppression of mitochondrial and glycolytic metabolism.

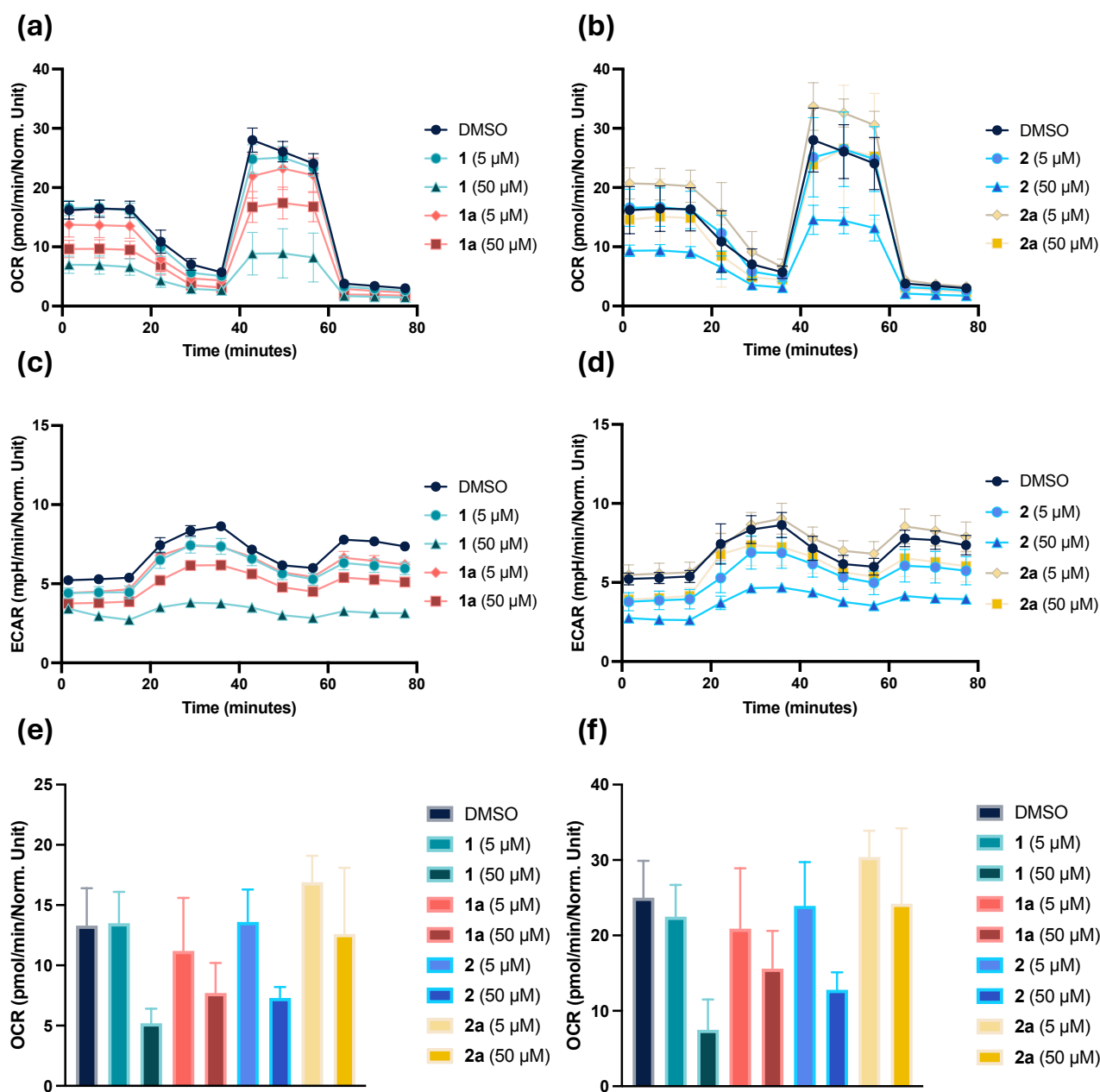


Figure 26. Seahorse Mito-Stress Test. Oxygen consumption rate (OCR) profiles over time for cells treated with compounds **1** and **1a** (a) and **2** and **2a** (b). (c,d) Extracellular acidification rate (ECAR) profiles over time for the same compound sets, reflecting glycolytic activity during mitochondrial stress. Quantification of basal respiration (e) and maximal respiration (f) extracted from the OCR curves for all compounds. OCR and ECAR measurements were normalized to cell number to account for variations in cell density across wells. Data are expressed as mean $\pm$ SD.

Finally, to deep investigate the biological effects associated with mitochondrial targeting, quantitative PCR analysis was conducted to measure the mRNA expression levels of the selected mitochondrial

genes. The analysis revealed a significant downregulation of the mitochondrial genes *ATP6*, *ND2*, and *ND3* in cells treated with compounds **1** or **2**, whereas the expression levels of the nuclear genes *MYC* and *BCL2* remained unaffected (Figure 27). This selective transcriptional repression of mitochondrial genes supports the hypothesis that the compounds interfere with mitochondrial DNA transcription, likely through stabilization of mitochondrial G4 structures. Additionally, these findings are consistent with the metabolic impairment previously observed in Seahorse analyses, reinforcing the proposed mitochondrial mechanism of action.

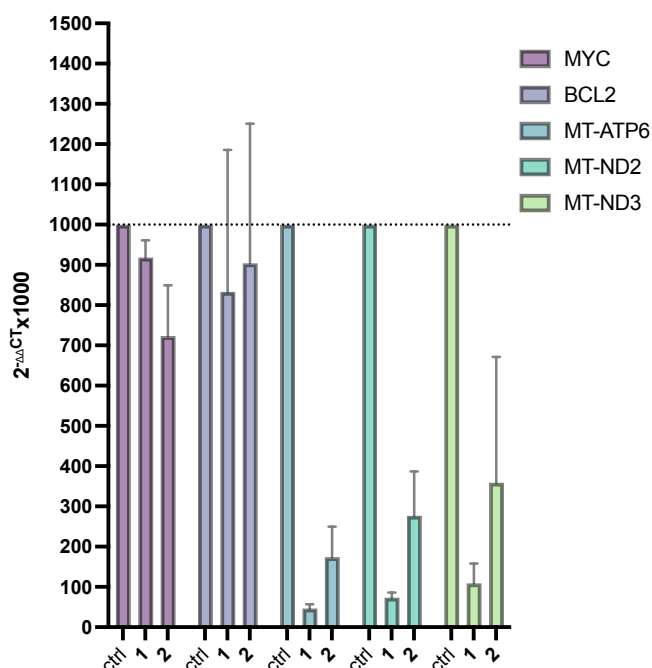


Figure 27. Relative mRNA expression levels of mitochondrial genes (*ATP6*, *ND2*, *ND3*) and nuclear genes (*MYC*, *BCL2*) in HCT116 cells treated with compounds **1** and **2**. Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method. RPL41 was used as housekeeping gene. Data, normalized to the untreated control, are expressed as mean $\pm$ SD.

To gain deeper insight into the mechanism of action of compounds **1** and **2**, a series of combination assays was conducted using **metformin** and **BAY-876**, (Figure 28) two metabolic modulators known to interfere with mitochondrial respiration and glucose uptake, respectively. **Metformin** is a widely prescribed antidiabetic drug, which has also shown promising anticancer. Preclinical studies demonstrate that **metformin** inhibits tumor growth, invasion, and metastasis in various cancers and enhances sensitivity to radio-, chemo-, and immunotherapy. Its main anticancer mechanism involves inhibition of mitochondrial complex I and activation of the AMPK/mTOR pathway, leading to altered energy metabolism.¹⁵⁵

The glucose transporter GLUT-1 is overexpressed in several cancer types, including colon, ovarian, breast, and brain tumors, among others. Because glucose uptake represents the rate-limiting step of

the glycolytic pathway that sustains cancer cell metabolism, its inhibition has emerged as a promising anticancer strategy. **BAY-876** has been identified as one of the most potent and selective inhibitors of GLUT-1.¹⁵⁶ This compound has demonstrated robust anticancer activity both in vitro and in vivo, underscoring its effectiveness as a GLUT-1 inhibitor and its promise in combination regimens or as a monotherapy in solid tumors.^{157–159}

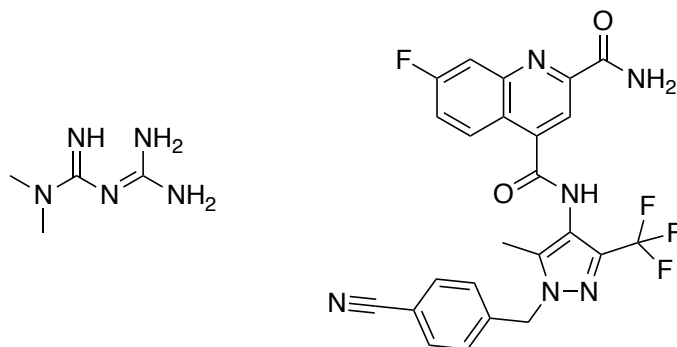


Figure 28. Chemical structure of **metformin** (left) and **BAY-876** (right).

Firstly, co-treatment experiments were performed to assess the potential synergistic interaction between the ruthenium complexes and metformin. In conventional 2D cultures, MTT viability assays revealed that the combination of compound **2** with metformin produced a markedly stronger antiproliferative effect than either agent alone. In contrast, compound **1** did not show significant synergy under the same conditions (Figure 29).

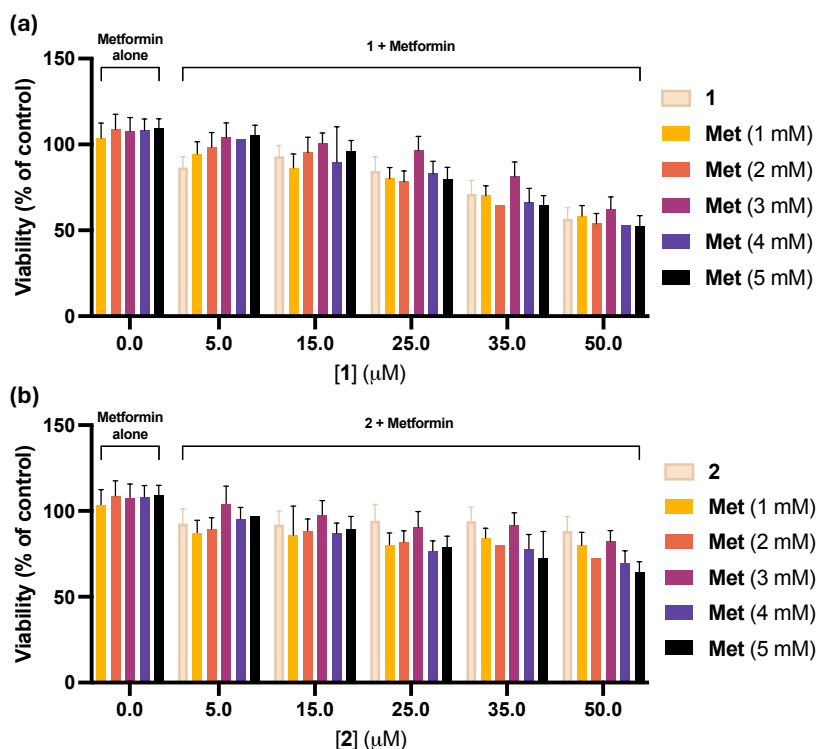


Figure 29. HCT116 cell viability measured with MTT assay after 72 h treatment with compounds **1** (a) or **2** (b), alone or in combination with **metformin**, at the indicated concentrations. Data are presented as the mean $\pm$ SD, expressed as a percentage of the untreated control.

Quantitative analysis using the Bliss independence model yielded a Bliss score of +0.38, indicating a clear synergistic interaction at the higher concentrations tested ($[2] = 50 \mu\text{M}$; $[\text{metformin}] = 5 \text{ mM}$). To better illustrate the interaction landscape between compound **2** and metformin, the calculated Bliss scores were represented as a heat map, which highlighted a distinct region of synergy at the highest concentrations of both agents (Figure 30).

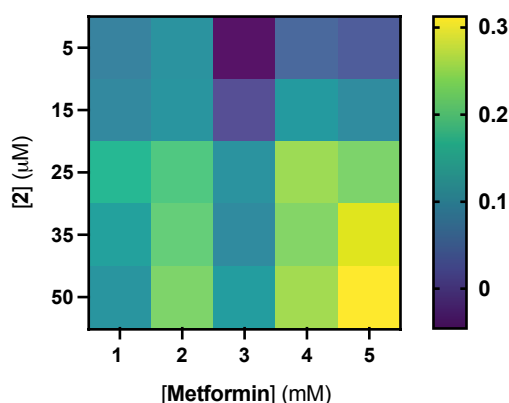


Figure 30. Heat map representation of the calculated Bliss scores for all combinations of compound **2** and **metformin**. Positive Bliss values (green-yellow) indicate synergistic interactions, values near zero (dark blue) reflect additivity.

When the same combinations were evaluated in three-dimensional spheroid cultures, a different trend emerged for compound **2**. In fact, in the spheroid growth assay, while compound **1** again showed no synergistic interaction with **metformin**, compound **2** displayed a shift toward antagonism (Bliss score = -0.34), as determined from the AUC values of the spheroid growth curves (Figure 31). Comparable results were obtained when considering the endpoint measurements, leading to similar conclusions (*data not shown*).

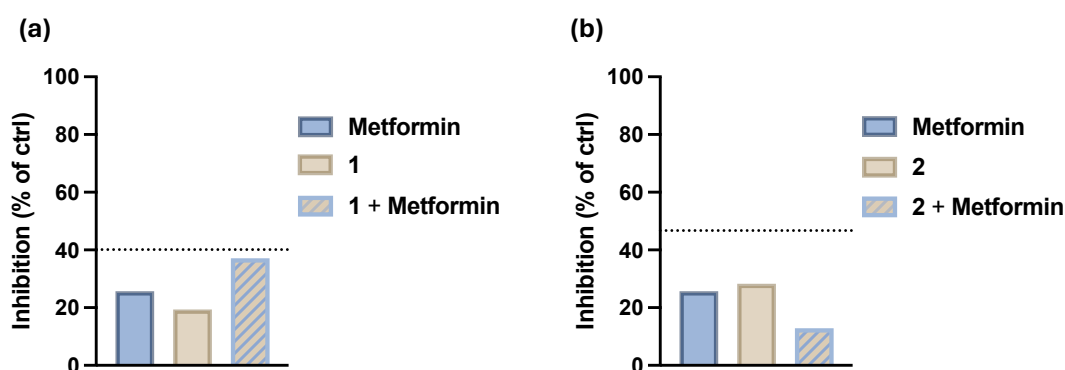


Figure 31. Percentage of inhibition calculated from the AUC values of the HCT116 spheroid growth curves (size over time) for compounds (a) **1** or (b) **2** ($10 \mu\text{M}$), alone or in combination with **metformin** (5 mM). The AUC values were derived from the normalized size-versus-time plots and expressed as % inhibition relative to the untreated control. The dotted horizontal line represents the expected additive inhibition calculated according to the Bliss independence model.

To further investigate the invasive potential of the treated cells, spheroids were replated under adherent conditions to monitor cell outgrowth over time. Quantitative analysis of the AUC values confirmed that compound **2** maintained its synergistic interaction with **metformin** (Bliss score = 0.14), consistent with the MTT assay results, whereas compound **1** remained additive in this experimental setting (Figure 32). Similar values were obtained when considering the endpoint measurements (*data not shown*). The transition from synergy in 2D monolayers to antagonism in 3D spheroids, followed by restored synergy upon replating, highlights the profound influence of the tumor microenvironment on drug interactions. In compact spheroids, metabolic gradients, restricted diffusion, and cellular adaptation can attenuate or even reverse synergistic effects that are otherwise evident under uniform 2D exposure. Upon replating, as cells regain metabolic homogeneity and drug accessibility, the combined mitochondrial stress induced by compound **2** and metformin re-establishes a synergistic inhibition of growth.

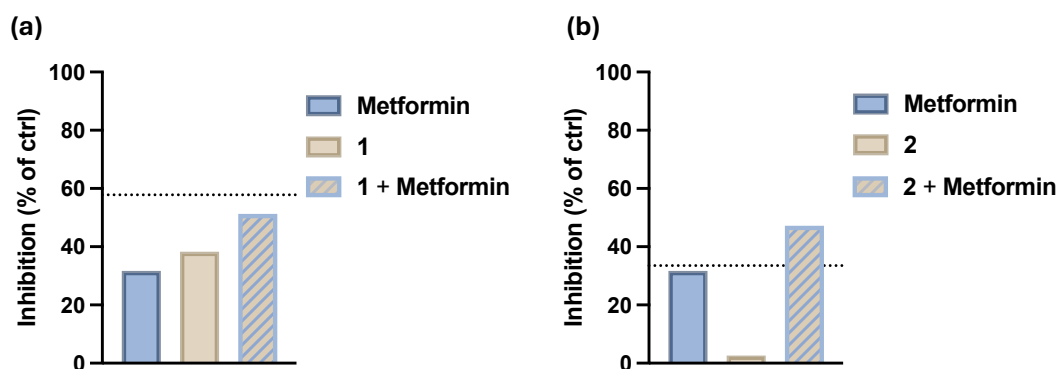


Figure 32. Percentage of inhibition calculated from the AUC values of the HCT116 spheroid outgrowth curves (cell expansion over time) for compounds **1** (a) or **2** (b) (10 μ M), alone or in combination with **metformin** (5 mM). The AUC values of the raw curves was expressed as % inhibition relative to the untreated control. The dotted horizontal line represents the expected additive inhibition calculated according to the Bliss independence model.

After evaluating the combinations with **metformin**, the study next focused on **BAY-876**, to explore whether simultaneous inhibition of glycolysis and mitochondrial function could enhance the overall antiproliferative effect of the ruthenium complexes. In MTT assays, at the highest concentration tested (**1** or **2** = 50 μ M; **BAY-876** = 10 μ M), Bliss scores of +0.34 and +0.40 were calculated for **1** and **2**, respectively (Figure 33).

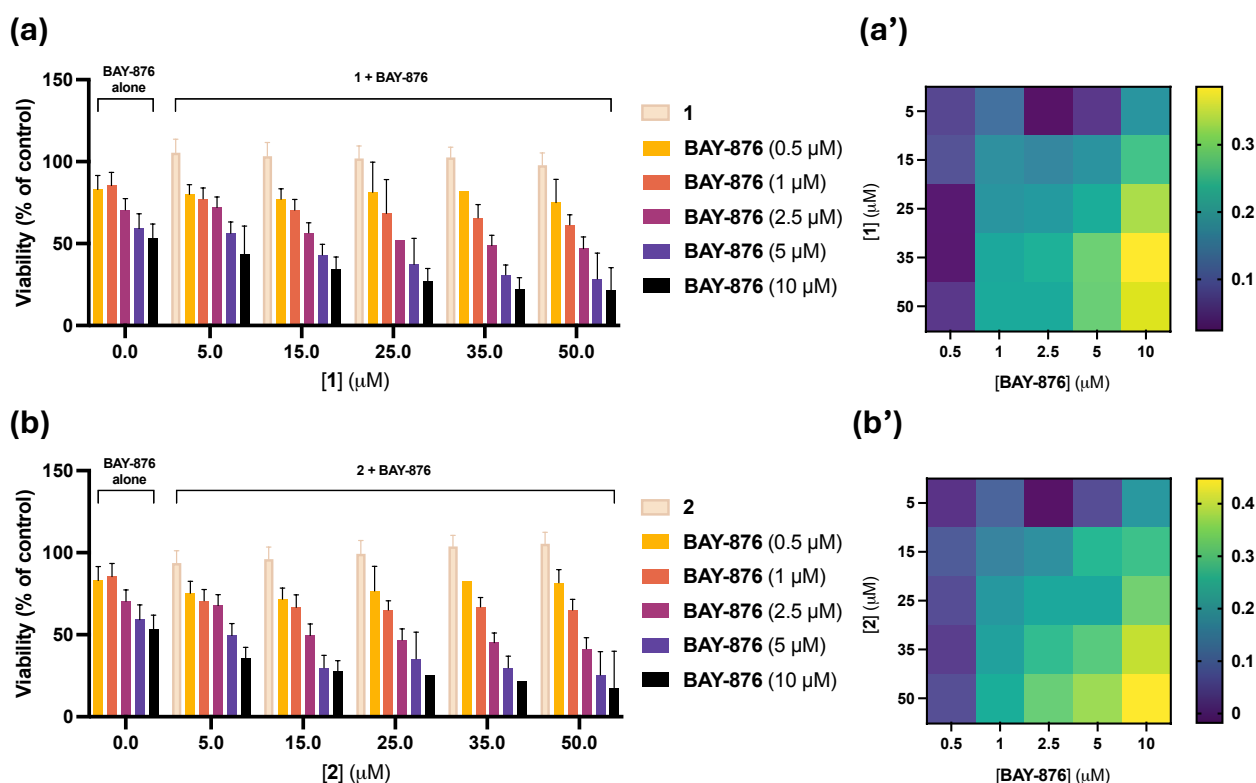


Figure 33. (a,b) HCT116 viability measured by MTT assay after 72 h of treatment with compounds **1** or **2**, respectively, alone or in combination with **BAY-876**, at the indicated concentrations. Data are presented as the mean $\pm$ SD, expressed as a percentage of the untreated control. (a',b') Corresponding Bliss synergy heat maps, showing the calculated Bliss scores for all drug combinations.

To further validate the synergistic interaction observed in short-term viability assays, cell proliferation was monitored in real time through confluency measurements over a six-day period. Under these conditions, compound **1** alone displayed higher activity (as also shown in Figure 21), whereas **BAY-876** and compound **2** alone produced only minor inhibition. Notably, all co-treatment combinations markedly enhanced the antiproliferative effect, thereby confirming the presence of a synergistic interaction. Quantitative analysis of cumulative proliferation, expressed as the area under the confluency curves, yielded Bliss scores of +0.29 and +0.41 for compounds **1** and **2**, respectively, at the highest concentration tested (Figure 34). Notably, in this assay, the extent of synergy was primarily driven by the increasing concentration of the ruthenium compounds, whereas escalating **BAY-876** levels produced only marginal additional effects. This finding suggests that the synergy arises mainly from potentiation of the mitochondrial impairment induced by the ruthenium compounds rather than from stronger GLUT-1 inhibition.

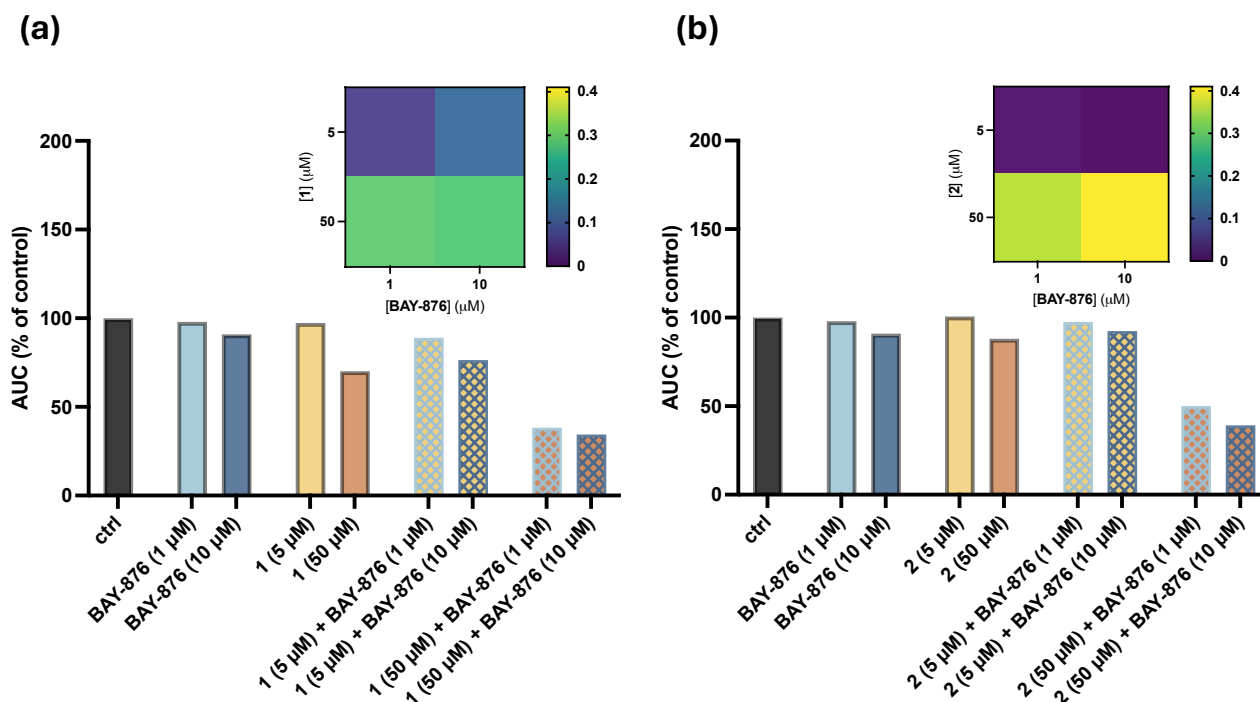


Figure 34. Quantification of HCT116 proliferation expressed as AUC values from real-time confluency measurements over six days for cells treated with compound **1** (a) or **2** (b), alone or in combination with **BAY-876**, at the indicated concentrations. Data are presented as the mean $\pm$ SD, expressed as percentage of the untreated control. Insets show the corresponding Bliss synergy heat maps for the four tested combinations.

The potential synergistic effects of the combinations were also evaluated using the clonogenic assay, to measure the long-term proliferative capacity of the treated cancer cells. Consistent with the previous results, co-treatment with **BAY-876** produced a further reduction in colony formation compared to the individual treatments (Figure 35). The concentrations were reduced in this assay because of the intrinsically higher potency of the compounds under prolonged exposure. Since the complexes alone already exerted a strong inhibitory effect at 25 μ M, the window for additional synergistic interaction was limited, resulting in modest Bliss values at the highest concentrations tested. Nevertheless, synergistic trends were more evident at lower compound concentrations, where the individual treatments were only partially active and therefore allowed clearer detection of combination effects.

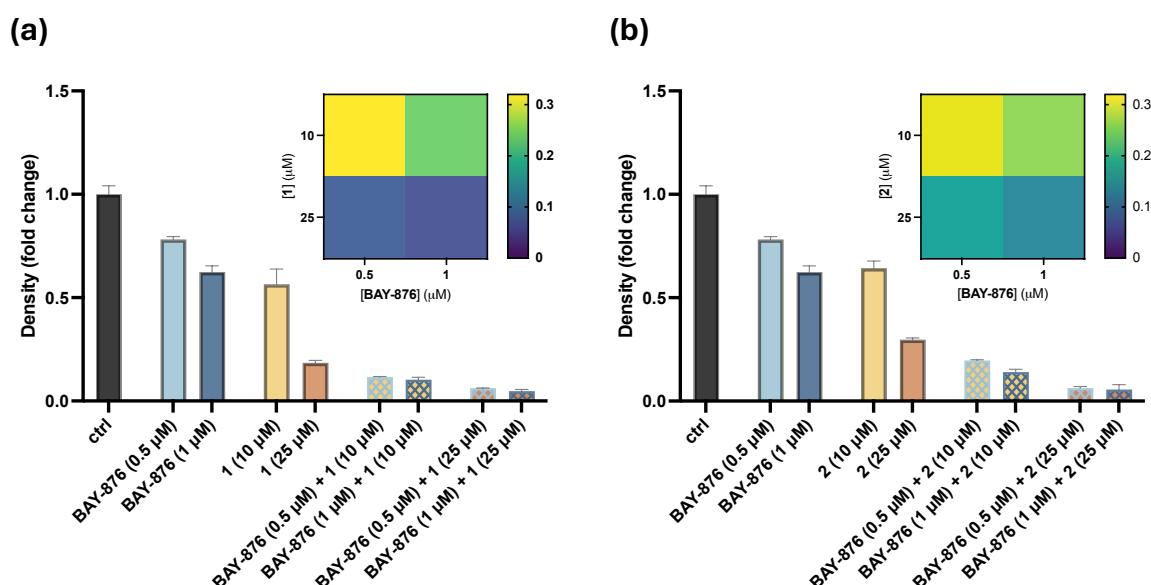


Figure 35. Quantification of colony density in HCT116 cells treated with compound **1** (a) or compound **2** (b), alone or in combination with **BAY-876**, at the indicated concentrations. Data are expressed as normalized colony density relative to the untreated control. Insets show the corresponding Bliss synergy heat maps for the four tested combinations.

The combined effects of compounds **1** and **2** with **BAY-876** were next evaluated in three-dimensional spheroid cultures, to assess whether the synergistic trends observed in monolayer assays extended to 3D tumor models. Co-treatment with **BAY-876** further reduced spheroid growth compared to the individual treatments. However, quantitative analysis using the Bliss independence model revealed no synergistic interaction, with Bliss scores approximating zero at the highest concentrations tested ($[1]$ or $[2] = 10 \mu\text{M}$; $[\text{BAY-876}] = 5 \mu\text{M}$), indicating an additive effect. The percentage of inhibition, calculated from both the AUC (Figure 36) and the endpoint size (*data not shown*) supported this interpretation. This outcome likely reflects the intrinsically higher drug resistance of 3D spheroids, as previously discussed in this section.

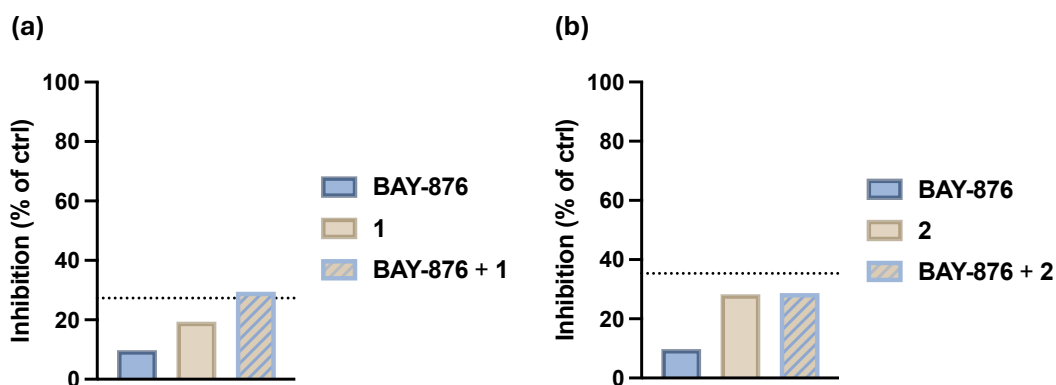


Figure 36. Percentage of inhibition calculated from the AUC values of the HCT116 spheroid growth curves (size over time) for compounds (a) **1** or (b) **2** (10 μ M), alone or in combination with **BAY-876** (5 μ M). The AUC values were derived from the normalized size-versus-time plots and expressed as % inhibition relative to the untreated control. The dotted horizontal line represents the expected additive inhibition calculated according to the Bliss independence model.

To further investigate the effect of the combined treatments on the invasive potential of cancer cells, the spheroids were replated in adherent wells and monitored for cellular outgrowth. In this assay, the combination with **BAY-876** produced a pronounced synergistic effect, as determined by the AUC analysis (Figure 37).

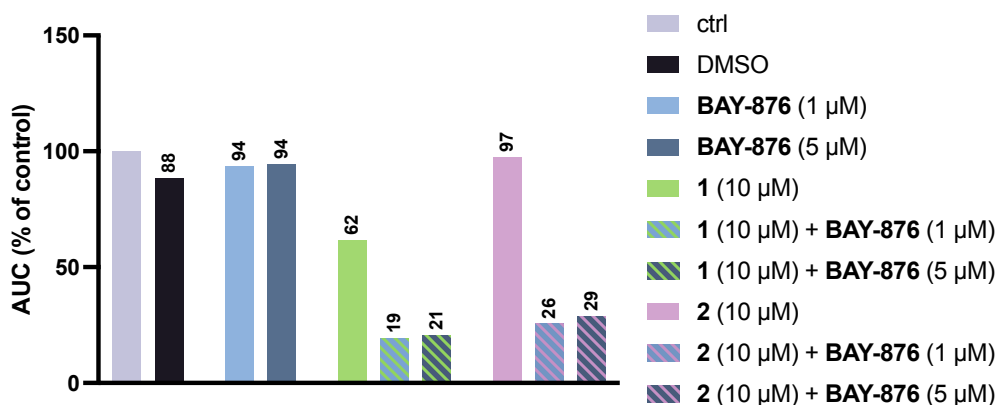


Figure 37. Quantification of the AUC obtained from the raw curves of HCT116 spheroid outgrowth size over time after replating. AUC values were normalized to the untreated control and expressed as percentage (% of control).

The calculated Bliss scores were +0.38 for compound **1** and +0.61 for compound **2**, indicating strong synergy, at the highest concentration tested ([**1**] or [**2**] = 10 μ M, [**BAY-876**] = 5 μ M). Interestingly, this behavior parallels the replating results obtained with **metformin**, where synergy was also restored after the loss of cooperative effects observed in compact 3D spheroids. Together, these findings reinforce the notion that cellular context and metabolic conditions critically modulate drug

interactions. In both combinations, once spheroids were replated and metabolic uniformity was regained, the joint effect of **BAY-876** or metformin with complexes **1** or **2** markedly suppressed cell outgrowth. This supports a common mechanism in which energy-metabolism interference under uniform exposure reinstates synergistic growth inhibition that is otherwise masked in the 3D spheroid environment.

Finally, to extend the biological evaluation beyond HCT116 cells and to explore potential cross-resistance profiles, the cytotoxic activity of the ruthenium complexes and their non-targeted analogues was assessed in the A2780 (cisplatin-sensitive) and A2780cis (cisplatin-resistant) ovarian cancer cell lines, by MTT assay after 72 hours of treatment (Figure 38). Overall, **1** and **2** complexes exhibited moderate antiproliferative activity within the tested concentration range, while the corresponding non-targeted analogues **1a** and **2a** showed negligible cytotoxicity. Interestingly, compound **1** displayed slightly higher toxicity in the A2780cis cells compared to the parental A2780 line, suggesting a possible ability to circumvent cisplatin resistance mechanisms. In contrast, compound **2** was more active in A2780 cells, indicating a different interaction profile or uptake behavior between the two complexes.

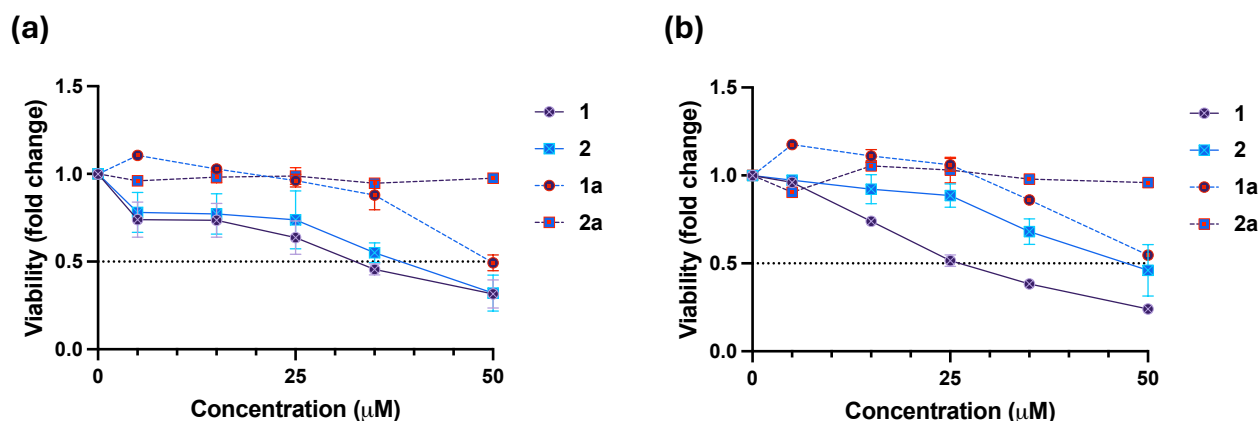


Figure 38. MTT-based cell viability after 72 h treatment with compounds **1**, **2**, **1a** or **2a** in (a) A2780 and (b) A2780cis cells. Data, normalized to the untreated control, are expressed as mean $\pm$ SD.

3. Experimental

Chemicals and solvents were purchased from Sigma Aldrich and Thermo Fisher Scientific and used without further purification. NMR spectra were recorded on Bruker AC-E series 400 MHz. Mass spectra were recorded on an Agilent 6540 QTOF LC/MS Mass Spectrometer by direct infusion. Elemental analyses were performed using an Eurovector EA3000 elemental analyzer at the Microanalytical Laboratory of the University of Vienna. The sequences of the ODNs used in this work are reported below (Table 2). Concentrations are reported per strand or per bases depending on

the experiment, as indicated in the captions. Lyophilized calf thymus DNA (ct-DNA) was obtained from Sigma-Aldrich and dissolved in 1 mM Tris-HCl buffer (pH 7.5). The final DNA concentration, expressed in nucleotide bases, was determined by UV spectrophotometry at 260 nm using a molar extinction coefficient of 6600 M⁻¹ cm⁻¹.

ODN	Nucleotide sequence
dsDNA	5'-TATAGCTA-Heg-TATAGCTATA-3'
ds26	5'-CAATCGGATCGAATTCGATCCGATTG-3'
atp6	5'-GGTTAGGCGTACGGCCAGGGCTATTGG-3'
nd2	5'-AGGGCGATGAGTGTGGGGAGGAATGGGGTGGGT-3'
nd3	5'-TGGAGAAAGGGACGCGGGCGGGGGATATAGGGT-3'

Table 2. Name of the ODNs used in this work and corresponding nucleotide sequences. In dsDNA, Heg linker is [(-CH₂-CH₂-O)₆]. The ODNs used for FRET melting assay are labelled at the 5' end with FAM (6-carboxyfluorescein) and at the 3' end with TAMRA (6-carboxy-tetramethyl-rhodamine).

For biological assays, compounds were dissolved in DMSO to prepare 10 mM stock solutions. These stocks were subsequently diluted in culture medium, ensuring that the final DMSO concentration did not exceed 1%. In the combinatorial assays, the Bliss score (ΔE) was calculated as the difference between the observed inhibition effect (E_{obs}) and the expected inhibition effect (E_{exp}), according to the Bliss independence model. The expected effect for a combination of drug A and drug B was defined as:

$$E_{\text{exp}} = E_A + E_B - (E_A \times E_B)$$

where E_A and E_B represent the fractional inhibition values of the individual treatments.

Synthesis and characterization.

dppz. 1,10-Phenanthroline-5,6-dione (105.8 mg, 0.5 mmol) was dissolved in methanol, and o-phenylenediamine (55.0 mg, 0.5 mmol) was added dropwise to the solution. The reaction mixture was refluxed for 2 h, after which it was allowed to cool to room temperature. The resulting white crystalline solid was collected by filtration. (59.2 mg, 42.9%). ¹H NMR (400 MHz, CDCl₃) δ 9.65 (dd, J = 8.1, 1.8 Hz, 2H), 9.28 (dd, J = 4.5, 1.8 Hz, 2H), 8.43 – 8.31 (m, 2H), 8.01 – 7.88 (m, 2H), 7.80 (dd, J = 8.1, 4.5 Hz, 2H).

[Ru(phen)₂Cl₂]. 1,10-phenanthroline (540.9 mg, 3 mmol), RuCl₃·3H₂O (392.5 mg, 1.5 mmol), and LiCl (48 mg, 1.05 mmol) were dissolved in 15 mL of anhydrous DMF and refluxed under inert nitrogen atmosphere for 24 h. After this time, acetone (35 mL) was added, and the mixture was stored in a freezer overnight. The following day, the mixture was filtered under vacuum, affording a black precipitate. The solid was washed with cold distilled water and diethyl ether and then dried to remove residual solvents. The product was obtained as a black powder (398.8 mg, 49.9%). ¹H NMR (400 MHz, DMSO) δ 10.28 (dd, J = 5.3, 1.3 Hz, 2H), 8.71 (dd, J = 8.2, 1.3 Hz, 2H), 8.29 (d, J = 8.9 Hz, 2H), 8.26 – 8.18 (m, 4H), 8.14 (d, J = 8.9 Hz, 2H), 7.75 (dd, J = 5.4, 1.2 Hz, 2H), 7.33 (dd, J = 8.1, 5.4 Hz, 2H).

[Ru(bpy)₂Cl₂]. The complex was prepared by reacting 2,2'-bipyridine with RuCl₃·3H₂O in a 2:1 molar ratio, following a procedure analogous to that used for the phenanthroline analogue. Specifically, 2,2'-bipyridine (63.1 mg, 0.4 mmol), RuCl₃·3H₂O (53.7 mg, 0.2 mmol), and LiCl (43.7 mg, 1.0 mmol) were dissolved in 10 mL of anhydrous DMF and refluxed under a nitrogen atmosphere for 24 h. After cooling, acetone (20 mL) was added, and the mixture was stored in a freezer overnight. The resulting suspension was filtered under vacuum to afford a black precipitate, which was washed with cold distilled water and diethyl ether and dried under vacuum to remove residual solvents (59.1 mg, 61.2 %). ¹H NMR (400 MHz, DMSO) δ 10.01 – 9.95 (m, 2H), 8.63 (dt, J = 8.2, 1.1 Hz, 2H), 8.50 – 8.45 (m, 2H), 8.06 (td, J = 7.8, 1.6 Hz, 2H), 7.77 (ddd, J = 7.3, 5.6, 1.3 Hz, 2H), 7.71 – 7.64 (m, 2H), 7.51 (dt, J = 6.1, 1.0 Hz, 2H), 7.10 (ddd, J = 7.3, 5.7, 1.4 Hz, 2H).

[Ru(phen)₂(phendione)](PF₆)₂. In a reaction flask, [Ru(phen)₂Cl₂] (78.6 mg, 0.15 mmol) and 1,10-phenanthroline-5,6-dione (40.5 mg, 0.19 mmol) were dissolved in 6 mL of a 1:1 H₂O:EtOH mixture. The reaction was stirred for 5 h, after which an excess of NH₄PF₆ was added. Following overnight storage in a refrigerator, a brown precipitate was obtained. The solid was collected by vacuum filtration, washed with portions of cold H₂O, EtOH, and diethyl ether, and dried (123.2 mg, 86%). ¹H NMR (400 MHz, DMSO) δ 8.87 (dd, J = 8.4, 1.3 Hz, 2H), 8.75 (dd, J = 8.3, 1.3 Hz, 2H), 8.55 – 8.48 (m, 2H), 8.45 – 8.35 (m, 4H), 8.27 (dd, J = 5.2, 1.2 Hz, 2H), 8.02 – 7.94 (m, 4H), 7.90 (dd, J = 5.6, 1.3 Hz, 2H), 7.73 (dd, J = 8.3, 5.3 Hz, 2H), 7.58 (dd, J = 7.9, 5.6 Hz, 2H).

[Ru(bpy)₂(phendione)](PF₆)₂. The bipyridine analogue complex was synthesized following a procedure similar to that described for [Ru(phen)₂(phendione)₂], starting from [Ru(bpy)₂Cl₂] (193.2 mg 70%). ¹H NMR (400 MHz, DMSO) δ 8.85 (dd, J = 7.9, 2.9 Hz, 4H), 8.54 (dd, J = 8.0, 1.3 Hz, 2H), 8.20 (tdd, J = 8.0, 4.1, 1.5 Hz, 4H), 7.95 (dd, J = 5.7, 1.4 Hz, 2H), 7.82 – 7.66 (m, 6H), 7.56 (dtd, J = 7.3, 5.7, 1.2 Hz, 4H).

[Ru(phen)₂(dppz-COOH)](PF₆)₂. [Ru(phen)₂(phendione)₂] (30.1 mg, 0.030 mmol) and 3,4-diaminobenzoic acid (4.1 mg, 0.027 mmol) were dissolved in 5 mL of ethanol, and the mixture was

refluxed for 3 h. After cooling to room temperature, the resulting precipitate was collected by filtration and washed with portions of cold ethanol and diethyl ether (28.0 mg, 96.3%). ^1H NMR (400 MHz, DMSO) δ 9.63 (ddd, J = 10.6, 8.2, 1.3 Hz, 2H), 8.87 (s, 1H), 8.79 (td, J = 7.6, 1.3 Hz, 4H), 8.45 (dd, J = 8.8, 2.6 Hz, 1H), 8.40 (s, 4H), 8.29 (dd, J = 5.3, 1.2 Hz, 2H), 8.19 (dt, J = 5.4, 1.4 Hz, 2H), 8.06 (dt, J = 5.3, 1.4 Hz, 2H), 7.91 (dt, J = 8.3, 5.7 Hz, 2H), 7.85 – 7.73 (m, 4H).

$[\text{Ru}(\text{bpy})_2(\text{dppz-COOH})](\text{PF}_6)_2$. The bipyridine analogue complex was synthesized following a procedure similar to that described for $[\text{Ru}(\text{phen})_2(\text{dppz-COOH})]$, starting from $[\text{Ru}(\text{phen})_2(\text{phendione})_2]$ (79.0 mg, 84%). ^1H NMR (400 MHz, DMSO) δ 9.66 (ddd, J = 9.3, 8.2, 1.3 Hz, 2H), 8.97 – 8.80 (m, 5H), 8.59 (dd, J = 8.8, 1.8 Hz, 1H), 8.50 (d, J = 8.9 Hz, 1H), 8.27 – 8.18 (m, 4H), 8.13 (t, J = 7.9 Hz, 2H), 8.03 (dt, J = 8.2, 5.5 Hz, 2H), 7.83 (d, J = 5.5 Hz, 2H), 7.77 (dd, J = 5.8, 1.4 Hz, 2H), 7.60 (ddd, J = 7.2, 5.6, 1.3 Hz, 2H), 7.39 (dd, J = 7.5, 5.9 Hz, 2H).

1a. $[\text{Ru}(\text{phen})_2\text{Cl}_2]$ (106.5 mg, 0.2 mmol) and dppz (56.5 mg, 0.2 mmol) were dissolved in approximately 9 mL of an $\text{H}_2\text{O}/\text{EtOH}$ mixture (1:3). The reaction mixture was stirred for 4 h, after which the addition of an excess of NH_4PF_6 induced precipitation of the product as a bright red solid. The precipitate was collected by filtration, washed with small portions of cold water, ethanol, and diethyl ether, and dried (138.7 mg, 67.1%). ^1H NMR (400 MHz, DMSO) δ 9.62 (dd, J = 8.3, 1.3 Hz, 2H), 8.79 (ddd, J = 8.5, 7.4, 1.3 Hz, 4H), 8.53 (dd, J = 6.6, 3.4 Hz, 2H), 8.40 (s, 4H), 8.27 (dd, J = 5.4, 1.2 Hz, 2H), 8.23 – 8.13 (m, 4H), 8.06 (dd, J = 5.3, 1.2 Hz, 2H), 7.91 (dd, J = 8.2, 5.4 Hz, 2H), 7.79 (ddd, J = 15.8, 8.3, 5.3 Hz, 4H).

2a. The dppz (92.0 mg, 0.2 mmol) was dissolved in 8.5 mL of a $\text{MeOH}/\text{H}_2\text{O}$ mixture (1:1) and brought to reflux. The precursor $[\text{Ru}(\text{phen})_2\text{Cl}_2]$ (52.8 mg, 0.2 mmol) was then added, and the mixture was stirred under reflux for 3 h. After cooling to room temperature, the addition of an excess of NH_4PF_6 induced precipitation of the product as a bright orange solid. The precipitate was collected by filtration, washed with water, and subsequently crystallized from acetonitrile/diethyl ether (133.7 mg, 67.8%). ^1H NMR (400 MHz, DMSO) δ 9.65 (dd, J = 8.3, 1.3 Hz, 4H), 8.87 (dd, J = 11.9, 8.3 Hz, 2H), 8.53 (dt, J = 6.0, 3.0 Hz, 6H), 8.27 – 8.18 (m, 2H), 8.14 (td, J = 7.9, 1.5 Hz, 2H), 8.03 (dd, J = 8.1, 5.5 Hz, 2H), 7.80 (ddd, J = 24.1, 6.2, 1.5 Hz, 2H), 7.60 (ddd, J = 7.4, 5.7, 1.3 Hz, 2H), 7.39 (ddd, J = 7.4, 5.7, 1.3 Hz, 2H).

(2-aminoethyl)triphenylphosphonium. In a Schlenk flask 2-bromoethylamine hydrobromide (204.9 mg, 1 mmol) and triphenylphosphine (262.9 mg, 1 mmol) were dissolved in acetonitrile (11 mL) and the mixture was refluxed overnight. The resulting white precipitate was collected, dissolved in methanol, and reprecipitated with diethyl ether. This purification step was repeated three times (197.4 mg, 51.1%). ^1H NMR (400 MHz, DMSO) δ 7.95 (ddt, J = 9.8, 4.9, 1.7 Hz, 3H), 7.89 – 7.75 (m, 12H), 3.99 – 3.74 (m, 2H), 3.17 – 2.90 (m, 2H).

General procedure for compounds 1 and 2. The ruthenium precursor (1 equiv.), the (2-aminoethyl)triphenylphosphonium (1 equiv.), in the presence of HOBt·H₂O (3 equiv.) and EDC·HCl (3 equiv.), were dissolved in anhydrous DMF (ca. 2–3 mL). The reaction mixture was stirred at room temperature under an inert atmosphere (N₂) for 7 days. After this period, a large excess of NaPF₆ acetone (2 mL), and water were added to afford a precipitate. The solid was stirred in water for 30 min, collected by filtration, and washed with small portions of cold diethyl ether.

1. Compound **1** was obtained following the general procedure reported above. The ruthenium precursor [Ru(phen)₂(dppz-COOH)] (53.4 mg, 0.05 mmol, 1 equiv.), (2-aminoethyl)triphenylphosphonium (1 equiv.), HOBt·H₂O (3 equiv.), and EDC·HCl (3 equiv.) were dissolved in anhydrous DMF (2 mL). The title compound was obtained as a bright red solid (55.3 mg, 72%). ¹H NMR (400 MHz, DMSO) δ 9.59 (ddd, J = 16.3, 8.2, 1.3 Hz, 2H), 9.44 (t, J = 5.7 Hz, 1H), 8.80 (td, J = 7.4, 1.7 Hz, 5H), 8.59 (d, J = 9.0 Hz, 1H), 8.43 (d, J = 2.0 Hz, 1H), 8.41 (s, 4H), 8.27 (ddd, J = 8.7, 5.3, 1.2 Hz, 2H), 8.23 – 8.18 (m, 2H), 8.08 – 8.04 (m, 2H), 7.97 – 7.85 (m, 11H), 7.85 – 7.74 (m, 10H), 3.97 (dt, J = 13.8, 7.1 Hz, 2H), 3.76 (s, 2H). ESI-MS m/z calculated for [M]⁺: 358.7510, found: 358.7561. CHN for **1** + 3 H₂O C: 48.35% H: 3.28% N: 8.05%, exp C: 48.30% H: 3.14% N: 8.32%.

2. Compound **2** was obtained following the general procedure reported above. The ruthenium precursor [Ru(bpy)₂(dppz-COOH)] (62.8 mg, 0.06 mmol, 1 equiv.), (2-aminoethyl)triphenylphosphonium (1 equiv.), HOBt·H₂O (3 equiv.), and EDC·HCl (3 equiv.) were dissolved in anhydrous DMF (2.5 mL). The title compound was obtained as an orange solid (48.1 mg, yield 55%). ¹H NMR (400 MHz, DMSO) δ 9.66 – 9.55 (m, 2H), 9.45 (s, 1H), 8.88 (dd, J = 12.2, 8.1 Hz, 4H), 8.80 (s, 1H), 8.59 (d, J = 8.9 Hz, 1H), 8.44 – 8.40 (m, 1H), 8.29 – 8.21 (m, 4H), 8.14 (t, J = 7.9 Hz, 2H), 8.05 (dt, J = 8.3, 5.2 Hz, 2H), 7.97 – 7.86 (m, 9H), 7.85 – 7.73 (m, 10H), 7.63 – 7.58 (m, 2H), 7.39 (t, J = 6.7 Hz, 2H), 3.97 (dd, J = 13.8, 7.0 Hz, 2H), 3.76 (s, 2H). ESI-MS m/z calculated for [M]⁺: 342.7509, found 342.7511. CHN for **2** + H₂O C: 47.85% H: 3.20% N: 8.51%, exp C: 48.07% H: 3.20% N: 8.53%.

FRET melting assay. FRET melting experiments were performed in 96-well plates using an Applied Biosystems™ QuantStudio 6 PCR cycler equipped with a FAM filter. Dual-labeled oligonucleotides (FAM at the 5' end and TAMRA at the 3' end) were diluted to the desired concentrations in 60 mM potassium cacodylate buffer (pH 7.4). Oligonucleotides were folded into their respective B-DNA or G-quadruplex topologies by heating to 95 °C for 5 min, followed by slow cooling to room temperature overnight. The final oligonucleotide concentration was 0.2 μM in a total reaction volume of 30 μL per well. Metal complexes were dissolved in DMSO to prepare 2 mM stock solutions and then diluted

in buffer to the desired concentration, maintaining a final solvent content below 1% (v/v). Fluorescence data were recorded in duplicate over the 25–95 °C range, with a 1 °C increment every 30 s. Emission values were normalized from 0 to 1 to enable cross-experimental comparison. The melting temperature ($T_{1/2}$) was defined as the temperature at which the normalized FAM fluorescence reached 0.5. DNA concentrations are expressed in strands.

UV-Vis. UV-Vis absorption spectra were recorded at room temperature using a Cary 1E double-beam spectrophotometer. For stability studies, DMSO stock solutions of the complexes were diluted to approximately 50 μ M in Tris–HCl (50 mM) / KCl (100 mM) buffer (pH 7.4), and spectra were collected every hour over a 24 h period. For titration experiments, the concentration of the metal complex was kept constant while increasing amounts of G4-DNA or B-DNA were added to monitor binding interactions. To maintain the overall complex concentration during titration, each DNA addition was balanced with an equal volume of a double-concentrated metal complex solution. ODNs were resuspended in Tris–KCl buffer (50 mM Tris–HCl, 100 mM KCl, pH 7.4) to obtain 100 μ M stock solutions, expressed in strand concentration. G4 folding was achieved following the procedure described in the FRET-melting section.

Fluorescence. Fluorescence experiments were carried out on a Jasco FP-8300 spectrofluorometer using 1 cm quartz cuvettes. Parameters were set as follows: excitation bandwidth 5 nm, emission bandwidth 10 nm, response 0.1 s, medium sensitivity, and scan speed 1000 nm/min. Emission spectra were collected in the 265–700 nm range, using excitation wavelengths scanned every 10 nm. Measurements were performed on 5 μ M solutions of the ruthenium complex. Subsequently, 100 μ M of DNA (expressed in bases concentration) was added to the same solution, and the fluorescence spectrum was recorded again under identical instrumental conditions. Stock solutions of the complexes were prepared in DMSO and diluted in Tris–KCl buffer (50 mM Tris–HCl, 100 mM KCl, pH 7.4).

Circular Dichroism. CD spectra were acquired at 25 °C using a Jasco J-715 spectropolarimeter. In these experiments, the DNA concentration was fixed while increasing amounts of the metal complexes were added. Spectra were recorded in the 220–450 nm range, with a response time of 0.5 s, a scan speed of 200 nm·min^{−1}, and data averaged over four accumulations. As for UV-Vis experiments, ODNs were dissolved in Tris–KCl buffer (50 mM Tris–HCl, 100 mM KCl, pH 7.4) to yield 100 μ M strand-equivalent stocks and folded into G4 structures as described in the FRET-melting section.

MTT assay. HCT116 (colorectal cancer cells), A2780 and A2780cis ovarian cancer cells were seeded in 96-well plates at a density of 4×10^3 cells per 100 μ L and allowed to adhere overnight. Cells were then exposed to the compounds at the indicated concentrations for 72 h. Combination studies were carried out by treating the cells simultaneously with both compounds at the indicated concentrations. Cell viability was determined using the MTT-based EZ4U assay (Biomedica, Vienna, Austria), following the manufacturer's instructions. Absorbance was recorded at 450 nm with a reference wavelength of 620 nm using a Spark® multimode microplate reader (Tecan, Switzerland). Data were analyzed in GraphPad Prism. Data represent at least three independent biological replicates per condition (n = 3-6, depending on the compound).

ATP assay. ATP release was measured using the RealTime-Glo™ Extracellular ATP Assay (Promega), following the manufacturer's instructions. Briefly, HCT116 cells were seeded in tissue-culture treated 96-well plates at a density 4×10^3 cells per 100 μ L and allowed to adhere overnight. On the day of treatment, cells were incubated with serial dilutions of the compounds – the ATP reagent was not added at this stage. The lyophilized reagent was solubilized with the Kit buffer. After 72h of drug exposure, 120 μ L of the SN were discarded, 50 μ L of ATP solution was added to each well and then 80 μ L of the mixture was transferred to opaque white 96-well plates. This was also measured at the Spark® multimode microplate reader (Tecan, Switzerland). In the presence of the RealTime-Glo™ reagent, freshly prepared at a 4X working concentration in culture medium. Untreated cells and vehicle controls were included in parallel. After 72 hours, luminescence was recorded using a plate reader Asys Expert Plus microplate reader (v1.4, Asys Hitech GmbH, Eugendorf, Austria). Raw data (relative luminescence units, RLU) were normalized to untreated controls.

ICP-MS. HCT116 cells were seeded at a density of 7.5×10^5 cells/mL in 6-well plates (2 mL per well) and allowed to adhere overnight. Cells were treated with the compounds at the specified concentrations for 5 or 24 h, with fresh culture medium serving as the solvent control. Following incubation, cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) at room temperature, detached by trypsinization, and counted. Cell pellets were then washed twice with PBS and left to dry overnight at room temperature. The next day, dried pellets were digested in 65% HNO₃ for 1 h and subsequently diluted with double-distilled water (ddH₂O) prior to ICP-MS analysis. Ruthenium levels were normalized to the corresponding cell counts for each condition.

Colony Formation Assay. Cells were seeded in 24-well plates at a density of 1,000–2,500 cells per well in 500 μ L of growth medium and allowed to recover for 24 h in the incubator. Subsequently, cells were treated with the indicated drugs at increasing concentrations. After 7–10 days (depending on the colony formation capacity of the control), cells were fixed with ice-cold methanol for 30 min at 4 °C and stained with 0.01% crystal violet in PBS. Fluorescence from the stained colonies was detected using a Typhoon TRIO Variable Mode Imager (GE Healthcare Life Sciences) with excitation at 633 nm. Quantification was performed using ImageJ software.

Seahorse. Cells were seeded at a density of 150,000 cells/mL in 80 μ L in Seahorse XFp Cell Culture Microplates to reach ~80–90% confluence and incubated overnight. The sensor cartridge was hydrated with Calibrant and incubated at 37 °C in a non-CO₂ incubator. On the following day, the assay medium (1 mM pyruvate, 2 mM glutamine, 10 mM glucose) was prepared and added to each well after one washing steps. For the Seahorse XF Mito Stress Test, inhibitor solutions were prepared to achieve final concentrations of 1.5 μ M oligomycin, 2 μ M FCCP, and 0.5 μ M rotenone/antimycin A, and were loaded into the designated injection ports of the sensor cartridges (as 10x stocks) Hoechst 33258 Staining Dye Solution (Abcam, Cambridge, UK) was added to port C with rotenone/antimycin A. at a final concentration of 4 μ M (1 mg ml⁻¹ in PBS, for quantification of cell numbers) Hoechst 33258 staining was used to normalize data to cell number using the Cytation imaging system and Gen5 software. Data acquisition and analysis were carried out with Wave Pro software (version 10.1.0.1) using the Seahorse XF Cell Mito Stress Test protocol.

Wound healing assay. Cell migration was evaluated using the Incucyte® 96-Well Scratch Wound Cell Migration and Invasion Assay (Sartorius). HCT116 cells were seeded in 100 μ L aliquots (50000 c/well) in 96-well ImageLock plates (Sartorius) and incubated overnight. The following day, uniform wounds were generated in each well using the Incucyte® WoundMaker tool. Detached cells were removed by gentle washing with phosphate-buffered saline (PBS), and fresh culture medium containing the test compounds at the indicated concentrations (or vehicle control) was added. Plates were placed in the Incucyte® Live-Cell Analysis System and imaged every hour over a period of 60 hours. Wound confluence and migration kinetics were automatically quantified using the Incucyte® Scratch Wound Cell Migration Analysis Software Module. Migration inhibition was calculated relative to untreated controls.

Spheroid formation assay. HCT116 cells were seeded in 100 μ L aliquots (3500 c/well) in 96-well round-bottomed ultra-low attachment plates (Corning 7007) and treated with the substances at the

same time. Spheroid formation was monitored by IncuCyte® (Sartorius) for 5 days, imaged at regular intervals (every 2 hours). Untreated spheroids were used as controls. Spheroid size was quantified automatically using the Incucyte® software module for spheroid analysis, and growth curves were plotted over time. For spheroid replating assays, mature spheroids were harvested and replated in standard 2D culture conditions. Cell outgrowth and viability were monitored using the Incucyte® system.

Quantitative RT-PCR. HCT116 cells were seeded at a density of 350,000 cells per well in six-well plates. After 24 h of recovery, cells were treated with the compounds at the indicated concentrations. Following 24 h of exposure, total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, MA, USA), according to the manufacturer's protocol. Quantitative real-time PCR was performed using the GoTaq® RT-qPCR system (Promega) with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). Gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, with RPL41 serving as the housekeeping control. Human-specific primers had the following sequences: RPL41: fwd (5'-CAAGTGGAGGAAGAAGCGA-3'), rev (5'-TTACTTGGACCTCTGCCTC-3'); MYC fwd (5'-ACCCTTGCCGCATCCACGAAAC-3'), rev (5'-CGTAGTCGAGGTCATAGTTCCTGTTGG-3'); BCL2 fwd (5'-CTTCGCCGAGATGTCCAGCCA-3'), rev (5'-CGCTCTCCACACACATGACCC-3'); ATP6 fwd (5'-CCCTAGCAATATCAACCATTAACC-3'), rev (5'-TAGAAGTGTGAAAACGTAGGCTTG-3'); ND2 fwd (5'-CCGGACAATGAACCATAACC-3'), rev (5'-TCAGAAGTGAAAGGGGGGCTA-3'); ND3 fwd (5'-CCCTCCTTTTACCCCTACCA-3'), rev (5'-GGCCAGACTTAGGGCTAGGA-3').

4. Conclusions

This chapter reported the design, synthesis, and characterization of two mitochondria-addressed Ru(II) polypyridyl-dppz conjugates bearing a triphenylphosphonium (TPP) moiety (**1** and **2**). The complexes are chemically stable in aqueous buffer and display the expected spectroscopic features, including a modest “light-switch” response upon DNA addition. FRET-melting assays demonstrated concentration-dependent binding to G4 models derived from mitochondrial genes, along with detectable effects on a duplex reference, while CD spectroscopy revealed ICD signals indicative of direct binding and structural perturbation of both B-DNA and G4 structures.

Complexes **1** and **2** exert cytostatic effects across 2D and 3D biological models. Live-cell assays revealed impaired migration (scratch wound), suppression of clonogenic survival (colony formation), and reduced growth of 3D spheroids. The stabilization of mitochondrial G4s in solution, combined

with the transcriptional repression of mtDNA-encoded genes (*ATP6*, *ND2*, *ND3*), supports a mechanism involving interference with mitochondrial gene expression. The compounds impair both oxidative phosphorylation and glycolysis, leading to sustained inhibition of cancer cell growth and invasion without inducing acute cytotoxicity. Notably, metabolic co-treatments revealed that glucose uptake inhibition (via **BAY-876**) or mitochondrial respiration blockade (via **metformin**) can enhance these effects. When tested as single agents, compound **1** consistently exhibited a stronger cytostatic effect than compound **2**, in line with its higher cellular accumulation and more pronounced impact on mitochondrial function. In contrast, in combinatorial assays, both complexes displayed synergistic interactions with **BAY-876**, while only compound **2** showed synergy with **metformin**. Overall, the Bliss synergy scores were higher for compound **2** than for compound **1**, indicating a greater propensity of compound **2** to potentiate its activity under metabolic co-treatment conditions.

Collectively, these results validate **1** and **2** as dual-mode agents that recognize and stabilize G4 motifs relevant to the mitochondrial genome and disrupt mitochondrial bioenergetics in cancer cells, with exploitable synergy with metabolic modulators. These findings motivate ligand and scaffold refinement to enhance G4 selectivity and organelle targeting, and they provide the rationale for the subsequent exploration of complementary Ru(II) architectures (Chapter II) to delineate structure-activity relationships and broaden therapeutic potential. Importantly, some additional studies on this project are still ongoing, and the present conclusions should be viewed as an evolving body of evidence.

Chapter II: Ruthenium(II) Arene Complexes with Mitochondrial Targeting Moieties for G-Quadruplex Binding

1. Introduction

Building on the previous work with Ru(II) polypyridyl complexes functionalized with triphenylphosphonium moieties for mitochondrial targeting (see Chapter I), the same strategy was extended to a different Ru(II) coordination scaffold. While octahedral polypyridyl complexes provide well-established platforms for DNA recognition and photophysical studies, half-sandwich Ru(II) p-cymene “piano-stool” complexes offer complementary features, including enhanced stability, modularity, and distinct modes of biological interaction and still remain relatively unexplored in the G4 field. In these systems, the η^6 -arene ligand (such as p-cymene) provides structural rigidity, lipophilicity, and stability under physiological conditions, while the remaining coordination sphere accommodates extended aromatic ligands for nucleic acid binding. In particular, incorporation of dipyrrophenazine (dppz) derivatives into Ru(II) arene scaffolds yields complexes with the potential for intercalation into DNA. Functionalization of the dppz unit with mitochondria-targeting groups further enhances organelle accumulation, leveraging the negative mitochondrial membrane potential to achieve selective delivery. Such designs are especially relevant given the emerging therapeutic interest in mitochondrial G4s structures.

This work reports the synthesis of the first Ru(II) p-cymene-dppz complex functionalized for mitochondrial targeting and evaluate its DNA-binding properties across B-DNA and mtDNA-derived G4 models. In parallel, its biological activity in cancer cell models was evaluated, with emphasis on mitochondrial function.

2. Results and Discussion

Synthesis and characterization.

A new mitochondria-targeted Ru(II) p-cymene complex was synthesized, featuring Ru(II) coordinated to a TPP-functionalized dppz ligand and a labile chlorido ligand. The precursor $[[\text{Ru}(\eta^6\text{-p-cymene})(\text{dppzCOOH})]^+$ was first prepared from the corresponding Ru dimer. Subsequently, following the amide-coupling strategy described in Chapter I, the carboxylic group of the Ru complex was conjugated to a TPP-bearing primary amine, affording the final compound $\text{Ru}(\eta^6\text{-p-cymene})(\text{dppz-TPP})^{2+}$ (**3**) (Figure 39). Compound **3** was isolated as the hexafluorophosphate salt and fully characterized by ^1H NMR spectroscopy (Figure A.3, Appendix A), ESI-MS (Figure B.3, Appendix B), and elemental analysis, which confirmed the expected structure and purity.

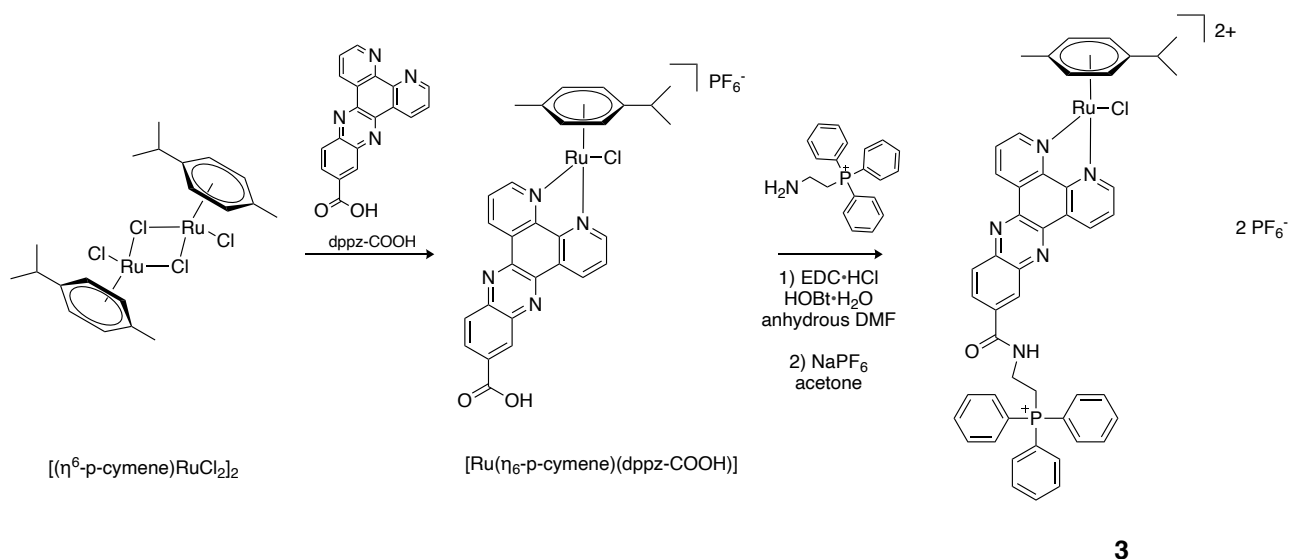


Figure 39. Synthetic route for compound **3**.

As a benchmark for this study, the corresponding complex bearing an unfunctionalized dppz ligand, $[\text{Ru}(\eta^6\text{-p-cymene})(\text{dppz})]^+$ (**3a**) (Figure 40), was prepared following established literature procedures, as detailed in the Experimental Section.

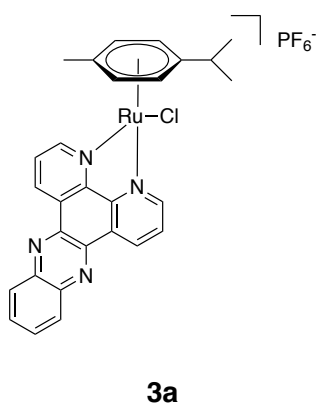


Figure 40. Chemical structure of $[\text{Ru}(\eta^6\text{-p-cymene})(\text{dppz})]^+$ (**3a**), used as benchmark in this work.

Stability in aqueous solutions.

The stability of compound **3** was assessed by UV-Vis spectroscopy, showing no significant spectral changes over 24 hours in Tris-KCl buffer (Figure 41). Notably, compound **3** exhibits an absorption band centered around 390 nm, which can be attributed to metal-to-ligand charge transfer (MLCT) transitions.

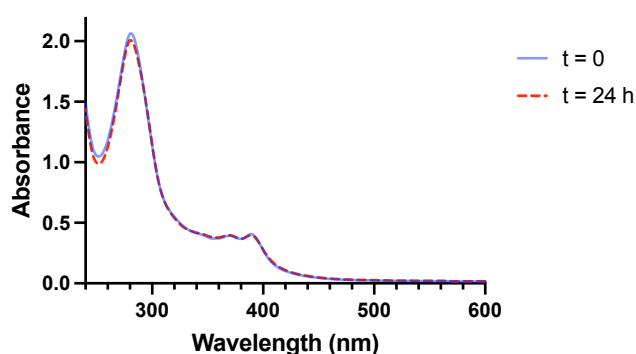


Figure 41. UV-Vis spectra of compound **3** in Tris–KCl buffer (50 mM Tris–HCl, 100 mM KCl, pH 7.4) at the two indicated time-points.

DNA interaction studies.

The interaction of compound **3** with DNA was evaluated by FRET-melting experiments using three mitochondrial G4-forming sequences, namely atp6, nd2, and nd3, derived from the corresponding mitochondrial genes.²⁴ In addition, three nuclear G4-forming sequences were included: kit1 and kit2, from the promoter region of the *KIT* oncogene, and htelo, from human telomeric DNA.^{151,152} A self-complementary duplex-forming sequence (dsDNA) was also tested as a B-DNA reference control. Compound **3** induced a clear stabilization of all tested oligonucleotides, with a notable preference for the G4 structures, particularly htelo and atp6, over duplex DNA (Figure 42a). Among the G4s, comparable ΔT_m increases were observed for both mitochondrial and nuclear sequences, indicating that compound **3** efficiently recognizes diverse quadruplex topologies. In contrast, stabilization of the duplex dsDNA was less pronounced, confirming selective affinity toward G4 conformations. Furthermore, the mitochondrial G4s were incubated with increasing concentrations of compound **3**, revealing a strong concentration-dependent stabilization. At the highest tested concentration, ΔT_m values between 20 and 40 °C were recorded, highlighting the strong G4-stabilizing capacity of the compound (Figure 42b).

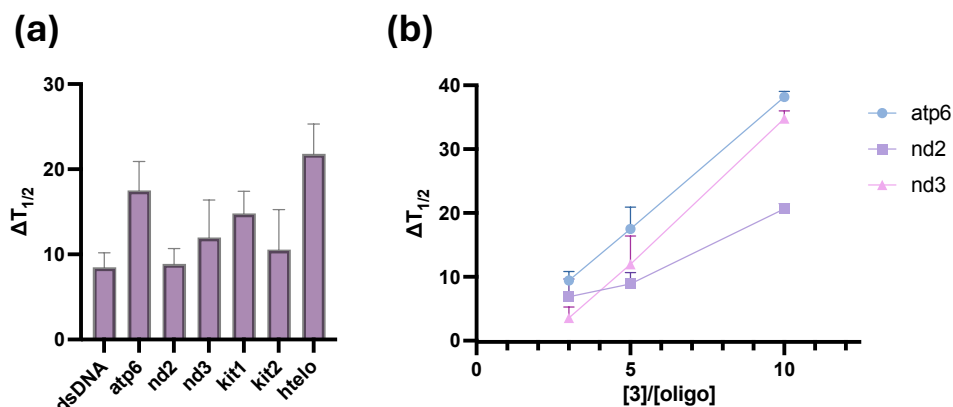


Figure 42. FRET-melting analysis of the interaction between compound **3** and DNA oligonucleotides. (a) ΔT_m values for mitochondrial (atp6, nd2, nd3) and nuclear G4-forming sequences (kit1, kit2, htelo), and for a duplex control (dsDNA) after incubation with **3** (1 μ M complex, 0.2 μ M oligonucleotide, expressed per strands). (b) Concentration-dependent stabilization (ΔT_m) of mitochondrial G4 sequences upon increasing concentrations of **3**.

To further investigate the structural impact of compound **3** on DNA, CD titrations were performed using the same set of oligonucleotides analyzed by FRET-melting. For the B-DNA reference sequence (ds26), only minor spectral variations were detected upon addition of **3**, indicating the absence of significant conformational perturbation in duplex DNA even at higher compound-to-DNA ratios (Figure 43a). In light of the dramatic B-DNA effects previously observed for **1** and **2** (see Chapter I), these data further support the notion that the dppz–TPP ligand does not intercalate between base pairs. In this piano-stool scaffold, the p-cymene arene and overall steric profile appear to disfavor duplex intercalation, thereby preserving B-DNA topology. In contrast, all three mitochondrial G4-forming sequences exhibited alterations in their characteristic CD profiles upon titration with the **3** (Figure 43b-d), primarily reflected in changes in band intensity. Among the tested sequences, atp6 displayed the most pronounced modifications, consistent with the higher stabilization recorded in the FRET-melting assay and pointing to a preferred interaction with the antiparallel topology. For nd2 (parallel), the appearance of a shoulder near ~ 290 nm may indicate an increased antiparallel contribution, suggestive of a topology shift induced by **3**. For the hybrid nd3 sequence, **3** enhanced the parallel component relative to the antiparallel one. Overall, these observations indicate that compound **3** is selective for mitochondrial G4s over duplex DNA, with a marked preference for the atp6 G4. As with **1** and **2**, it cannot be excluded that the observed spectral changes partly arise from ICD signals overlapping DNA's intrinsic near-UV CD, or from a combination of ICD effects and topology rearrangements. Nevertheless, the magnitude and consistency of the atp6 response underscore a higher affinity/selectivity of **3** for the antiparallel atp6 quadruplex.

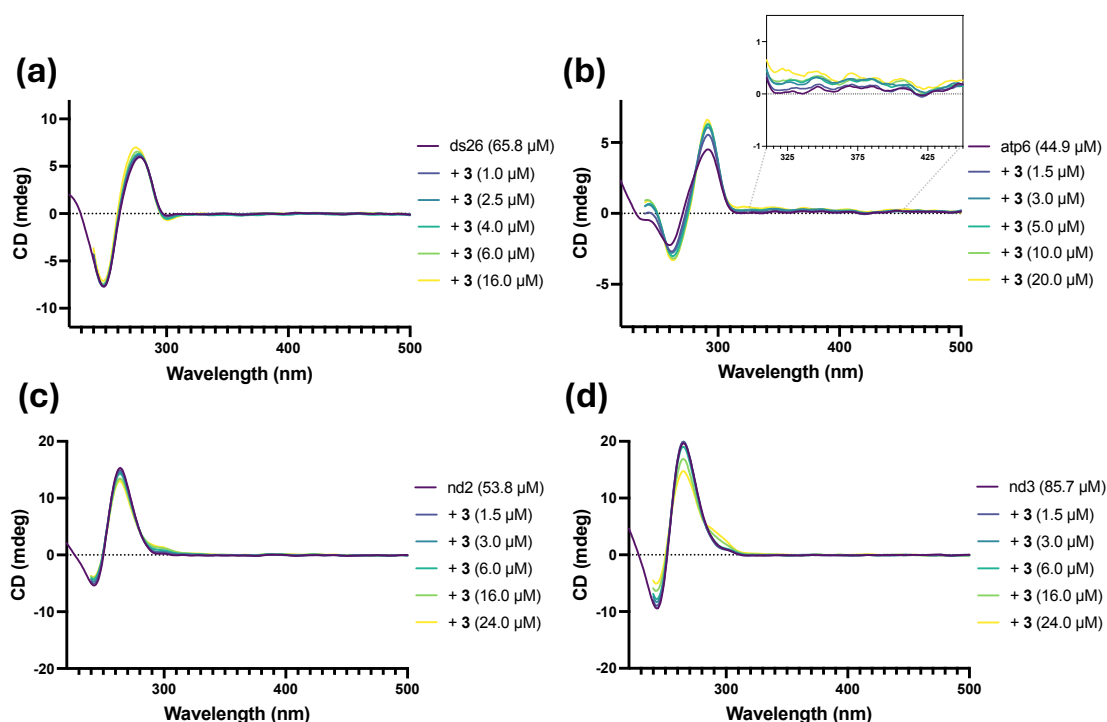


Figure 43. CD spectra of ds26, atp6, nd2 and nd3 with increasing amount of **3**. Inset: magnified view of the ICD region (310-450 nm). ODN concentrations are reported per bases.

The emission intensity of compound **3** was first measured in Tris-KCl buffer and then in the presence of B-DNA to assess its potential for a light-switch effect. Upon DNA addition, only a modest enhancement of fluorescence was observed ($\sim 4.1\%$), confirming the relatively low affinity of the compound towards the B conformation (Figure 44).

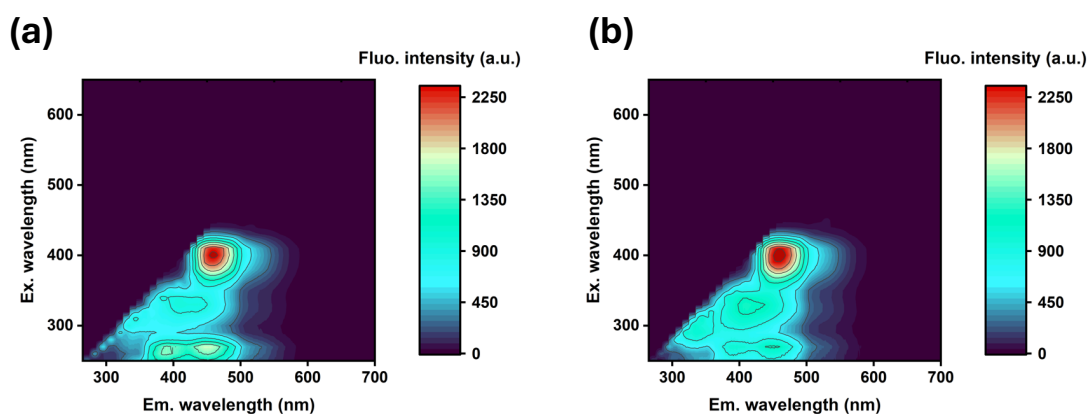


Figure 44. Emission contour plots of **3** recorded in Tris-KCl buffer (50 mM Tris-HCl, 100 mM KCl, pH 7.4) before (a) and after addition of ct-DNA (b). $[\mathbf{3}] = 5 \mu\text{M}$, $[\text{ct-DNA}] = 100 \mu\text{M}$ (expressed per strand).

Biological evaluation.

To determine whether the mitochondrial-targeting design of compound **3** translates into biological activity, cellular uptake and cytotoxicity in cancer cell lines were firstly evaluated. The intracellular accumulation of ruthenium was quantified in HCT116 cells following incubation with compound **3** at three different concentrations (10, 25, and 50 μM) and two time points (5 and 24 h) (Table 3). The cellular uptake was found to be both concentration- and time-dependent, with markedly higher Ru levels detected at longer incubation times. When compared with the previously investigated polypyridyl complexes **1** and **2** (Chapter I), compound **3** exhibited substantially higher cellular accumulation, indicating improved internalization efficiency.

Compound	Concentration (μM)	Ru(ng)/ 10^6 cells	
		5 h	24 h
3	10	2.2 ± 0.1	3.2 ± 0.1
	25	5.9 ± 1.0	9.2 ± 0.2
	50	11.0 ± 0.1	<i>n.d.</i>

Table 3. Intracellular ruthenium uptake in HCT116 after incubation with compound **3** at the indicated concentration and times (*n.d.*, not determined). Values are reported as mean $\pm$ SD.

The cytotoxicity of compound **3** and **3a** was first evaluated in HCT116 cells across the 0-50 μM concentration range using the MTT assay. While compound **3a** displayed measurable activity, with an IC_{50} of 20.0 μM (*data not shown*), compound **3** produced no significant reduction in cell viability within the same concentration range (Figure 45a). However, ATP quantification assays revealed an intrinsic cytotoxic activity of compound **3**, characterized by a marked, concentration-dependent decrease in intracellular ATP levels relative to untreated controls, yielding an IC_{50} value of 4.8 μM (Figure 45b). Notably, the ATP response curve did not approach complete depletion, but instead reached a plateau at higher concentrations, indicating a saturation of the compound's effect. This behavior suggests that compound **3** exerts a cytostatic rather than fully cytotoxic response. Moreover, the discrepancy between the MTT and ATP assays likely reflects an early impact of compound **3** on mitochondrial bioenergetics, consistent with its designed mitochondrial targeting properties and similar to what was previously observed for compounds **1** and **2**, where metabolic impairment preceded the loss of overall cell viability.

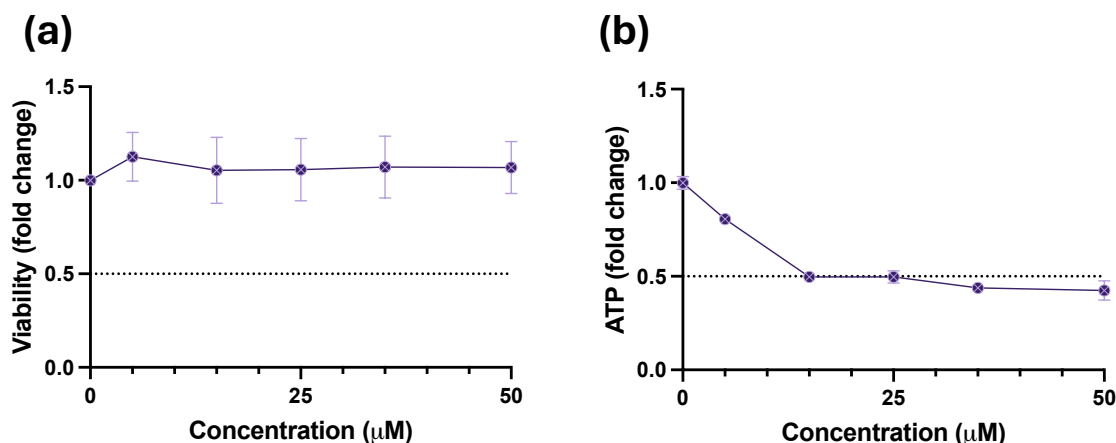


Figure 45. (a) MTT assay and (b) ATP assay measured after 72 hours incubation with **3**. Data, normalized to the untreated control, are expressed as mean $\pm$ SD.

After confirming cellular uptake and activity of complex **3**, its impact on cell proliferation was subsequently assessed through real-time confluency measurements using the IncuCyte® live-cell imaging system. At 5 μM , **3** produced no appreciable effect on cell confluency, whereas at 50 μM it caused a delay in the increase of confluency relative to the untreated control. This behavior is consistent with a cytostatic rather than cytotoxic response. Over time, the treated cells gradually resumed proliferation and ultimately reached confluency levels comparable to the control at the endpoint, attaining nearly full confluency (Figure 46a). Quantitative analysis of cumulative proliferation, expressed as the area under the confluency curves (Figure 46b), confirmed the transient nature of the effect, with the AUC for compound **3** at 50 μM reduced by approximately 18% relative to the untreated control.

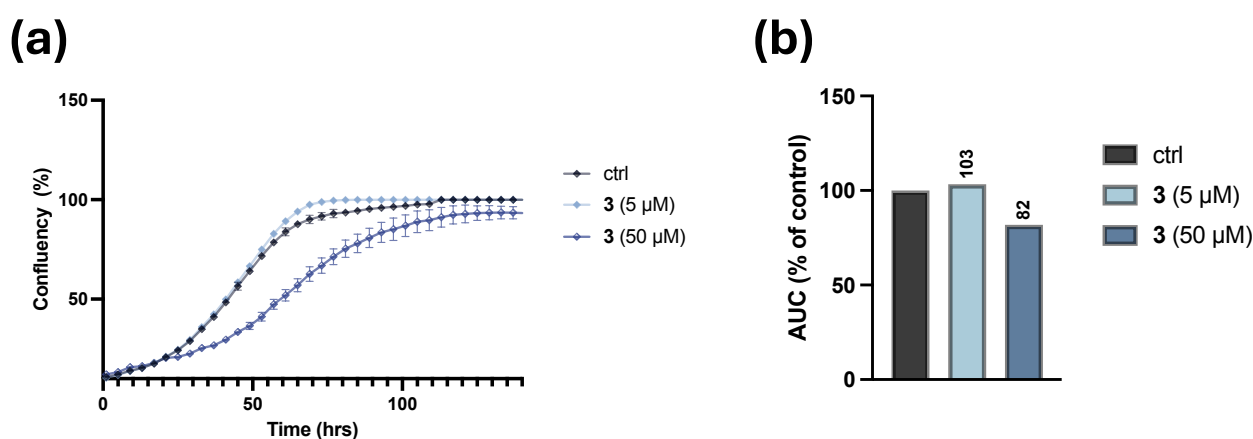


Figure 46. (a) Time-course of confluency (%) in HCT116 cells treated with **3** at the two indicated concentration, over 6 days; data are expressed as mean $\pm$ SD. (b) Quantification of proliferation expressed as the area under the confluency curves (AUC), normalized to the untreated control (%).

To further evaluate the long-term antiproliferative potential of compound **3**, a colony formation (clonogenic) assay was performed to assess the ability of treated cells to survive and form colonies after prolonged exposure. In this assay, compound **3** exerted a pronounced cytostatic effect, resulting in a significant reduction in the overall staining density of colonies after one week of incubation. At the lowest concentration (10 μ M), compound **3** produced only a minor reduction in colony formation, whereas at the highest concentration tested (25 μ M), the colony density decreased by approximately 72% relative to the untreated control (Figure 47). This marked decrease in colony-forming efficiency indicates that compound **3** compromises the long-term proliferative capacity of HCT116 cells, even in the absence of pronounced acute cytotoxicity.

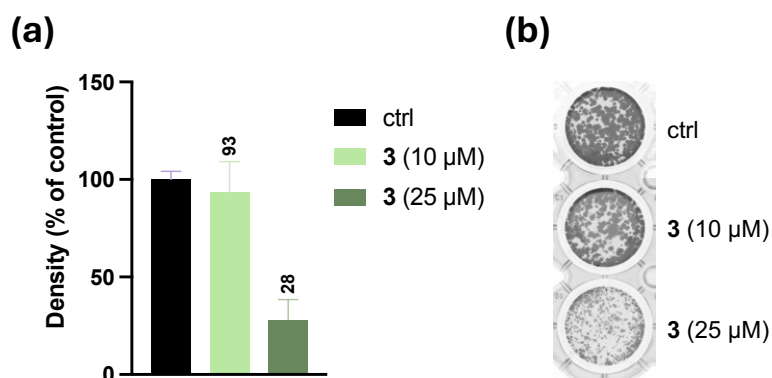


Figure 47. (a) Colony formation assay in HCT116 cells after treatment with compound **3**. Data, normalized to the untreated control, are expressed as mean $\pm$ SD (b) Representative stained wells of control and treated cells. Quantification of colony number and area was performed using ImageJ.

Furthermore, spheroid formation and subsequent replating assays were conducted to assess the impact of treatment on tumor-like growth, recovery capacity, and clonogenic potential under more physiologically relevant conditions. In the spheroid formation assay, cells were seeded and treated simultaneously to determine whether drug exposure affected spheroid development. Spheroid growth was then monitored over four days using the Incucyte® live-cell imaging system. (Figure 48a). To enable a meaningful comparison between the two compounds, the spheroid growth assay was performed using a lower concentration of **3a** (5 μ M) relative to **3** (10 μ M), reflecting the higher intrinsic potency of the non-targeted analogue observed in 2D viability assays. Under these conditions, compound **3** did not markedly affect spheroid growth during the early phase of the experiment, as indicated by the similar shape of the growth curves and the comparable AUC values extrapolated from them (Figure 48b). However, at the endpoint, spheroids treated with compound **3** were approximately 19% smaller than the untreated control, suggesting a measurable late-onset growth-inhibitory effect (Figure 48c). This delayed response likely reflects a cumulative cytostatic effect emerging at later stages of growth. Conversely, spheroid growth was progressively affected by

compound **3a** throughout the entire duration of the experiment, although it ultimately led to a comparable reduction in spheroid size at the endpoint.

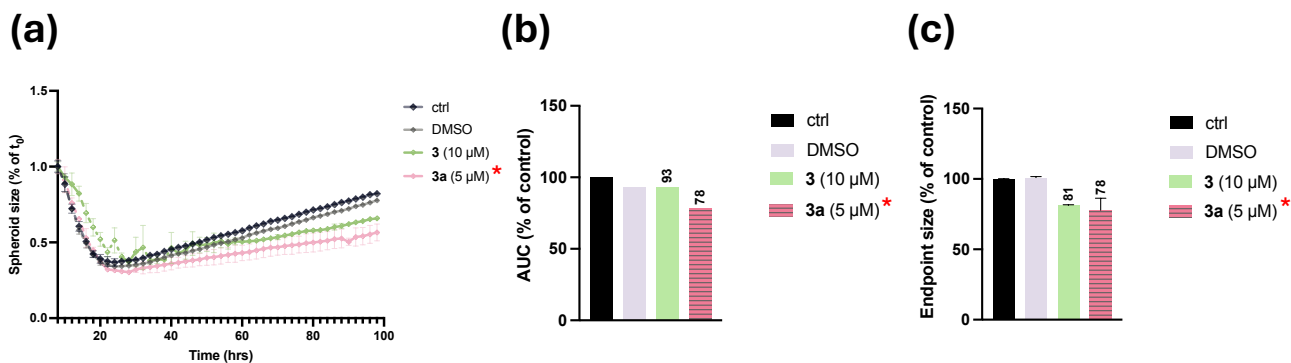


Figure 48. Spheroid formation assay with HCT116. (a) Representative spheroid growth curves normalized to the first measurable time point (8 h, set to 1), data are expressed as mean $\pm$ SD. (b) AUC values calculated from the normalized curves in (a) and expressed as percentage of the untreated control. (c) Endpoint spheroid size at the final time point, expressed as percentage of the control. *Note: a lower concentration of **3a** (5 μ M) was used due to its higher intrinsic activity.

To further evaluate the invasive potential of the treated cells, spheroids previously exposed to compounds **3** and **3a** were replated in adherent conditions, and the outgrowth of invading cells was monitored over ~90 hours (Figure 49a). Treatment with compound **3** produced a modest reduction in cell outgrowth, with both AUC and endpoint values decreased by approximately 20% relative to the untreated control. Conversely, the inhibitory effect previously observed in spheroid growth upon treatment with compound **3a** was not maintained after replating. In this case, the outgrowth kinetics of **3a**-treated spheroids closely overlapped with those of the untreated control, indicating a loss of inhibitory activity under adherent conditions, at later time points following treatment (Figure 49b,c).

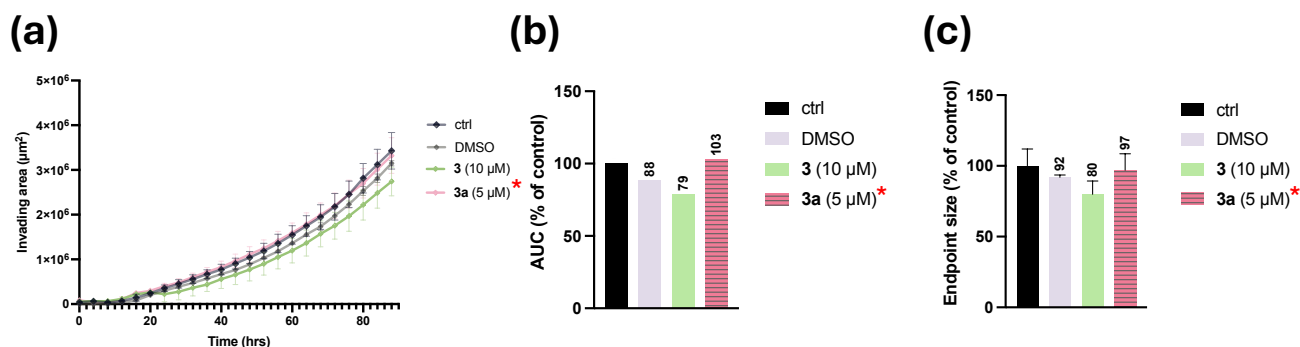


Figure 49. Spheroid replating assay with HCT116. (a) Raw invasion kinetics showing the expansion of the invading cell area over time; data are expressed as mean $\pm$ SD. (b) Quantification of the cumulative invasion, expressed as the area under the curve (AUC) from panel (a) and normalized to the untreated control. (c) Endpoint invading area at the final time point, expressed as percentage of the control.

The impact of **3** and **3a** on cell migration was further assessed through scratch-wound assays, where the rate of wound closure served as an indicator of cell migratory capacity under the different treatment conditions (Figure 50). The experiment was conducted using three concentrations of compound **3** (10, 25, and 50 μM) and a single concentration of **3a** (10 μM). Compound **3** produced only a minor effect on wound closure, with a measurable reduction in migration observed at 50 μM , corresponding to a 16% decrease in the AUC compared to the untreated control. In contrast, treatment with **3a** (10 μM) led to a more pronounced inhibition of migration, resulting in a 27% reduction in AUC relative to the control.

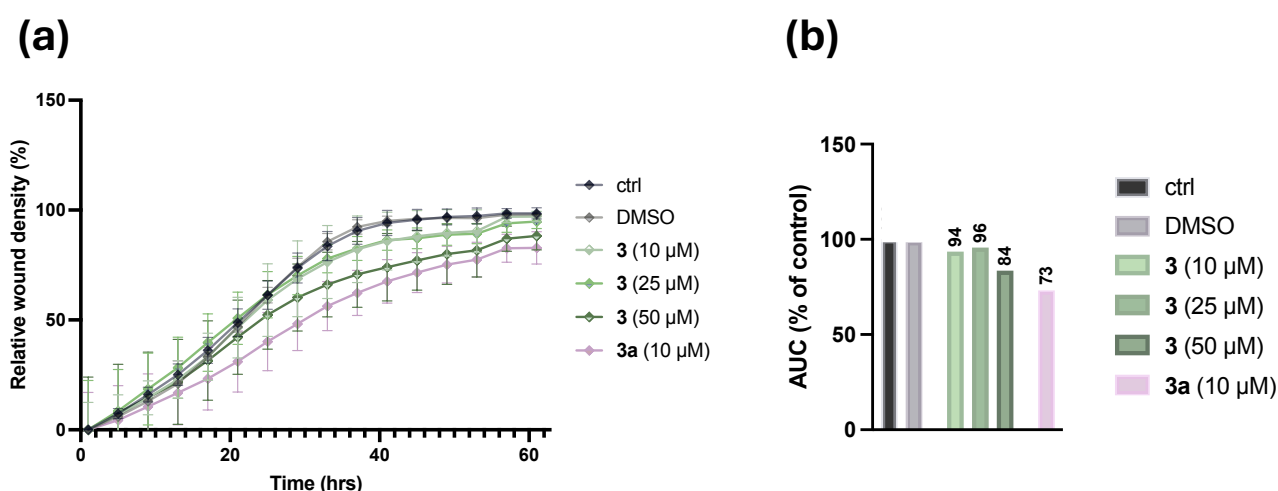


Figure 50. Scratch (migration) assay performed with HCT116 cells after treatment with compound **3** (10, 25, and 50 μM) or **3a** (10 μM). Time-course of wound closure is represented as relative wound density, automatically normalized to cell confluence outside the wound. Additionally, relative wound density was normalized to the initial time point to correct for minor differences in scratch width. Data are expressed as mean $\pm$ SD. (b) AUC values from (a), expressed as percentage of the control.

Consistent with the mitochondrial-targeting rationale guiding the design of compounds **3**, its effects on mitochondrial respiration and glycolytic activity were assessed in HCT116 cells using the Seahorse XF Mito Stress Test. HCT116 cells were treated with two increasing concentrations of compound **3** (5 and 50 μM) and with a single concentration of **3a** (5 μM), as higher doses of the latter caused complete loss of cell viability. As shown in Figure 51, compound **3** induced a reduction in the OCR, with a marked decrease in both basal and maximal respiration at 50 μM , indicating an impairment of mitochondrial oxidative phosphorylation. The effect on the extracellular acidification rate (ECAR) was less pronounced, suggesting that glycolytic compensation remained only partially affected. Compound **3a** produced a similar pattern of metabolic inhibition, but at substantially lower concentrations, consistent with its higher intrinsic potency.

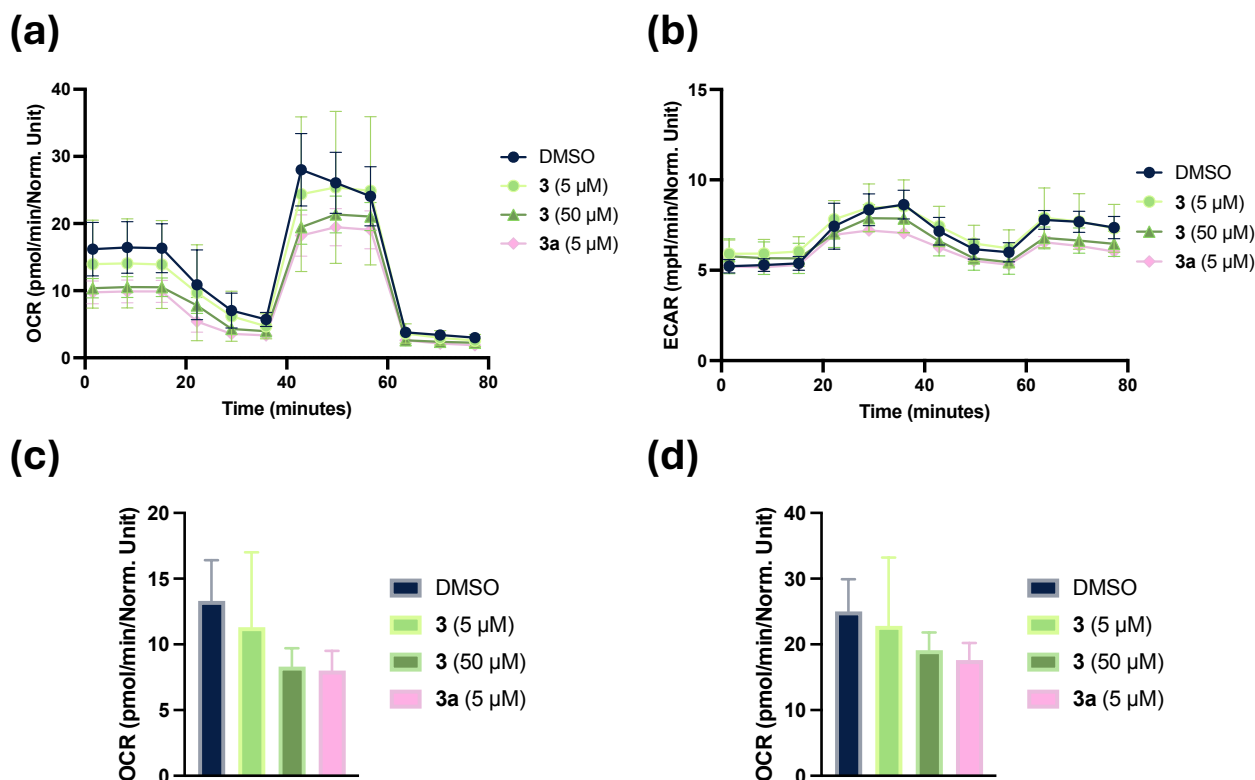


Figure 51. Seahorse Mito-Stress Test. (a) Oxygen consumption rate (OCR) profiles over time for cells treated with compounds **3** and **3a**. (b) Extracellular acidification rate (ECAR) profiles over time for the same compound sets, reflecting glycolytic activity during mitochondrial stress. Quantification of basal respiration (c) and maximal respiration (d) extracted from the OCR curves for all compounds. OCR and ECAR measurements were normalized to cell number to account for variations in cell density across wells. Data are expressed as mean $\pm$ SD.

To further explore the biological consequences of mitochondrial targeting, quantitative PCR experiments were performed to assess the mRNA expression levels of the selected mitochondrial genes. Quantitative RT-PCR analysis revealed that treatment with compound **3** caused a marked downregulation of the mitochondrial genes *ATP6*, *ND2*, and *ND3*, whose expression levels were almost completely suppressed. However, compound **3** produced also a moderate decrease in the expression of the nuclear genes *MYC* and *BCL2*, indicating a broader, less selective impact on gene regulation (Figure 52). The pronounced repression of mitochondrial transcripts further supports the strong interference of compound **3** with mtDNA transcription, supporting the initial hypothesis of stabilization of mitochondrial G4s, while the partial effect on nuclear genes suggests an extended interaction spectrum or secondary metabolic consequences.

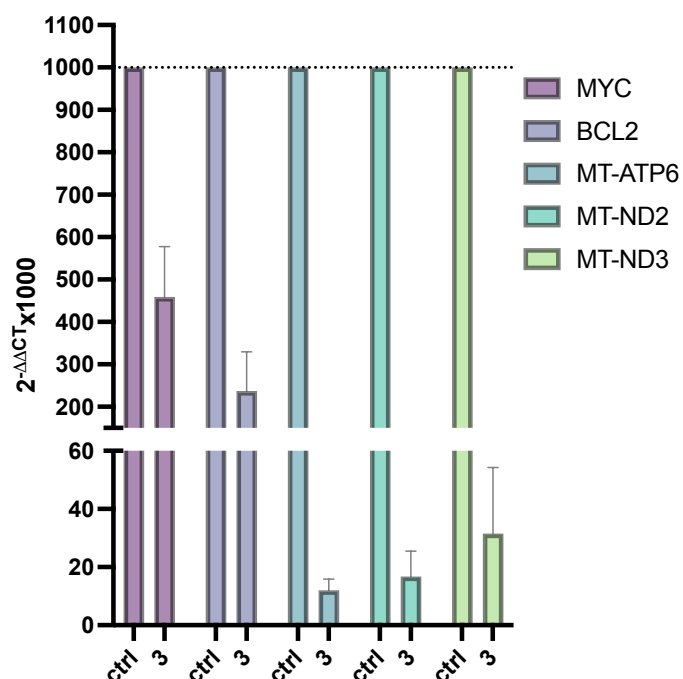


Figure 52. Relative mRNA expression levels of mitochondrial genes (ATP6, ND2, ND3) and nuclear genes (MYC, BCL2) in HCT116 cells treated with compound **3**. Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method. RPL41 was used as housekeeping gene. Data, normalized to the untreated control, are expressed as mean $\pm$ SD.

Building on the approach described in Chapter I for compounds **1** and **2**, combination assays were performed with the metabolic modulators **metformin** and **BAY-876** to further investigate the mechanism of action and potential metabolic interactions of compound **3**. These experiments aimed to determine whether compound **3** could act synergistically with metabolic inhibitors to enhance anticancer efficacy, as previously observed for its polypyridyl analogues.

The combined effects of compound **3** and **metformin** were firstly evaluated by MTT assay after 72 hours of co-treatment. The assay revealed a clear reduction in cell viability compared to the single-agent treatments (Figure 53a). Quantitative analysis using the Bliss independence model confirmed a synergistic interaction, with Bliss scores reaching up to +0.36 at the highest concentrations of both drugs (**[3]** = 50 μ M; **[metformin]** = 5 mM). The corresponding heat map visualization of Bliss values further highlights this cooperative effect, indicating that the combination enhances cytostatic activity beyond the sum of individual effects (Figure 53b).

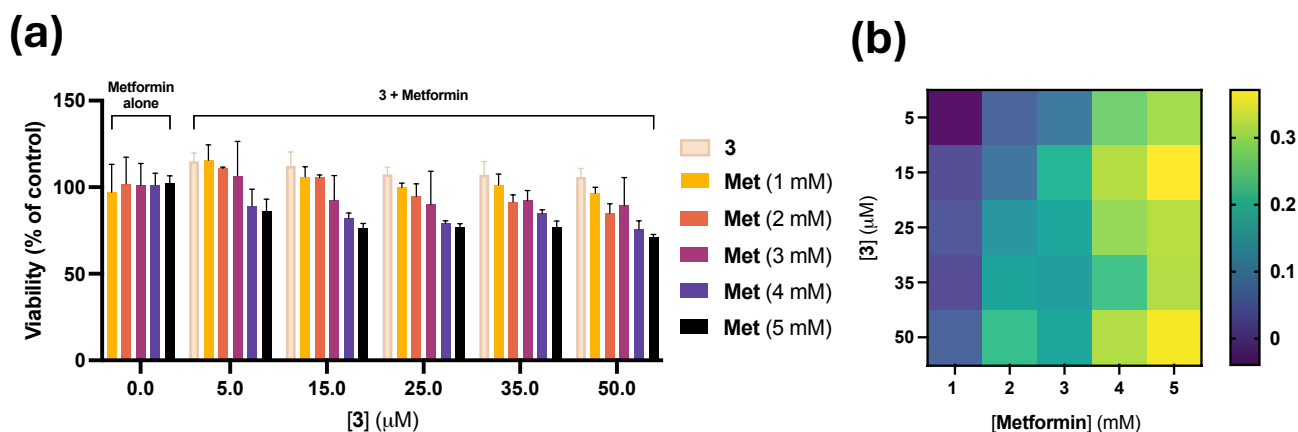


Figure 53. (a) HCT116 cell viability measured with MTT assay after 72 h treatment with **3**, alone or in combination with **metformin**, at the indicated concentrations. Data are presented as the mean $\pm$ SD, expressed as a percentage of the untreated control. (b) Heat map representation of the calculated Bliss scores for all combinations of compound **3** and **metformin**. Positive Bliss values (green-yellow) indicate synergistic interactions, values near zero (dark blue) reflect additivity.

The combinatorial effects of compounds **3** (10 μ M) or **3a** (5 μ M) with **metformin** (5 mM) were next assessed in three-dimensional spheroid cultures to determine whether the synergistic behavior observed in monolayer conditions extended to 3D tumor models. In this setting, the combination of compound **3** with **metformin** resulted in a slightly antagonistic effect, whereas the **3a**–**metformin** combination displayed an additive response (Figure 54). Comparable results were obtained when considering the endpoint measurements, leading to similar conclusions (*data not shown*). These results indicate that the cooperative interaction observed in simpler models is attenuated in the more physiologically relevant 3D environment, likely due to limited drug penetration or metabolic adaptation within the spheroids.

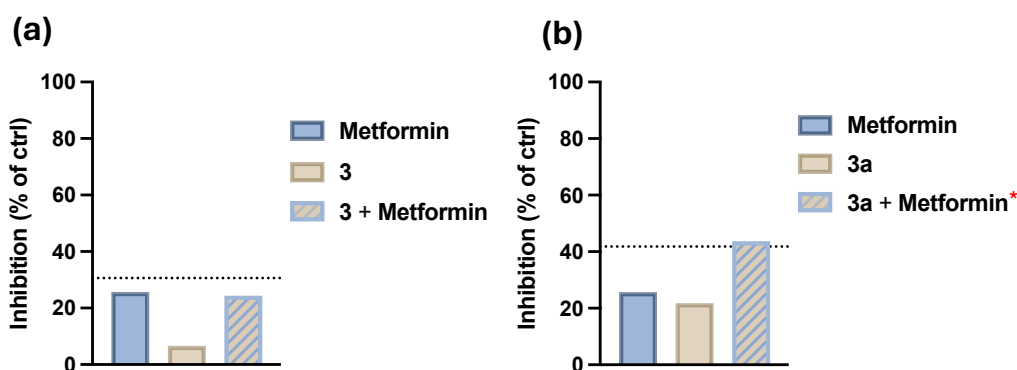


Figure 54. Percentage of inhibition calculated from the AUC values for HCT116 spheroid growth upon co-treatment of **metformin** (5 mM) with compound (a) **3** and (b) **3a**, respectively at 10 and 5 μ M. The AUC values were derived from the normalized size-versus-time plots and expressed as % inhibition relative to the untreated control. The dotted horizontal line indicates the expected additive inhibition predicted by the Bliss independence model.

To assess also the impact of the combined treatments on the invasive potential of cancer cells, the spheroids were replated under adherent conditions and monitored for cell outgrowth over time. In contrast to the behavior observed during spheroid formation, co-treatment with **metformin** produced a synergistic inhibitory effect on outgrowth for both compounds **3** and **3a**. Quantitative analysis of the AUC values, normalized to the untreated control, confirmed that the observed inhibition exceeded the expected additive effect predicted by the Bliss independence model (Figure 55). Similar values were obtained when considering the endpoint measurements (*data not shown*). In particular, Bliss scores of +0.10 were calculated for both combinations. These results suggest that the combination of **metformin** with either compound enhances the suppression of post-spheroid proliferative and migratory behavior.

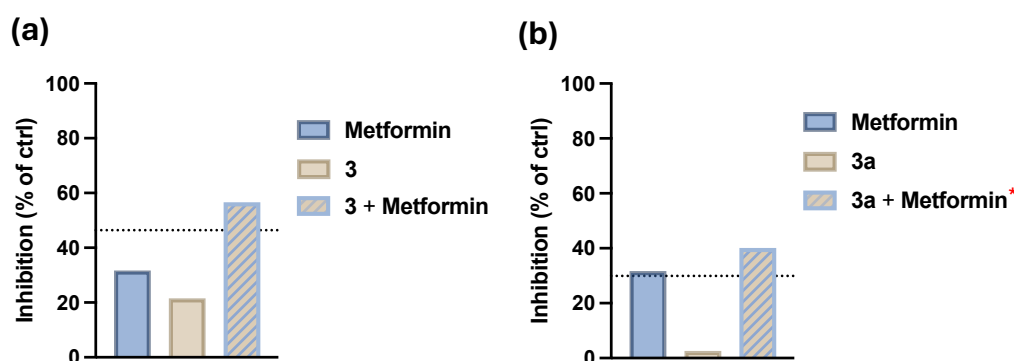


Figure 55. Percentage of inhibition calculated from the AUC values of the HCT116 spheroid outgrowth curves (cell expansion over time) after replating. The AUC values of the raw curves was expressed as % inhibition relative to the untreated control. The dotted horizontal line indicates the expected additive inhibition predicted by the Bliss independence model. [**Metformin**] = 5 mM; [**3**] = 10 μ M; [**3a**] = 5 μ M.

After assessing the combinations with **metformin**, attention then turned to **BAY-876**, to examine whether the activities of this modulator and compound **3** could mutually potentiate their antiproliferative effects. Firstly, the combined effects of compound **3** with **BAY-876** were evaluated by MTT assay after 72 hours of co-treatment. The combination led to a pronounced decrease in cell viability compared to either compound alone (Figure 56a). Quantitative analysis revealed a strong synergistic interaction, with Bliss scores reaching up to +0.49 at the highest concentrations tested ([**3**] = 50 μ M; [**BAY-876**] = 10 μ M) (Figure 56b).

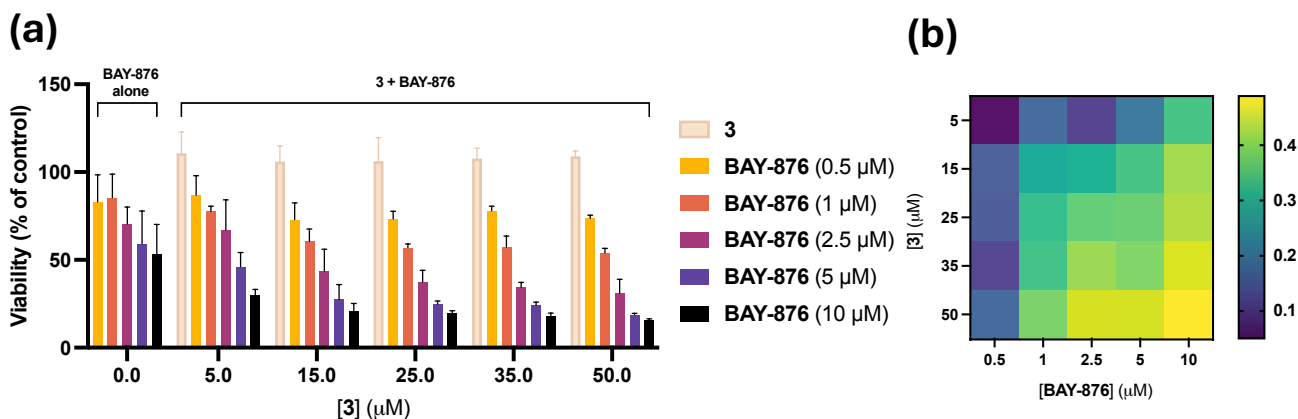


Figure 56. (a) Cell viability measured with MTT assay after 72 h treatment with **3**, alone or in combination with **BAY-876**, at the indicated concentrations. Data are presented as the mean $\pm$ SD, expressed as a percentage of the untreated control. (b) Heat map representation of the calculated Bliss scores for all combinations of compound **3** and **BAY-876**. Positive Bliss values (green-yellow) indicate synergistic interactions, values near zero (dark blue) reflect additivity.

To confirm and extend the observed synergy, cell proliferation was monitored in real time by Incucyte® confluency measurements over six days. The combination treatment markedly reduced the increase in confluency compared to either agent alone, confirming a sustained growth-inhibitory effect (Figure 57). Quantitative analysis of cumulative proliferation, expressed as the AUC, corroborated the strong synergistic interaction, consistent with the MTT results. The calculated Bliss score reached values comparable to those obtained in the short-term assay, indicating that the combined inhibition of glycolytic and mitochondrial pathways exerts a prolonged effect over time.

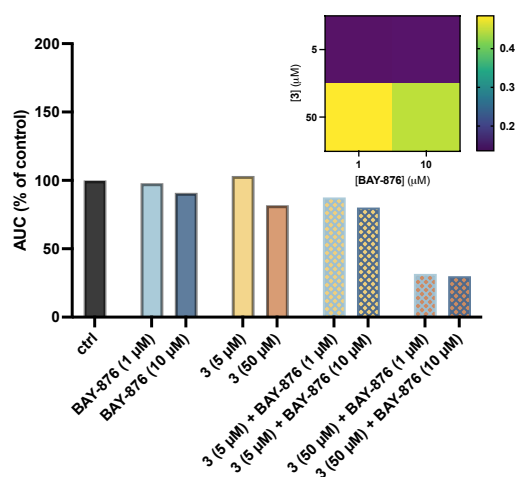


Figure 57. Quantification of HCT116 proliferation expressed as AUC values from real-time confluency measurements over six days for cells treated with compound **3**, alone or in combination with **BAY-876**, at the indicated concentrations. Data are presented as the mean $\pm$ SD, expressed as percentage of the untreated control. Insets show the corresponding Bliss synergy heat maps for the four tested combinations.

The potential synergistic effects of compound **3** and **BAY-876** were further examined in the colony formation assay. Also in this setting, the combination treatment further reduced colony number and staining density compared to single-agent exposures (Figure 58). However, because the highest concentrations of **3** produced a strong inhibition, synergy was most evident at lower doses. Quantitative analysis revealed Bliss scores of +0.48 and +0.40 for [**3**] = 10 μ M and [**BAY-876**] = 0.5-1 μ M, respectively.

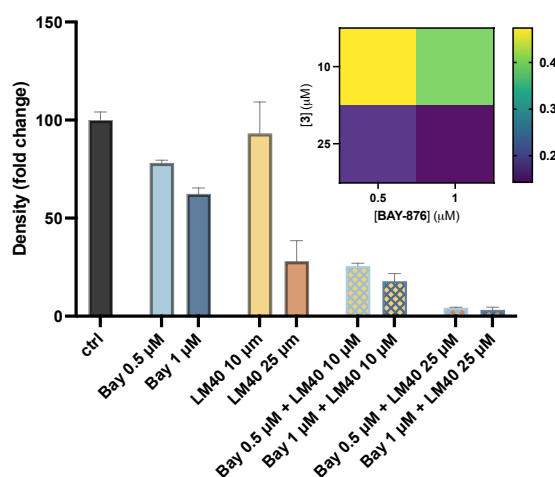


Figure 58. Quantification of colony density in HCT116 cells treated with compound **3** alone or in combination with **BAY-876**, at the indicated concentrations. Data are presented as the mean $\pm$ SD, expressed as percentage of the untreated control. Insets show the corresponding Bliss synergy heat maps for the four tested combinations.

To assess whether the synergistic interaction persisted under more physiologically relevant conditions, the combinatorial effects of **3** and **BAY-876** were also evaluated in three-dimensional spheroid cultures. Co-treatment further reduced spheroid growth compared to either compound alone. Quantitative analysis of the AUC values and endpoint spheroid sizes confirmed that the synergistic effect was maintained in 3D culture (Figure 59). The observed inhibition exceeded the additive response predicted by the Bliss independence model, with a Bliss score of +0.10 at the highest **BAY-876** concentration (1 μ M), while the lowest concentration resulted only in an additive effect (*data not shown*).

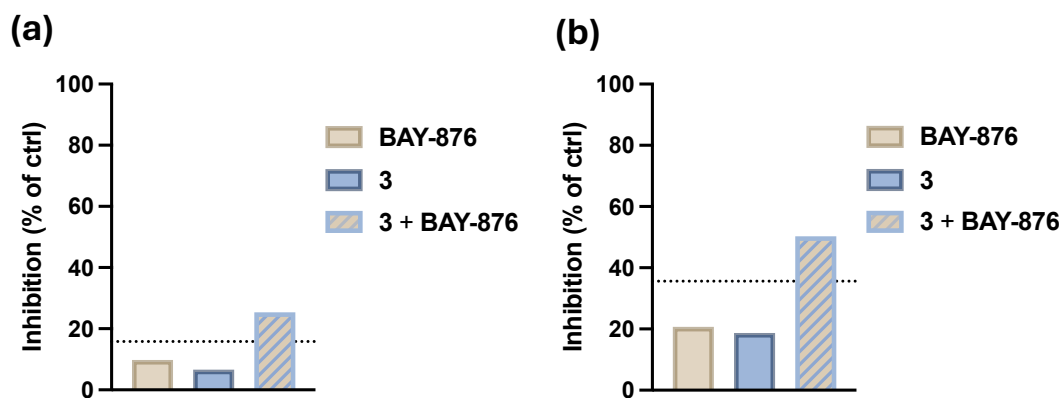


Figure 59. Quantitative analysis of HCT116 spheroid growth inhibition following co-treatment with **3** (10 μ M) and **BAY-876** (5 μ M). (a) Percentage of inhibition calculated from the AUC and (b) analysis done on corresponding endpoint spheroid sizes. The dotted horizontal line represents the expected additive inhibition calculated according to the Bliss independence model.

Complementary, spheroids previously exposed to compound **3** and **BAY-876** were and replated in adherent conditions, to evaluate the ability of surviving cells to reattach and proliferate. As expected, the synergistic effect observed during the initial treatment was maintained upon replating. This was reflected by the markedly reduced spheroid regrowth and overall size compared to single-agent treatments, indicating that the combination induced a sustained impairment of clonogenic and self-renewal potential (Figure 60). Notably, Bliss scores of +0.27 and +0.40 were calculated from the AUC values for the combinations of compound **3** (10 μ M) with **BAY-876** at 1 μ M and 5 μ M, respectively.

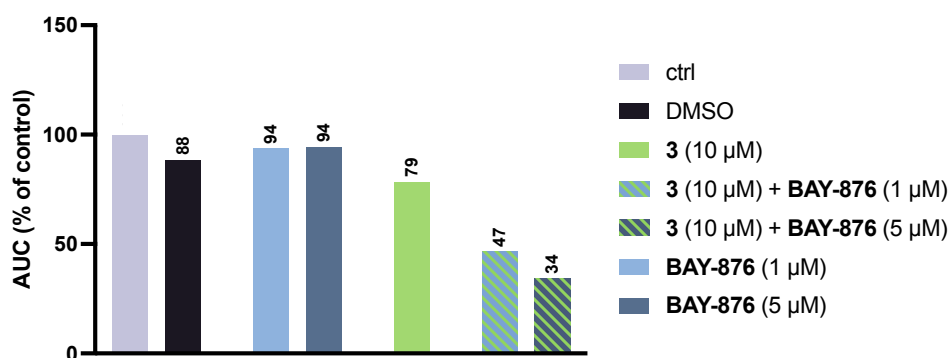


Figure 60. Quantification of the area under the curve (AUC) obtained from the raw curves of HCT116 spheroid outgrowth size over time after replating. AUC values were normalized to the untreated control and expressed as percentage (% of control).

To gain deeper insight into the metabolic consequences of treatment, intracellular ATP levels were quantified after 72 hours of exposure to compound **3**, **BAY-876**, and their combination. A clear synergistic effect was again observed, with high Bliss scores of +0.45 and +0.43, at 5 μ M of

compound **3** combined with 2.5 μM and 1 μM of **BAY-876**, respectively. These results indicate that the combined treatment led to a marked depletion of cellular ATP content, consistent with a cooperative impairment of mitochondrial and glycolytic energy production pathways (Figure 61).

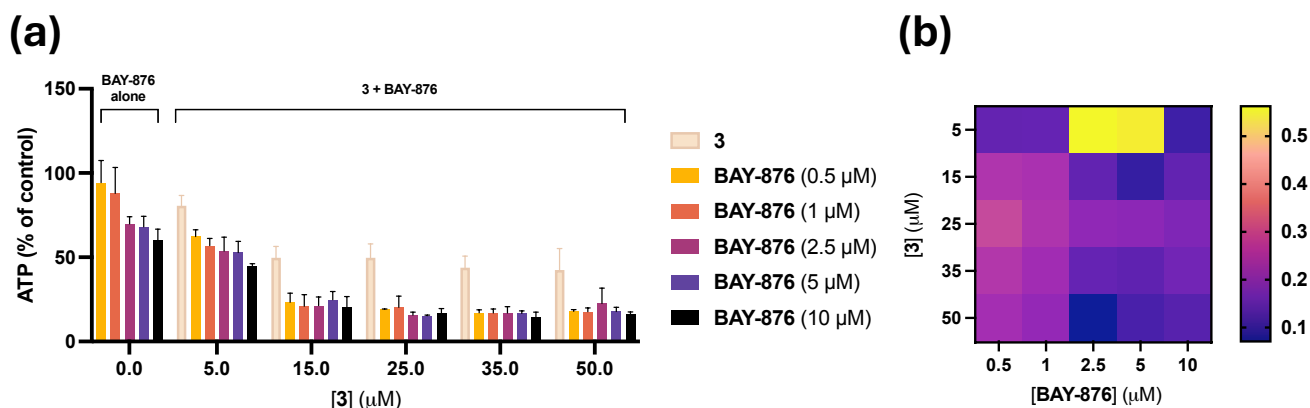


Figure 61. (a) ATP assay after 72 h treatment with **3**, alone or in combination with **BAY-876**, at the indicated concentrations. Data are presented as the mean $\pm$ SD, expressed as percentage of the untreated control. (b) Heat map representation of the calculated Bliss scores for all combinations of compound **3** and **BAY-876**.

Finally, to broaden the biological evaluation beyond HCT116 cells and investigate potential cross-resistance profiles, the cytotoxic activity of compound **3** and its non-targeted analogue **3a** was examined in A2780 (cisplatin-sensitive) and A2780cis (cisplatin-resistant) ovarian cancer cell lines using the MTT assay after 72 hours of treatment. Compound **3** showed slightly higher cytotoxicity compared to HCT116 cells, with a marginally increased sensitivity in the cisplatin-resistant A2780cis line. Moreover, the related compound **3a** displayed pronounced toxicity in both models, with an IC_{50} of approximately 5 μM , confirming its strong antiproliferative potential, as demonstrated in HCT116 (Figure 62).

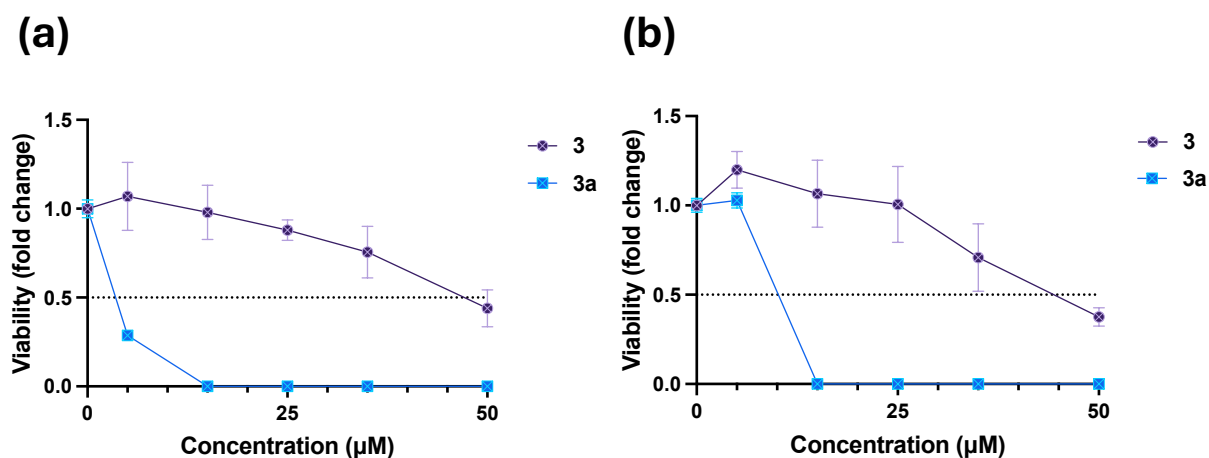


Figure 62. MTT-based cell viability after 72 h treatment with compounds **3** or **3a** in (a) A2780 and (b) A2780cis cells. Data are normalized to the control and presented as the mean $\pm$ SD.

3. Experimental

Chemicals and solvents were purchased from Sigma Aldrich and Thermo Fisher Scientific and used without further purification. The synthetic procedures of (2-aminoethyl)triphenylphosphonium and dppz ligand are reported in Chapter I. NMR spectra were recorded on Bruker AC-E series 400 MHz. Electrospray ionization mass spectrometry (ESI-MS) was performed with a dual electrospray interface and a quadrupole time-of-flight mass spectrometer (Agilent 6530 Series Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC-MS). Elemental analyses were performed using an Eurovector EA3000 elemental analyzer at the Microanalytical Laboratory of the University of Vienna. The ODN sequences used in this work are reported in Table 2 (Chapter I). DNA concentrations are expressed either per strand or per base, according to the specific experiment, as detailed in the corresponding figure captions. Lyophilized calf thymus DNA (ct-DNA) was purchased from Sigma-Aldrich and dissolved in 1 mM Tris-HCl buffer (pH 7.5). The final DNA concentration, calculated in nucleotide base equivalents, was determined spectrophotometrically at 260 nm using a molar extinction coefficient of $6600 \text{ M}^{-1}\text{cm}^{-1}$.

For biological assays, compounds were dissolved in DMSO to prepare 10 mM stock solutions. These stocks were subsequently diluted in culture medium, ensuring that the final DMSO concentration did not exceed 1%. In combination studies, the Bliss score (ΔE) was computed as the difference between the observed inhibition effect (E_{obs}) and the expected inhibition effect (E_{exp}), based on the Bliss independence model. The expected combined effect of drug A and drug B was defined as:

$$E_{\text{exp}} = E_A + E_B - (E_A \times E_B)$$

where E_A and E_B denote the fractional inhibition values obtained for each compound when tested individually.

Synthesis and characterization.

dppz-COOH. In 1.5 mL of ethanol, 1,10-phenanthroline-5,6-dione (84.1 mg, 0.4 mmol) was dissolved. Afterwards, 3,4-diaminobenzoic acid (60.9 mg, 0.4 mmol) was dissolved in 1 mL and added dropwise to the phenanthroline solution. The mixture was brought to reflux, and after 30 min the formation of a whitish precipitate was observed. The powder was collected by vacuum filtration and dispersed in boiling methanol. The product was then filtered again and washed with cold methanol (91.4 mg, 80.9%). ^1H NMR (400 MHz, DMSO) δ 9.60 (ddd, $J = 9.9, 8.1, 1.8 \text{ Hz}$, 2H), 9.23 (dd, $J = 4.4, 1.8 \text{ Hz}$, 2H), 8.78 (d, $J = 1.6 \text{ Hz}$, 1H), 8.50 (dd, $J = 8.7, 1.7 \text{ Hz}$, 1H), 8.32 (d, $J = 8.7 \text{ Hz}$, 1H), 7.97 (ddd, $J = 8.1, 5.4, 4.4 \text{ Hz}$, 2H).

$[\text{Ru}(\eta^6\text{-}p\text{-cymene})(\text{dppz-COOH})](\text{PF}_6)$. A solution of $[(\eta^6\text{-}p\text{-cymene})\text{RuCl}_2]_2$ (91.9 mg, 0.15 mmol) and the ligand dppz-COOH (97.8 mg, 0.3 mmol) in methanol was refluxed overnight. The following

day, the solvent was evaporated and the residue dissolved in water (6 mL). Addition of a saturated NaPF₆ solution led to the precipitation of the product as a yellow-green hexafluorophosphate salt (61.3 mg, 55.1%). The precipitate was collected by filtration, washed with several portions of cold water and diethyl ether, and residual solvents were removed under vacuum. ¹H NMR (400 MHz, DMSO) δ 10.03 (d, J = 5.3 Hz, 2H), 9.73 (dd, J = 8.3, 4.5 Hz, 2H), 8.92 (s, 1H), 8.60 – 8.43 (m, 1H), 8.32 (dt, J = 9.1, 4.7 Hz, 2H), 6.40 (d, J = 6.2 Hz, 2H), 6.17 (d, J = 6.2 Hz, 2H), 2.70 (p, J = 6.9 Hz, 1H), 2.22 (s, 3H), 1.00 (d, J = 6.8 Hz, 6H).

3. Compound **3** was obtained following the general procedure reported above. The ruthenium precursor [Ru(η⁶-*p*-cymene)(dppz-COOH)] ((37.9 mg, 0.05 mmol, 1 equiv.), (2-aminoethyl)triphenylphosphonium (1 equiv.), HOBt·H₂O (3 equiv.), and EDC·HCl (3 equiv.) were dissolved in anhydrous DMF (1.5 mL). The title compound was obtained as yellowish solid. (32.9 mg, 54.0%). ¹H NMR (400 MHz, DMSO) δ 10.04 (dt, J = 5.5, 1.3 Hz, 2H), 9.72 (ddd, J = 16.4, 8.3, 1.3 Hz, 2H), 9.41 (t, J = 5.5 Hz, 1H), 8.76 (d, J = 1.9 Hz, 1H), 8.56 (d, J = 8.9 Hz, 1H), 8.42 – 8.21 (m, 3H), 8.03 – 7.70 (m, 14H), 6.40 (d, J = 6.2 Hz, 2H), 6.16 (d, J = 6.0 Hz, 2H), 3.97 (dt, J = 14.0, 7.2 Hz, 2H), 3.86 – 3.64 (m, 2H), 2.77 – 2.63 (m, 1H), 2.22 (s, 3H), 0.99 (d, J = 6.9 Hz, 6H). ESI-MS m/z calculated for [M]⁺: 442.5968, found: 442.5968. CHN for **3** + 1 H₂O C: 48.26% H: 4.11% N: 5.71%, exp C: 48.46% H: 3.78% N: 6.16%.

3a. A solution of [(η⁶-*p*-cymene)RuCl₂]₂ (30.6 mg, 0.05 mmol) and dppz ligand (28.2 mg, 0.1 mmol) in methanol (8.5 mL) was refluxed overnight. The following day, the solvent was evaporated, and the residue was dissolved in water (6 mL). Addition of a saturated NaPF₆ solution induced precipitation of the product. The solid was collected by filtration, washed with several portions of cold water and diethyl ether, and dried under vacuum to remove residual solvents (28.3, 81.2%). ¹H NMR (400 MHz, DMSO) δ 10.01 (s, 2H), 9.73 (s, 2H), 8.49 (s, 2H), 8.32 (s, 2H), 8.17 (s, 2H), 6.39 (s, 2H), 6.17 (s, 2H), 2.70 (s, 1H), 2.21 (s, 3H), 1.00 (d, J = 6.9 Hz, 6H).

FRET melting assay. FRET melting studies were conducted in 96-well plates using an Applied Biosystems™ QuantStudio 6 Real-Time PCR System equipped with a FAM (6-carboxyfluorescein) detection filter. Dual-labelled oligonucleotides (FAM at the 5' end and TAMRA at the 3' end) were diluted to the desired concentration in 60 mM potassium cacodylate buffer (pH 7.4). To ensure proper folding into G4 or duplex conformations, oligonucleotides were heated at 95 °C for 5 min and allowed to cool gradually to room temperature overnight. The final strand concentration in each well was 0.2 μM in a total reaction volume of 30 μL. Stock solutions of the metal complexes (2 mM in DMSO) were diluted into buffer immediately before use, keeping the final DMSO content below 1% (v/v). Fluorescence signals were collected in duplicate from 25 °C to 95 °C, in 1 °C increments every 30 s.

Normalized fluorescence (0–1 scale) was used to determine the melting midpoint ($T_{1/2}$), defined as the temperature corresponding to 50% FAM fluorescence.

UV-Vis. UV-Vis spectra were acquired at room temperature on a Cary 1E double-beam spectrophotometer. For stability monitoring, 50 μ M solutions of the complexes were prepared by diluting DMSO stocks into Tris–HCl (50 mM) / KCl (100 mM) buffer, pH 7.4, and spectra were recorded hourly for 24 h. In titration experiments, the complex concentration was kept constant while increasing amounts of ODNs were added. To maintain total metal complex concentration, each DNA addition was compensated by an equal volume of double-strength complex solution. DNA oligonucleotides were dissolved in Tris–KCl buffer (50 mM Tris–HCl, 100 mM KCl, pH 7.4) to obtain 100 μ M strand stock solutions and folded into G4 structures as previously described for FRET experiments.

Fluorescence. Fluorescence measurements were performed on a Jasco FP-8300 spectrofluorometer using 1 cm quartz cuvettes. Instrumental settings were: excitation bandwidth 5 nm, emission bandwidth 10 nm, response time 0.1 s, and scan speed 1000 nm/min. Emission spectra were collected from 265 nm to 700 nm upon excitation at wavelengths incremented every 10 nm. Measurements were first recorded for 5 μ M solutions of the complexes alone and then after addition of 100 μ M DNA (expressed in base concentration). All samples were prepared in Tris–KCl buffer (50 mM Tris–HCl, 100 mM KCl, pH 7.4) with final DMSO content <1%.

Circular Dichroism. CD spectra were recorded at 25 °C on a Jasco J-715 spectropolarimeter. The DNA concentration was kept constant while increasing amounts of complex were added. Spectra were collected from 220–450 nm with a scan rate of 200 nm·min^{–1}, response time 0.5 s, and averaged over four accumulations. ODNs were prepared and folded as described for the FRET assay.

MTT assay. HCT116, A2780, and A2780cis cells were seeded in 96-well plates (4 × 10³ cells/100 μ L) and allowed to attach overnight. Cells were then treated with the indicated compounds, alone or in combination, for 72 h. Viability was assessed using the EZ4U (MTT-based) assay (Biomedica, Vienna, Austria) according to the manufacturer's protocol. Absorbance was measured at 450 nm with reference at 620 nm using a Spark® multimode microplate reader (Tecan, Switzerland). Data were processed in GraphPad Prism and represent at least three independent biological replicates (n = 3–6).

ATP assay. Extracellular ATP levels were quantified using the RealTime-Glo™ Extracellular ATP Assay (Promega). HCT116 cells (4×10^3 cells/100 μ L) were plated in 96-well plates and allowed to adhere overnight before exposure to serial dilutions of the compounds. After 72 h of incubation, 50 μ L of the ATP reagent solution (4 $\times$ working concentration in culture medium) was added to each well. Luminescence was recorded using a Spark® multimode microplate reader (Tecan, Switzerland). Untreated and vehicle controls were included in parallel. Raw luminescence values (RLU) were normalized to the untreated control.

ICP-MS. HCT116 cells were seeded at 7.5×10^5 cells/mL in 6-well plates (2 mL/well) and treated with the test compounds for 5 h or 24 h. After washing twice with PBS, cells were trypsinized, counted, and washed again in PBS. Dried cell pellets were digested in 65% HNO₃ for 1 h, diluted with ultrapure water, and analyzed by ICP-MS. Ruthenium content was normalized to cell count.

Colony formation assay. Cells (2,500 per well) were plated in 24-well plates and allowed to recover for 24 h before treatment with the indicated drugs. After 7, colonies were fixed with ice-cold methanol (30 min, 4 °C) and stained with 0.01% crystal violet. Stained colonies were imaged with a Typhoon TRIO Variable Mode Imager (GE Healthcare Life Sciences) and quantified using ImageJ.

Seahorse. Cells were seeded at 150,000 cells/mL (80 μ L/well) in Seahorse XFp microplates and incubated overnight. The next day, culture medium was replaced with assay medium (1 mM pyruvate, 2 mM glutamine, 10 mM glucose) after one wash. The Mito Stress Test was performed by sequential injection of oligomycin (1.5 μ M), FCCP (2 μ M), and rotenone/antimycin A (0.5 μ M each). Hoechst 33258 (4 μ M) was added for normalization of cell number. Data were acquired and analyzed using Wave Pro software (v10.1.0.1).

Wound-healing assay. Cell migration was monitored using the Incucyte® 96-Well Scratch Wound Assay (Sartorius). HCT116 cells (5×10^4 per well) were seeded in 96-well ImageLock plates and incubated overnight. Uniform wounds were generated with the Incucyte® WoundMaker, and cells were washed with PBS before adding fresh medium containing the test compounds. Plates were imaged every hour for 60 h in the Incucyte® Live-Cell system. Wound confluence was quantified automatically, and migration inhibition was calculated relative to untreated controls.

Spheroids. Spheroids were generated by seeding HCT116 cells (3,500 cells/100 μ L) in ultra-low-attachment 96-well round-bottom plates (Corning 7007) in the presence of the compounds. Formation

and growth were monitored by live-cell imaging using the Incucyte® system for up to 5 days, with images collected every 2 h. Untreated spheroids served as controls. Spheroid area was quantified using the Incucyte® software. For replating experiments, mature spheroids were collected, dissociated, and reseeded under adherent 2D culture conditions. Cell regrowth and viability were monitored over time by Incucyte® imaging.

Quantitative RT-PCR. HCT116 cells (3.5×10^5 cells/well) were seeded in 6-well plates and treated with the indicated compounds for 24 h. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, MA, USA) and reverse-transcribed with the GoTaq® RT-qPCR System (Promega). Amplifications were carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method using *RPL41* as the internal reference. Primers for *RPL41*, *MYC*, *BCL2*, *ATP6*, *ND2*, and *ND3* were the same as previously reported (Chapter I, experimental section).

4. Conclusions

In this chapter, the strategy of targeting mitochondrial G4s was extended from Ru(II) polypyridyl systems to a half-sandwich arene architecture. A novel Ru(II) p-cymene complex bearing a TPP-functionalized dppz ligand (**3**) was synthesized and fully characterized. The complex demonstrated chemical stability under physiological conditions and retained the capacity to interact with DNA. In particular, compound **3** stabilized mitochondrial G4 sequences and induced detectable conformational perturbations, as shown by FRET and CD analyses, respectively.

Biological evaluation revealed that compound **3** exhibited efficient cellular uptake, while the cytotoxicity was moderate. However, the compound impaired mitochondrial respiration and ATP production, consistent with a direct impact on bioenergetics. Furthermore, in 3D spheroid and colony assays, the compound reduced long-term growth potential, and synergy with **metformin** and **BAY-876** and metformin was registered across diverse models. Gene expression analysis supported mitochondrial dysfunction as a key component of its activity. Overall, these results highlight Ru(II) arene complexes as complementary scaffolds to octahedral polypyridyl derivatives for mitochondrial G4 targeting. The findings underscore the value of structural diversification in ruthenium-based drug design and provide a foundation for further optimization aimed at enhancing G4 selectivity, mitochondrial delivery, and anticancer efficacy. Finally, additional studies related to this project are still in progress.

Chapter III: Novel Ruthenium(II) Complexes with Polypyridyl and 1,2,4 Oxadiazole Ligands: From Synthesis to Biological Evaluation in Cancer Cells Lines

1. Introduction

As discussed before, ruthenium(II) polypyridyl complexes represent a promising class of anticancer agents and G4 DNA binders. In parallel, 1,2,4-oxadiazoles are versatile heteroaromatic scaffolds frequently found in bioactive molecules, including anticancer agents. However, only a few metal complexes bearing oxadiazole ligands have been reported, leaving their role in modulating metal-biomolecule interactions largely unexplored.¹⁶⁰ For instance, a Cu(II) complex bearing 1,2,4-oxadiazole ligands exhibited cytotoxic activity against HepG2 and HT29 cells, whereas the free ligand was inactive.¹⁶¹

Building on the complementary potential of Ru(II) polypyridyl complexes and oxadiazoles, and the largely unexplored activity of the latter's coordination compounds, we developed four new Ru(II) complexes (**4**, **5**, **6** and **7**) featuring bipyridine or phenanthroline ligands in combination with two different 1,2,4-oxadiazole moieties (**L1-L2**).

The four newly synthesized compounds were structurally characterized, and their DNA-binding properties towards both B-DNA and G4 structures were investigated using spectroscopic techniques. Their potential antiproliferative effects were subsequently assessed in vitro in A2780 ovarian cancer cells and their cisplatin-resistant counterpart (A2780cis).

2. Results and Discussion

Synthesis and characterization.

The four new compounds were synthesized by combining two Ru(II) polypyridyl scaffolds, [Ru(bpy)₂Cl₂] and [Ru(phen)₂Cl₂] with two pyridyl-oxadiazole ligands (**L1-L2**). In these ligands, the five-membered heterocycle carries a pyridyl substituent at the 3-position, enabling coordination to ruthenium through nitrogen and affording a stable chelate. The syntheses proceeded smoothly, with chloride ligands being replaced by pyridyl-oxadiazoles within 30 minutes in an ethanol-water mixture (Figure 63). The products were isolated as hexafluorophosphate salts by precipitation and purified using straightforward procedures, affording good overall yields of 40–53%.

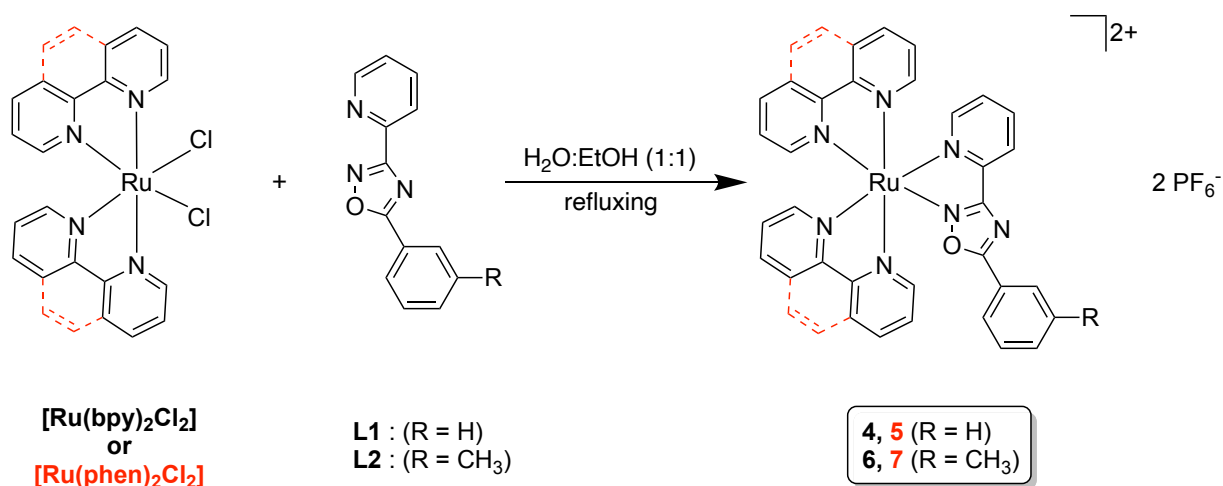


Figure 63. Synthetic pathway of the new four Ru(II) complexes (**4**, **5**, **6** and **7**) obtained by the combination of $[\text{Ru}(\text{bpy})_2\text{Cl}_2]$ and $[\text{Ru}(\text{phen})_2\text{Cl}_2]$ with **L1** and **L2**.

The compounds were fully characterized by standard analytical techniques, including ^1H NMR spectroscopy (Figures A5-7, Appendix A), electrospray ionization mass spectrometry (ESI-MS, Figures B-5-7, Appendix B), and elemental analysis. A single crystal of complex **6** was obtained by slow diffusion of diethyl ether into acetonitrile, and its X-Ray structure confirmed the expected octahedral geometry, with the ruthenium center coordinated to the heterocycle via the N2 atom (Figure 64).

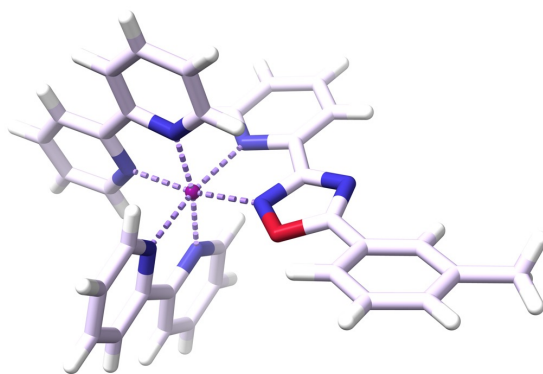


Figure 64. Crystal structure of complex **6** determined by single-crystal X-Ray diffraction.

In parallel, the coordination of the oxadiazole ligands was further investigated using a different ruthenium scaffold, namely the p-cymene “piano-stool” system. In this context, the oxadiazole **L2** was successfully coordinated to the Ru(II) p-cymene precursor by stirring the two components in methanol for 30 minutes (Figure 65a). The structure of the resulting complex, namely **RuL2**, was confirmed by single-crystal X-ray diffraction of crystals obtained directly from methanol solution (Figure 65b). Additional spectroscopic studies are currently underway to complete the full structural characterization of this compound. As a consequence, **RuL2** was discarded from further interaction and biological studies.

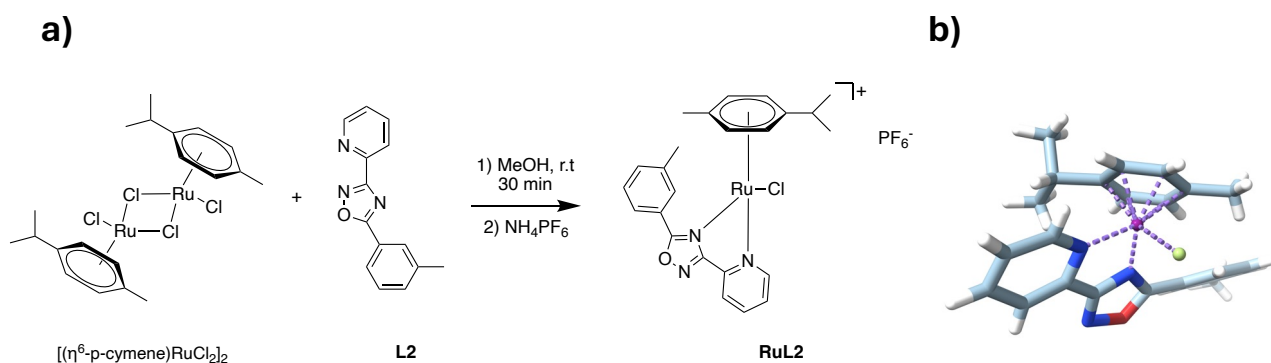


Figure 65. (a) Synthesis path for compound **RuL2**. (b) Crystal structure of compound **RuL2**, obtained by single-crystal X-Ray diffraction.

Stability.

The stability of the new compounds was evaluated by UV-Vis spectroscopy. All complexes remained stable in Tris-KCl buffer (50 mM Tris-HCl, 100 mM KCl, pH 7.4) for at least 24 hours (Figure 66a). Similarly, DMSO stock solutions displayed no significant spectral changes over the same period, confirming good stability under these conditions (Figure 66b).

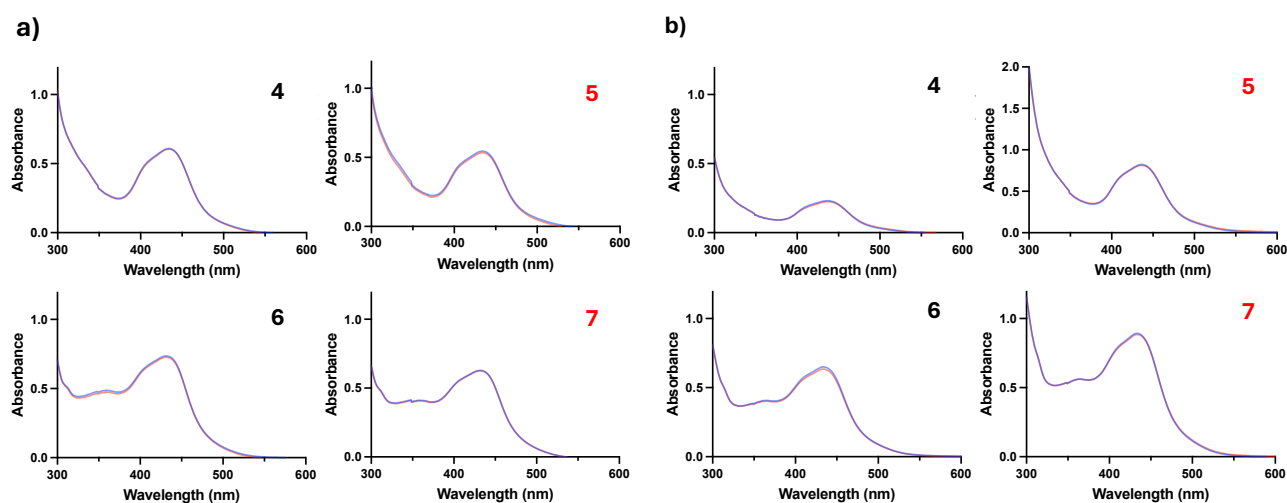


Figure 66. UV-Vis spectra acquired at the two different time points (blue: $t = 0$; red: $t = 24$ h) of compounds **4**, **5**, **6** and **7** in (a) Tris HCl 50 mM/KCl 100 mM buffer (DMSO <10% v/v) and in (b) DMSO.

DNA interaction studies.

To investigate the potential interaction of the new compounds with DNA, FRET melting assay, UV-Vis absorption, and CD spectroscopy were employed. Experiments were performed on DNA sequences capable of forming both G4 and B-DNA structures. In particular, the following G4-forming oligonucleotides were selected to assess the G4-binding properties of the complexes: myc, vegf, htelo, htert, kit1, kit2, and bcl2.

In FRET-melting assay, at the standard molar ratio (DNA:compound = 1:5), the four ruthenium complexes demonstrated no stabilization capacity toward dsDNA, htelo, bcl2, httert, kit1 and kit2 (Figure 67).

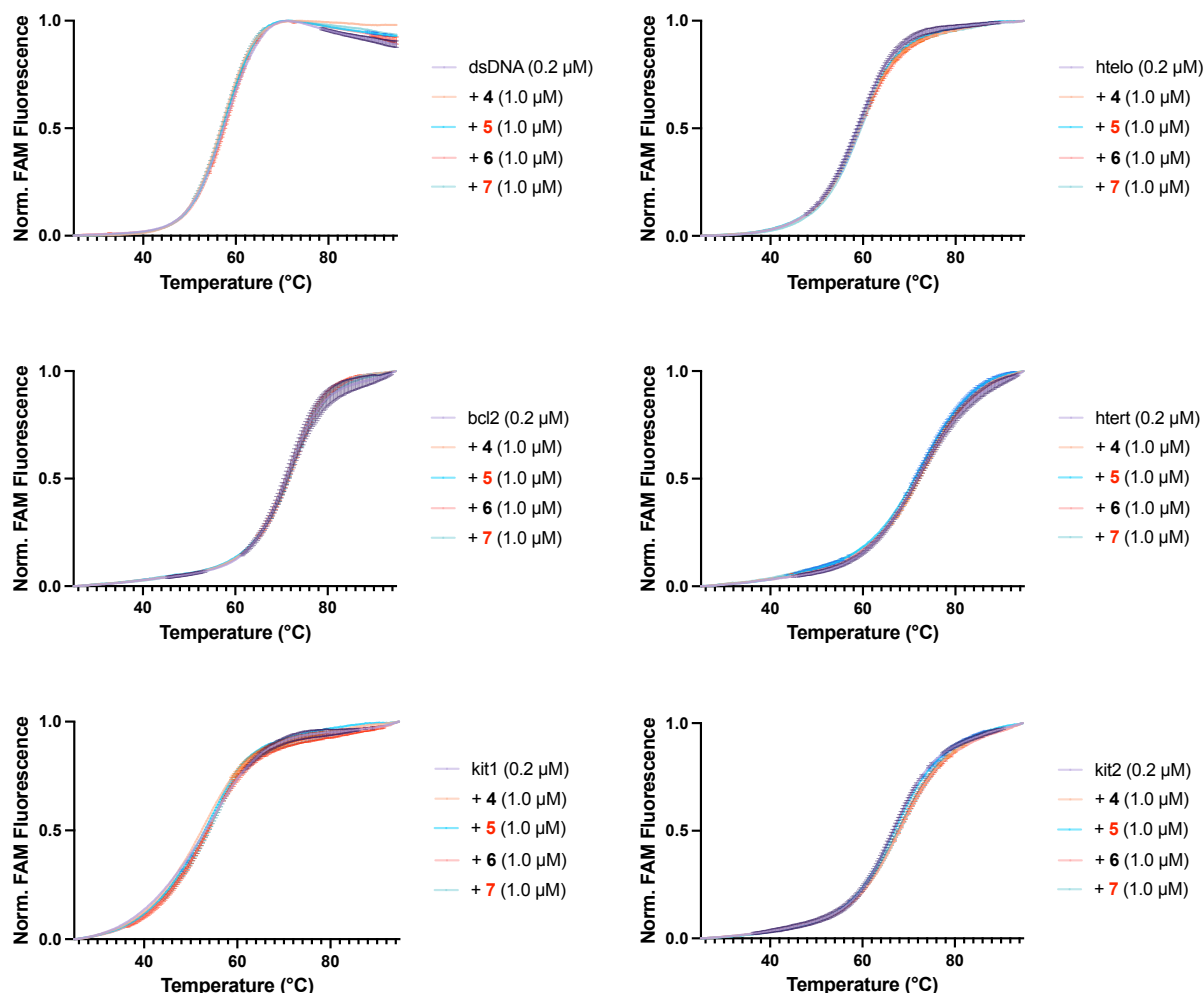


Figure 67. FRET-melting curves of different ODNs incubated with either **4**, **5**, **6** or **7** at the standard molar ratio (DNA:compound = 1:5). ODN concentrations are expressed per strands.

Nevertheless, minor variations were observed only for vegf and myc. For the latter, the assays were performed in 10 mM potassium cacodylate buffer rather than the commonly used (60 mM), as the melting temperatures of these G4s at higher potassium concentrations fell outside a practical range for detecting compound-induced shifts. When vegf was incubated with increasing concentrations of the compounds, a concentration-dependent increase in stabilization was observed (Figure 68a). To determine whether this effect was due to ionic strength rather than specific G4 recognition, measurements were repeated in the same buffer supplemented with 50 mM LiCl. These conditions matched the 60 mM monovalent ionic strength commonly used without adding further G4-stabilizing K⁺. Under these conditions, the apparent stabilization was abolished (Figure 68b).

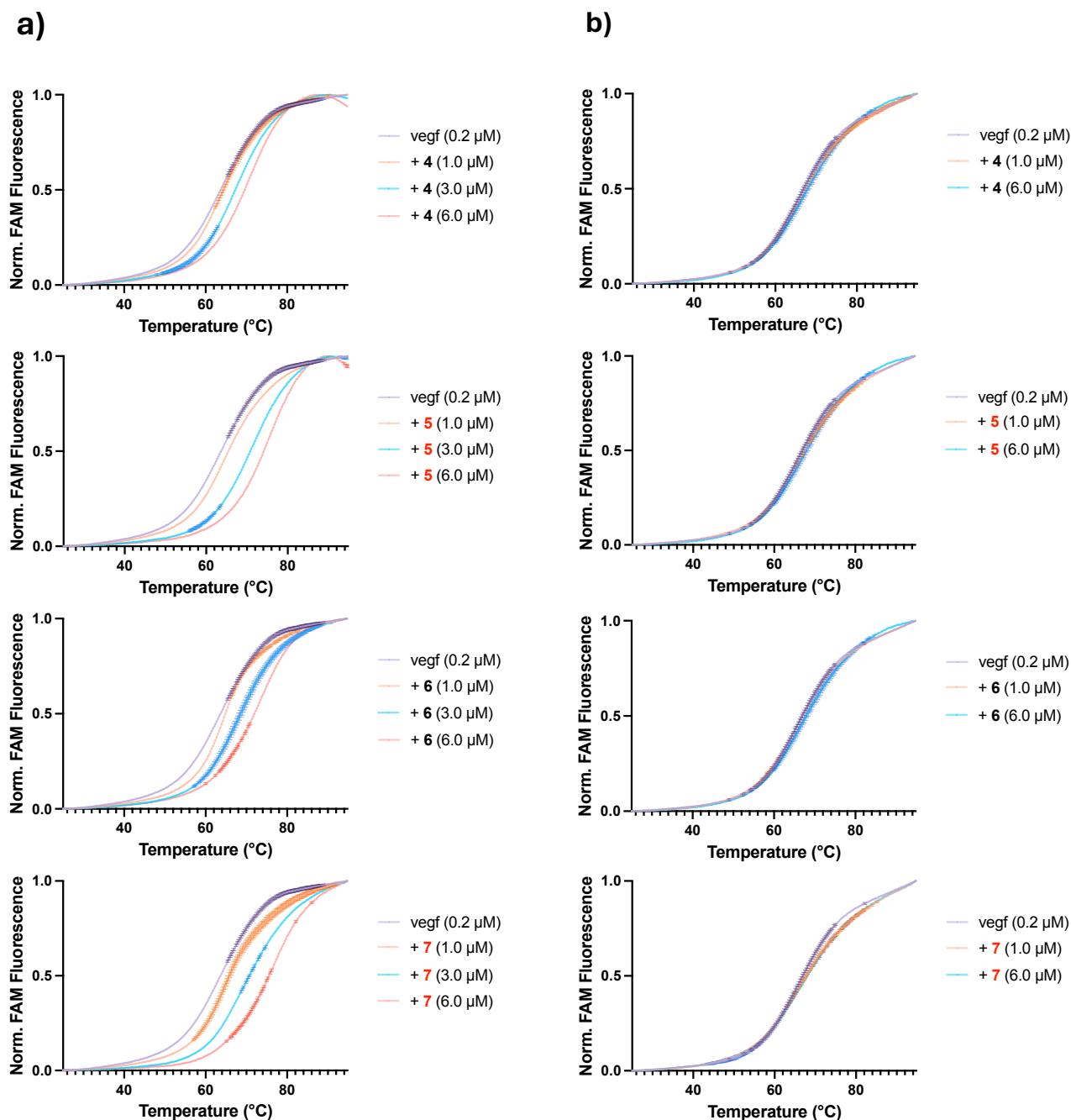


Figure 68. FRET-melting curves of vegf incubated with increasing concentration of **4**, **5**, **6** or **7** in 10 mM potassium cacodylate buffer (a) or in 10 mM potassium cacodylate buffer + 50 mM LiCl (b). ODN concentrations are expressed per strands.

The same experimental rationale was applied to the myc G4, initially using 10 mM potassium cacodylate buffer, which yielded a concentration-dependent stabilization effect (Figure 69a). However, upon supplementation with 50 mM LiCl, the results mirrored those observed for vegf, confirming that the apparent stabilization was not attributable to specific G4 recognition (Figure 69b).

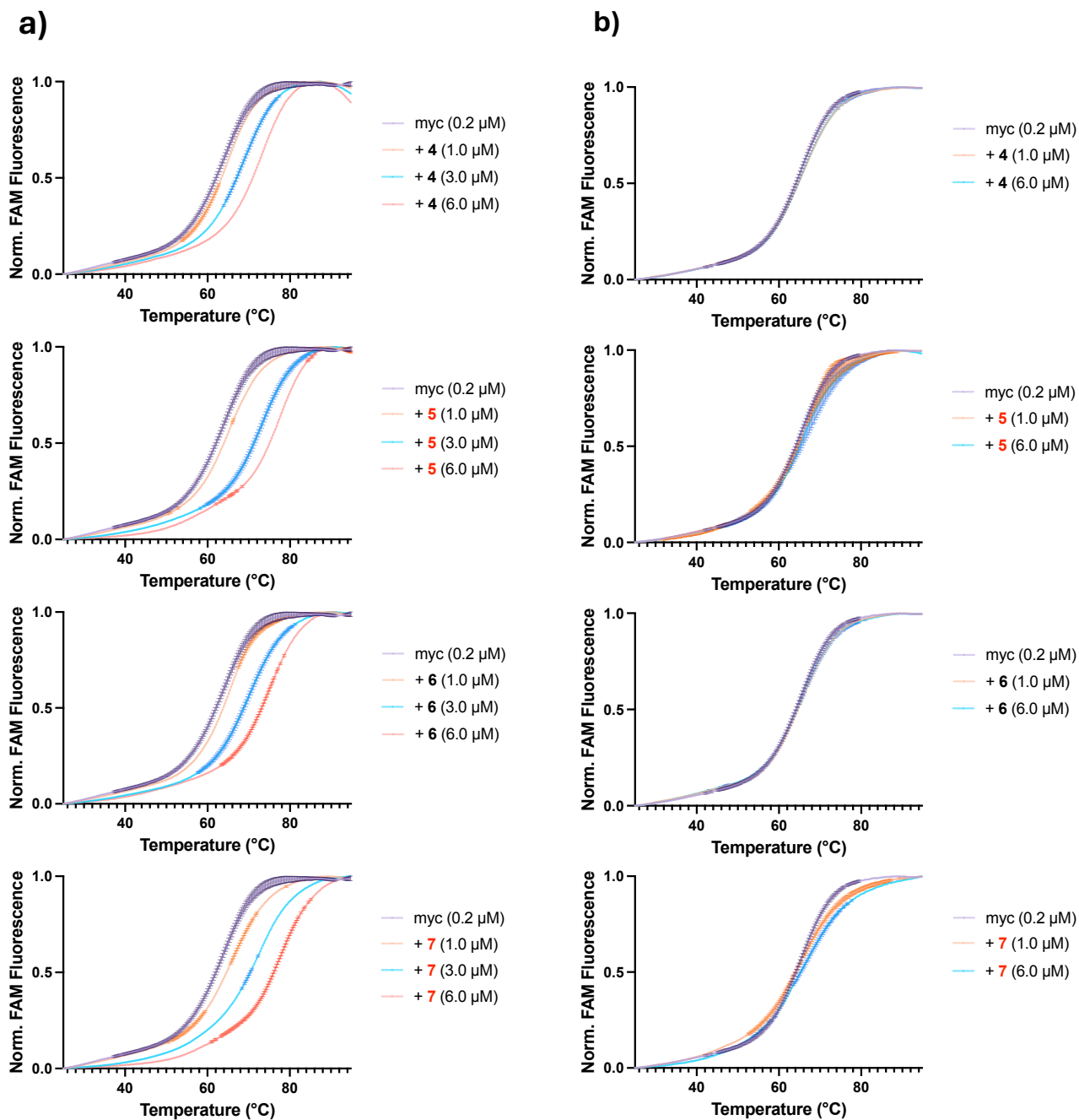


Figure 69. FRET-melting curves of myc incubated with increasing concentration of 4, 5, 6 or 7 in 10 mM potassium cacodylate buffer (a) or in 10 mM potassium cacodylate buffer + 50 mM LiCl (b). ODN concentrations are expressed per strands.

Further binding studies involved UV-Visible spectrophotometric titration of the complexes both duplex and quadruplex DNA, specifically vegf and c-myc. The titrations were conducted by maintaining a constant concentration of the complex while progressively increasing the amount of DNA added to the cuvette. No effects associated with the interaction of the complexes with the tested G4 sequences were observed (Figure 70).

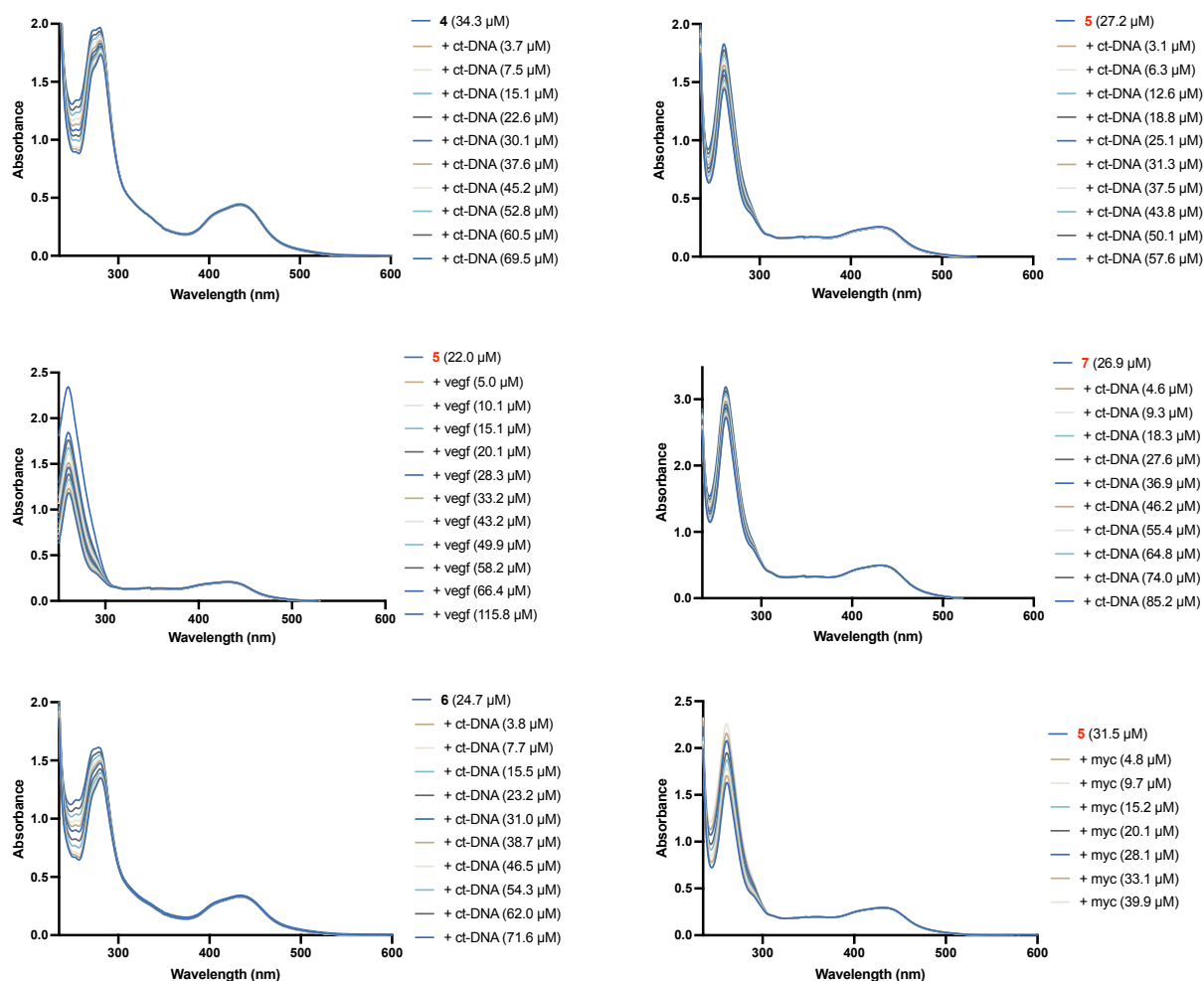


Figure 70. UV-Vis titrations of **4**, **5**, **6** or **7** with increasing amounts of ct-DNA (B-DNA model) or the G4-forming ODNs myc and vegf. ODN concentrations are expressed in strands.

Finally, CD spectroscopy titrations revealed that the characteristic CD spectra of both B-DNA and G4s remained largely unchanged after interaction with all metal complexes, indicating that the original topologies were preserved (Figure 71).

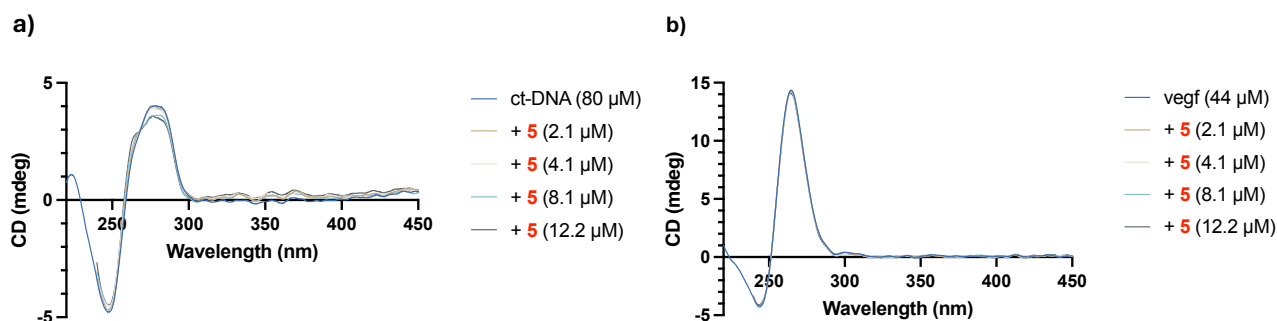


Figure 71. CD spectra of (a) ct-DNA and (b) vegf in presence of increasing amount of **5**. ODN concentrations are expressed in bases.

Cytotoxicity and cellular uptake.

The biological activity of the new Ru(II) complexes was evaluated in cancer cell models. Cellular uptake and cytotoxicity assays (MTT) were performed to assess the potential ability of the compounds to enter cells and exert antiproliferative effects. Their cytotoxicity was evaluated in A2780 ovarian cancer cells and their cisplatin-resistant variant (A2780cis). In particular, MTT assays were performed over a concentration range of 0-100 μM with a 72-hour treatment period. Among the tested complexes, **5** and **7** displayed the lowest IC_{50} values in both cell lines. Interestingly, A2780cis cells displayed increased sensitivity to the compound compared to the parental A2780 line, suggesting that the mechanism of cisplatin resistance does not confer cross-resistance. This enhanced response may reflect metabolic or mitochondrial vulnerabilities acquired during the development of cisplatin resistance (Figure 72).

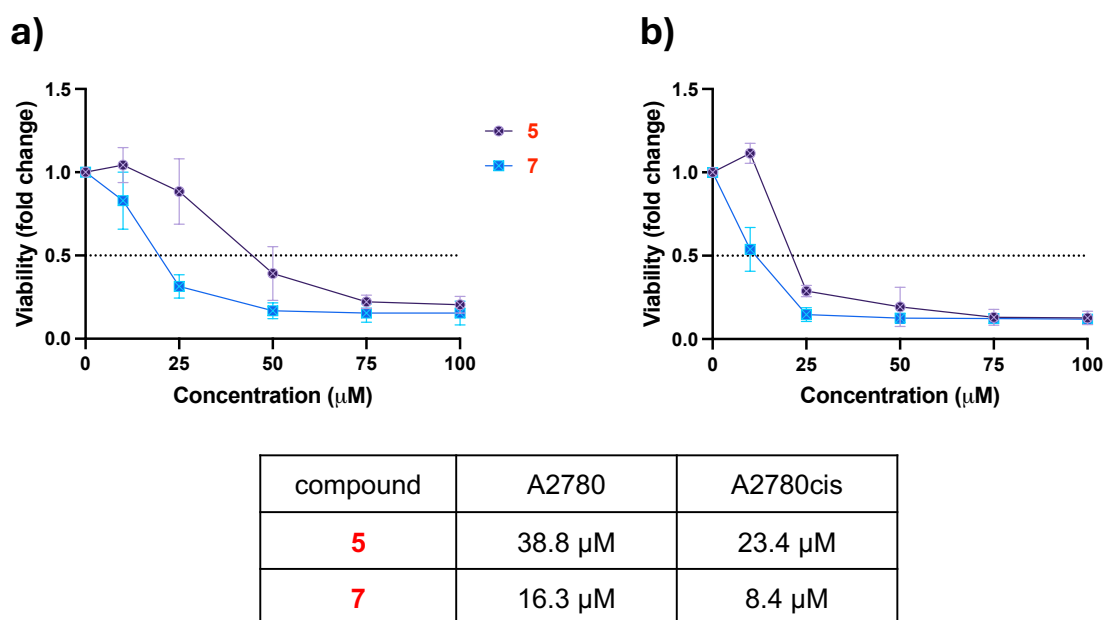


Figure 72. Dose–response curves obtained by MTT assay after 72 hours treatment of A2780 (a) and A2780cis (b) cells. Cell viability data are normalized to untreated controls. The IC_{50} values, reported in the table, were calculated using GraphPad Prism. Data are normalized to the control and presented as the mean $\pm$ SD.

The antiproliferative properties of the newly synthesized compounds were also evaluated in human colorectal cancer cells HCT116, and their BOLD-100 and oxaliplatin resistant variants (HCT116/BoldR and HCT116/OxaR, respectively). The IC_{50} values obtained in HCT116 were comparable to those measured in A2780, but no appreciable differences between compounds **5** and **7** was registered. The HCT116/BoldR variant showed no significant change in sensitivity compared to the parental cells, indicating that the mechanism underlying BOLD-100 resistance does not affect the

response to the ruthenium complexes **5** and **7**. In contrast, enhanced activity was observed in the oxaliplatin-resistant HCT116 variant, mirroring the increased sensitivity of A2780cis cells compared to A2780. These results indicate that the ruthenium complexes may circumvent resistance pathways typically associated with platinum chemotherapeutics, highlighting a distinct mode of action and a potential advantage in resistant cancer phenotypes (Figure 73).

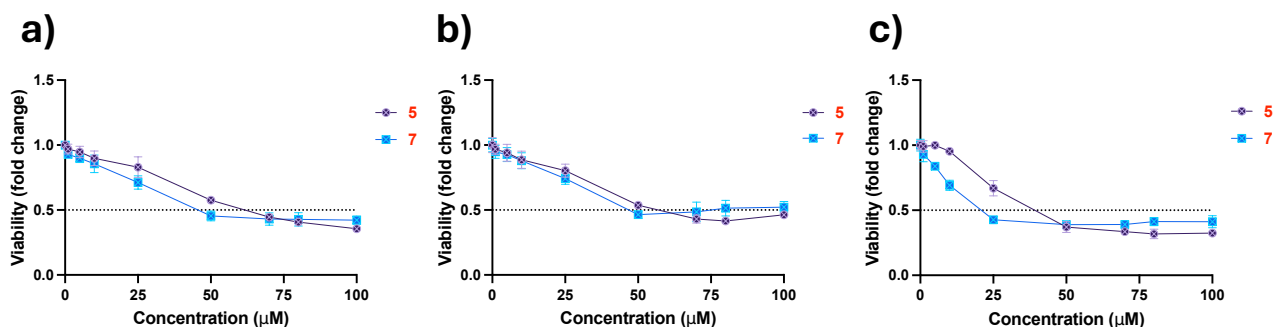


Figure 73. Dose–response curves obtained by MTT assay after 72 hours treatment of a) HCT116, b) HCT116/BoldR and c) HCT116/OxaR cells. Cell viability data are normalized to untreated controls. Dose-response curves were fitted using nonlinear regression (variable slope four parameters), and IC_{50} values were calculated using GraphPad Prism. Data are normalized to the control and presented as the mean $\pm$ SD.

Notably, in contrast to the A2780 and A2780cis cell lines, MTT assays performed on HCT116 cells, and their oxaliplatin- and BOLD-100-resistant derivatives revealed that the compounds did not reach complete cytotoxicity, as cell viability never dropped to zero even at the highest concentrations tested. This partial response suggests that the compounds exert predominantly cytostatic rather than fully cytotoxic effects in these colorectal cancer models.

To assess whether cellular uptake contributed to the observed activity profile, the intracellular ruthenium accumulation in A2780 and A2780cis cells was quantified by ICP-MS following treatment with the more active compounds **5** and **7**. Cells were exposed to two concentrations (10 and 25 μ M) for 5 and 24 hours. Both complexes exhibited a clear concentration-dependent uptake (Figure 74). Notably, only **7** showed a marked time-dependent increase in ruthenium accumulation and reached the highest overall intracellular levels. However, no significant differences were observed between the two cell lines, indicating that uptake does not account for the different sensitivities measured in the viability assays. Nonetheless, the higher intracellular accumulation of compound **7** may explain its stronger cytotoxic activity compared to compound **5**.

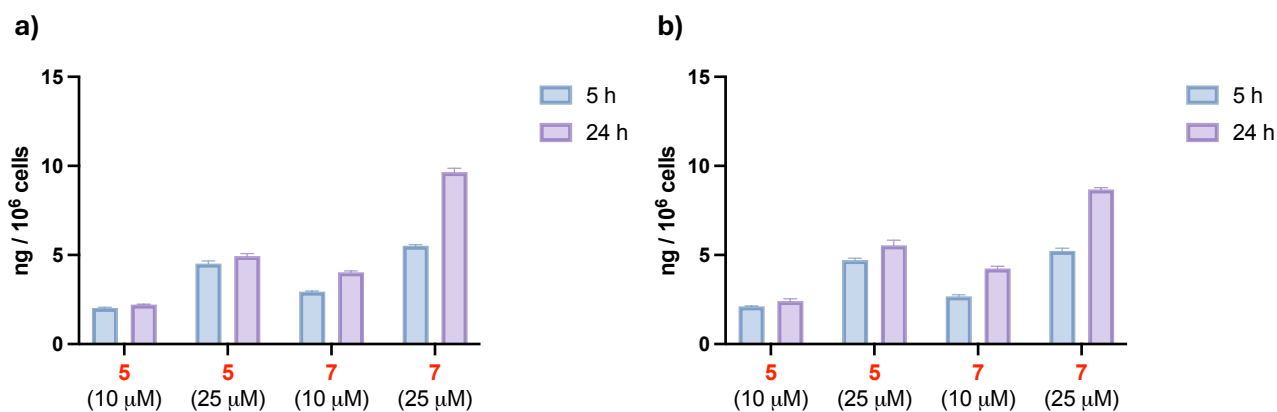


Figure 74. Cellular uptake of **5** and **7** at different concentrations and time points in a) A2780 and b) A2780cis cells. Cells were treated with the compounds at 10 and 25 μM for 5 h and 24 h. Intracellular ruthenium content was quantified by ICP-MS and is expressed as ng Ru per 10^6 cells. Data represent mean $\pm$ SEM.

3. Experimental

Chemicals and solvents were purchased from Sigma Aldrich and Thermo Fisher Scientific and used without further purification. NMR spectra were recorded on Bruker AC-E series 400 MHz. Mass spectra were recorded on an Agilent 6540 QTOF LC/MS Mass Spectrometer by direct infusion. Elemental analyses were performed using an Eurovector EA3000 elemental analyzer at the Microanalytical Laboratory of the University of Vienna. X-ray intensity data measured on STOE STADIVARI diffractometer equipped with multilayer monochromator, Mo K/ α and Cu K/ α micro focus sealed tube and Oxford cooling system at the University of Vienna. The ODN sequences used in this work are reported in Table 4. Buffers were prepared using MilliQ water and final pH values were measured by CREASON pH meter-GLP. In biological assay, stock solutions were prepared in DMSO and pre-diluted in culture medium to maintain a final DMSO concentration below 1%, thereby minimizing solvent-related cytotoxicity.

Name	Nucleotide sequence
dsDNA	5'-TATAGCTA-Heg-TATAGCTATA-3'
myc	5'-TGAGGGTGGGTAGGGTGGGTAA-3'
vegf	5'-CGGGGCGGGCCTTGGGCGGGGT-3'
htelo	5'-AGGGTTAGGGTTAGGGTTAGGG-3'
htert	5'-AGGGGAGGGGCTGGGAGGGC-3'
kit1	5'-AGGGAGGGCGCTGGGAGGAGGG-3'
kit2	5'-CGGGCGGGCGCGAGGGAGGGG-3'
bcl2	5'-AGGGGCGGGCGCGGGAGGAAGGGGGCGGGAGCGGGGCTG-3'

Table 4. Name of the ODNs used in this work and corresponding nucleotide sequences. In dsDNA, Heg linker is $[(-\text{CH}_2-\text{CH}_2-\text{O}-)_6]$. The ODNs used for FRET melting assay are labelled at the 5' end with FAM and at the 3' end with TAMRA.

Synthesis. Synthetic procedures used for **L1** and **L2** have already been reported elsewhere.^{162,163}

General procedure for **4**, **5**, **6** and **7**. The compounds were synthesized by mixing either $[\text{Ru}(\text{bpy})_2\text{Cl}_2]$ or $[\text{Ru}(\text{phen})_2\text{Cl}_2]$ (0.15 mmol) with the corresponding oxadiazole (**L1** or **L2**) (0.15 mmol) in 12 mL of an $\text{H}_2\text{O}/\text{EtOH}$ solution (1:1 volume ratio). The mixture was refluxed for 30 minutes and then allowed to cool to room temperature. To precipitate the desired product as a hexafluorophosphate salt, an excess of sodium hexafluorophosphate (NaPF_6) was added. The resulting solid was filtered and washed with small portions of cold distilled water, ethanol and diethyl ether. Finally, any remaining solvent was removed under vacuum.

Synthesis of **4**. The product was isolated as an orange powder with a yield of 47%. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 8.91 – 8.80 (m, 4H), 8.63 (d, $J = 7.8$ Hz, 1H), 8.55 (d, $J = 5.5$ Hz, 1H), 8.34 – 8.16 (m, 5H), 8.05 – 7.99 (m, 2H), 7.88 – 7.82 (m, 1H), 7.81 – 7.52 (m, 11H). ESI-MS m/z calculated for $[\text{M}]^+$: 318.5581, found: 318.5589. Elemental analysis calculated for $\text{C}_{33}\text{H}_{25}\text{F}_{12}\text{N}_7\text{OP}_2\text{Ru} + \frac{1}{2} \text{H}_2\text{O}$: C 42.36%, H 2.80%, N 10.48%; found: C 42.41%, H 2.65%, N 10.45%.

Synthesis of **5**. The product was isolated as an orange powder with a yield of 40%. ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 9.08 (dd, $J = 5.3, 1.2$ Hz, 1H), 8.88 (dt, $J = 8.3, 1.4$ Hz, 2H), 8.78 (ddd, $J = 12.2, 8.3, 1.2$ Hz, 2H), 8.67 – 8.61 (m, 1H), 8.46 – 8.31 (m, 4H), 8.31 – 8.22 (m, 2H), 8.08 (dd, $J = 5.3, 1.3$ Hz, 1H), 8.03 – 7.92 (m, 5H), 7.81 – 7.66 (m, 4H), 7.62 – 7.52 (m, 3H). ESI-MS m/z calculated for $[\text{M}]^+$: 342.5581, found: 342.5579. Elemental analysis calculated for $\text{C}_{37}\text{H}_{25}\text{F}_{12}\text{N}_7\text{OP}_2\text{Ru}$: C 45.6%, H 2.59%, N 10.06%; found: C 45.29%, H 2.76%, N 9.76%.

Synthesis of **6** The product was isolated as an orange powder with a yield of 51%. ¹H NMR (400 MHz, DMSO-d₆) δ 8.89 – 8.79 (m, 4H), 8.61 (dd, J = 7.8, 1.4 Hz, 1H), 8.53 (dd, J = 5.7, 1.4 Hz, 1H), 8.34 – 8.16 (m, 5H), 7.86 – 7.75 (m, 5H), 7.73 – 7.64 (m, 2H), 7.64 – 7.48 (m, 6H), 2.39 (s, 3H). ESI-MS m/z calculated for [M]⁺: 325.5659, found: 325.5682. Elemental analysis calculated for C₃₄H₂₇F₁₂N₇OP₂Ru: C 43.41%, H 2.89%, N 10.42%; found: C 43.11%, H 2.93%, N 10.20%.

Synthesis of **7**. The product was isolated as an orange powder with a yield of 53%. ¹H NMR (400 MHz, DMSO-d₆) δ 9.07 (dd, J = 5.4, 1.3 Hz, 1H), 8.89 (dt, J = 8.2, 1.6 Hz, 2H), 8.79 (ddd, J = 11.6, 8.3, 1.3 Hz, 2H), 8.67 – 8.61 (m, 1H), 8.46 – 8.32 (m, 4H), 8.32 – 8.24 (m, 2H), 8.09 (dd, J = 5.4, 1.2 Hz, 1H), 8.04 – 7.92 (m, 3H), 7.81 – 7.69 (m, 5H), 7.61 – 7.43 (m, 3H), 2.35 (s, 3H). ESI-MS m/z calculated for [M]⁺: 349.5660, found: 349.5688. Elemental analysis calculated for C₃₈H₂₇F₁₂N₇OP₂Ru + 1.5 H₂O: C 44.94%, H 2.98%, N 9.65%; found: C 44.94%, H 2.72%, N 9.60%.

FRET-melting assay. FRET melting experiments were performed using an Applied Biosystems™ QuantStudio 6 PCR cyclers in 96-well format, using a FAM (6-carboxyfluorescein) filter. Oligonucleotides labeled with FAM and TAMRA (6-carboxytetramethylrhodamine) were diluted to the desired concentrations in 60 mM potassium cacodylate buffer (pH 7.4). In the first place, for vegf and myc sequences, dilutions were performed in 10 mM potassium cacodylate (pH 7.4) to ensure a melting temperature range suitable for detecting compound-induced stabilization. Secondly, these ODNs were diluted in the same buffer supplemented with 50 mM LiCl. ODNs were folded into their respective B-DNA or G-quadruplex topologies by heating at 95 °C for 5 minutes, followed by slow cooling to room temperature overnight. Final oligonucleotide concentration was 0.2 μM in a total volume of 30 μL per well. Metal complexes were dissolved in DMSO to prepare 2 mM stock solutions and subsequently diluted in buffer, ensuring a final solvent concentration <1%. Fluorescence data were collected in duplicate across the 25–95 °C range, with a 1 °C ramp every 30 seconds. Emission values were normalized from 0 to 1 for cross-experiment comparability. The melting temperature (T_{1/2}) was defined as the temperature at which normalized FAM emission equals 0.5. DNA concentrations are expressed in strands.

UV-Vis and CD spectroscopy. UV-Vis spectra were collected at room temperature on a Cary 1E double-beam spectrophotometer. For stability studies, DMSO stock solutions were diluted to ~50 μM in Tris-HCl (50 mM) / KCl (100 mM) buffer (pH 7.4), and spectra were recorded every hour over a 24 h period. In UV-Vis titrations, metal complexes were kept at a constant concentration, while increasing amounts of G4-DNA or B-DNA were added to monitor binding interactions. To ensure the metal complex concentration remained unchanged throughout the titration, each addition of DNA

solution was matched with an equal volume of a double-concentrated metal complex solution. CD spectra were acquired on a Jasco J-715 spectropolarimeter at 25 °C. In this case, DNA was maintained at a fixed concentration and increasing amounts of the metal complexes were added. CD measurements were performed in the 220-450 nm range, with a response time of 0.5 s, a scan speed of 200 nm·min⁻¹, and data averaged over four accumulations.

Cell viability assay. HCT116, HCT116/BoldR, HCT116/OxaR, A2780 and A2780cis were plated at a density of 4×10^3 cells per 100 μ L in 96-well microtiter plates and allowed to adhere overnight. The cells were treated with the compounds at the specified concentrations for 72 hours. Cells were treated with the compounds at the indicated concentrations for 72 h. Cell viability was assessed using the MTT-based EZ4U assay (Biomedica, Vienna, Austria) according to the manufacturer's instructions. Absorbance was measured at 450 nm with a reference wavelength of 620 nm using an Asys Expert Plus microplate reader (v1.4, Asys Hitech GmbH, Eugendorf, Austria). Data were processed with GraphPad Prism and represent three independent biological replicates per condition (n = 3).

Cellular Ruthenium Uptake Measurements. A2780 and A2780cis cells were seeded at a density of 7.5×10^5 cells/mL in 6-well plates (2 mL per well) and allowed to adhere overnight. Cells were then treated with the compounds at the indicated concentrations for 5 or 24 hours, using fresh culture medium as the solvent control. After incubation, cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) at room temperature, detached using trypsin, and counted. Cell pellets were subsequently washed twice with PBS and allowed to dry overnight at room temperature. The following day, dried pellets were digested in 65% HNO₃ for 1 hour and then diluted with double-distilled water (ddH₂O) prior to ICP-MS analysis. The obtained values were then normalized to the number of cells for each condition.

4. Conclusions

In this study, the first four Ru(II) polypyridyl-oxadiazole complexes were synthesized and characterized. Their stability, nucleic acid interactions, and biological activity were assessed. Under the experimental conditions, the compounds displayed only modest and nonspecific effects on DNA and G4 stabilization, suggesting that their anticancer activity is unlikely to arise from direct G4 targeting. In ovarian cancer cell models, the complexes exhibited moderate antiproliferative effects, with phen-based derivatives showing the lowest IC₅₀ values. Notably, the cisplatin-resistant A2780cis line was more sensitive than the parental A2780 cells, indicating a mechanism of action distinct from

platinum drugs. ICP-MS analysis further revealed that **7** accumulated to the highest intracellular levels in a time-dependent manner, which may explain its greater cytotoxicity relative to **5**. Altogether, this work expands the small family of oxadiazole-coordinated metal complexes and highlights how oxadiazole ligands modulate Ru(II) polypyridyl properties. Although DNA binding appears limited, the observed biological activity and uptake behavior establish oxadiazole-functionalized Ru(II) complexes as promising scaffolds for further development and optimization. Finally, some aspects of this project are still under active investigation.

Chapter IV: 4-phenylthiazole derived Ru(II) metalacycles and their interaction with G-Quadruplex

1. Introduction

In collaboration with Prof. Wolfgang Kandioller (University of Vienna) and in close cooperation with his PhD student Paul Getreuer, who spent three months in Palermo as part of his doctoral project, the DNA-binding properties of novel 4-phenylthiazole-derived Ru(II) metalacycles were investigated as part of a broader study on their anticancer potential. This collaborative work resulted in two scientific publications: “Investigating the anticancer potential of 4-phenylthiazole derived Ru(II) and Os(II) metalacycles” (*Dalton Transactions*, 2024),¹⁶⁴ and “Exploring the Anticancer Properties of 4-Phenylthiazole-Based Ru(II) and Os(II) Metalacycles Featuring 1-Methylimidazole as N-Donor Functionality” (*Bioinorganic Chemistry and Applications*, 2025).¹⁶⁵

As outlined in section 1.4.2, under physiological conditions Ru(II) can coordinate π -arene moieties, giving rise to highly robust “piano-stool” architectures, which are promising candidates for anticancer drug development.^{166,167} These complexes, often featuring halide leaving groups, undergo aquation to enable biomolecular interactions, a process central to their activity.^{168,169} In this context, C,N-coordinated scaffolds have proven to be resilient and display notable cytotoxic properties.^{166,170}

Within this framework, a library of Ru(II) metalacycles bearing 4-phenylthiazole ligands was synthesized, along with derivatives in which the labile chlorido ligands were replaced by an N-donor group (1-methylimidazole) to improve solubility, stability, and biological performance.^{165,166} Notably, Ru–arene complexes have only rarely been studied against G4s.^{171–174}

The chemical structure of the complexes studied in this collaborative work, prepared at the University of Vienna, are reported below (Figure 75). My contribution in this study was to evaluate the new complexes’ DNA binding properties, including B- and G4- topologies. In particular, DNA binding studies using FRET melting, CD spectroscopy, mass spectrometry, and molecular docking were performed.

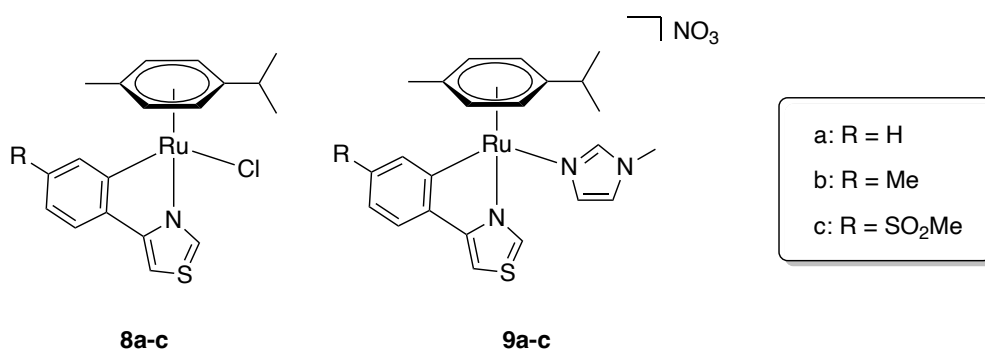


Figure 75. Chemical structure of the compounds **8a-c** and **9a-c**, studied in this work.^{164,165}

2. Results and Discussion

FRET-melting assay

The DNA binding properties of compounds **8a-c** were first assessed by FRET melting assays using G4 sequences (kit1 and myc) and a duplex reference (dsDNA). Both G4s form in the promoters of the corresponding oncogenes and regulate their transcription.^{175,176} Stabilization was quantified by increases in melting temperature ($\Delta T_{1/2}$). Notably, no stabilization was observed with dsDNA up to a 1:20 DNA/compound ratio (Figure 76).

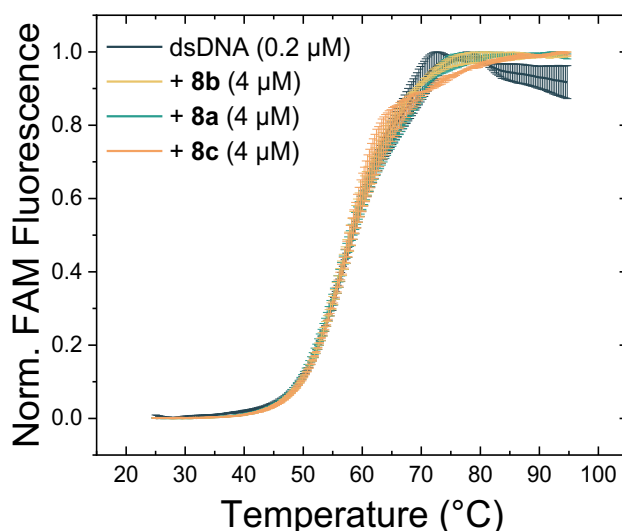


Figure 76. FRET melting profiles of dsDNA oligonucleotide (0.2 μ M, reported per strand) upon interaction with the indicated compounds at the indicated concentration. Buffer: 60 mM potassium cacodylate, pH 7.4.

On the other hand, a clear concentration-dependent stabilization was detected with G4s (Figure 77). In particular, the most pronounced effect was observed for myc with **8b**, which increased the melting temperature by ~ 15 $^{\circ}$ C at 1:20 and 18 $^{\circ}$ C at 1:30. By contrast, only modest stabilization of kit1 was achieved with **8a** and **8c** at higher ratios. Compared to other metal complexes,¹⁷⁷ these cyclometalates are moderate stabilizers but show marked selectivity for G4 over B-DNA.

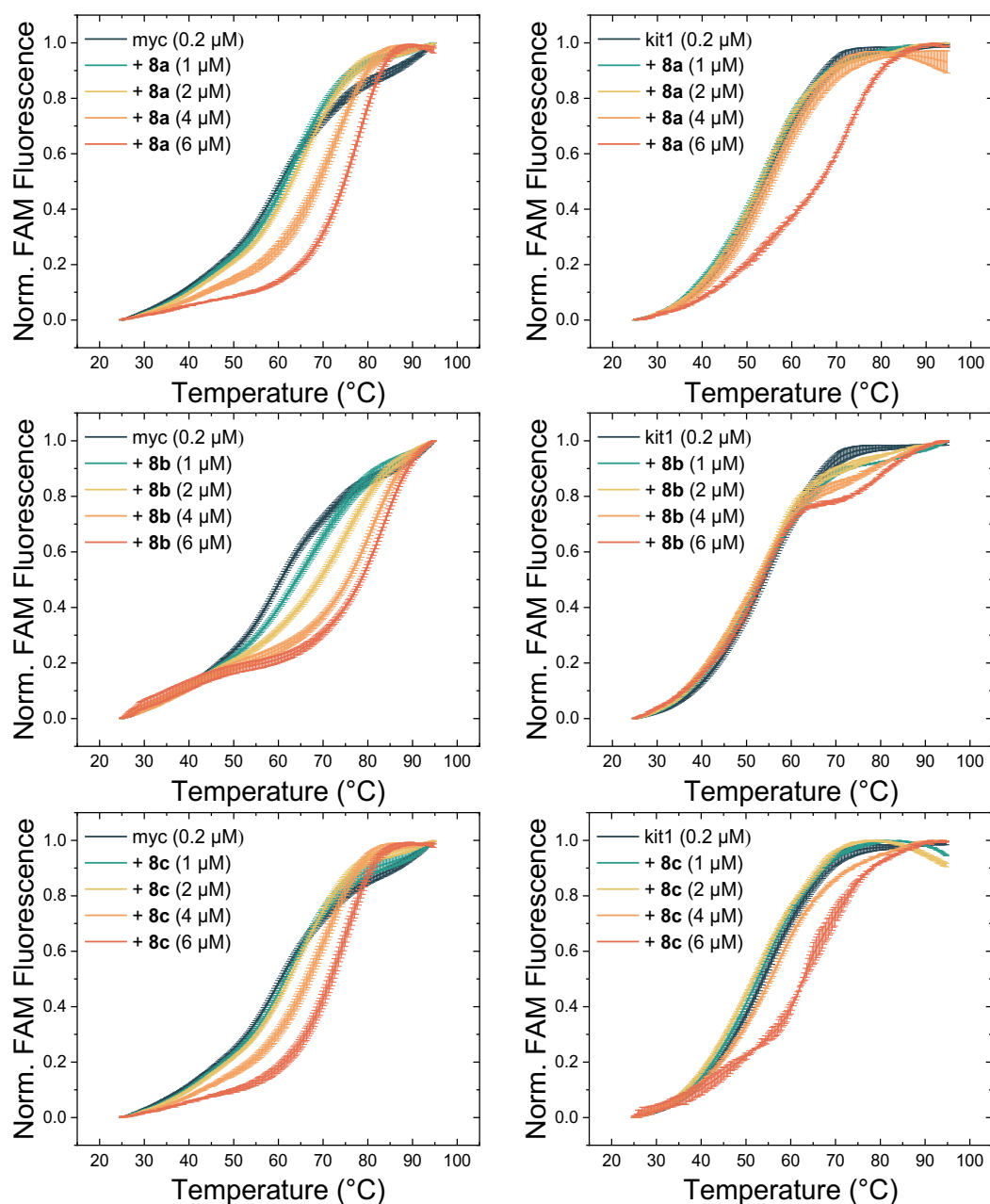


Figure 77. FRET melting profiles of myc (left) and kit1 G4s (right) at 0.2 μM , upon interaction with the indicated compounds at the indicated concentration values. Buffer: 60 mM potassium cacodylate, pH 7.4. ODN concentrations are reported per strand.

In contrast to the chlorido analogues, the methyl imidazole derivatives (**9a-c**) did not stabilize any of the tested G4s or B-DNA sequences. This indicates that the methyl imidazole ligand suppresses the ability of the Ru(II) complexes to undergo base coordination after aquation, thereby precluding DNA interaction under the experimental conditions employed (Figure 78). For this reason, no further investigation was conducted on these complexes.

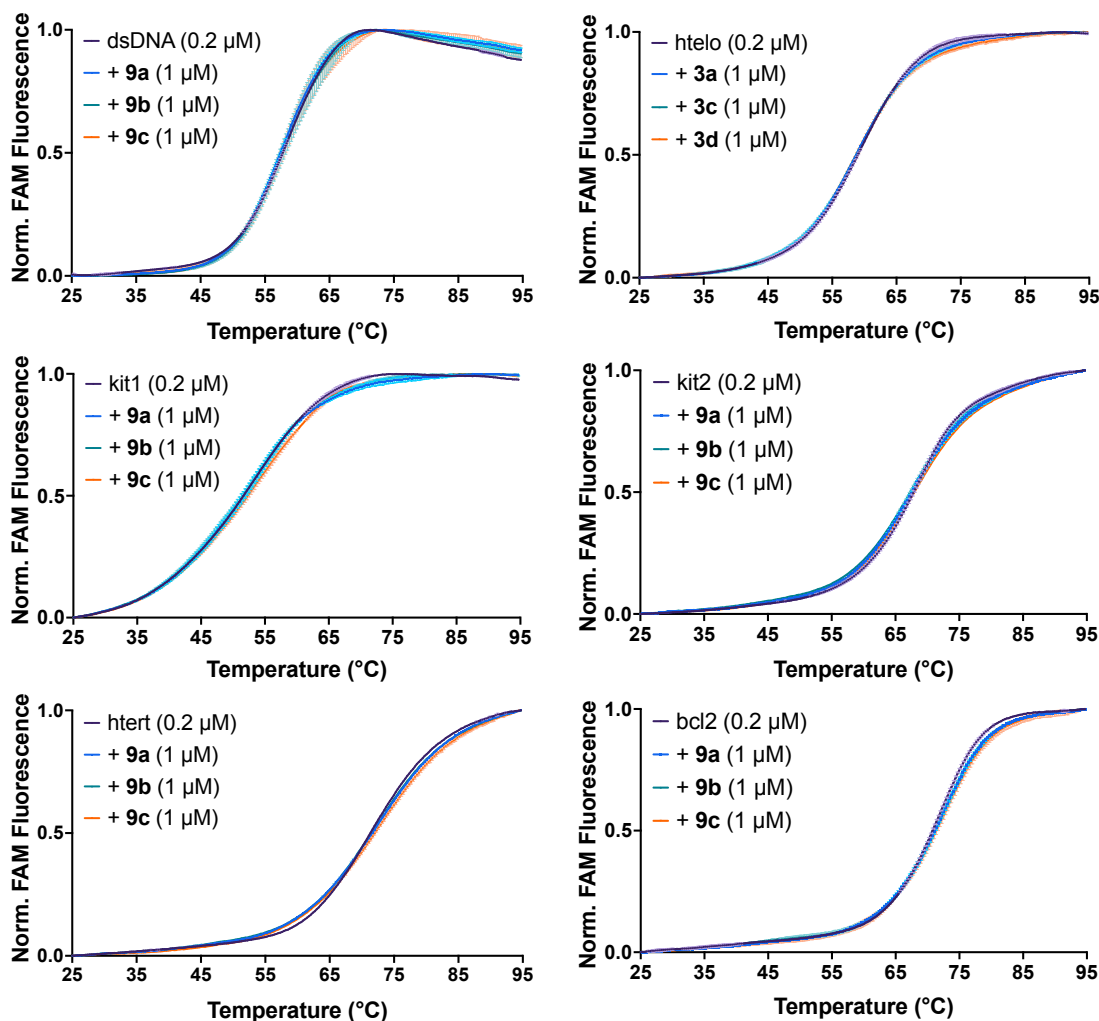


Figure 78. FRET melting curves of the indicated oligonucleotides when incubated with compounds **9a-c**. Buffer: 60 mM potassium cacodylate, pH 7.4. ODN concentrations are reported per strand.

Circular Dichroism

To further characterize the DNA interaction properties of compounds **8a-c**, CD titrations were performed using various G4 topologies and calf thymus DNA (ct-DNA) as a B-DNA model. The spectra displayed the characteristic parallel G4 signatures, which remained essentially unchanged upon addition of the complexes. These results indicate that **8a-c** do not induce detectable conformational perturbations of the G4 topology under the tested conditions (Figure 79).

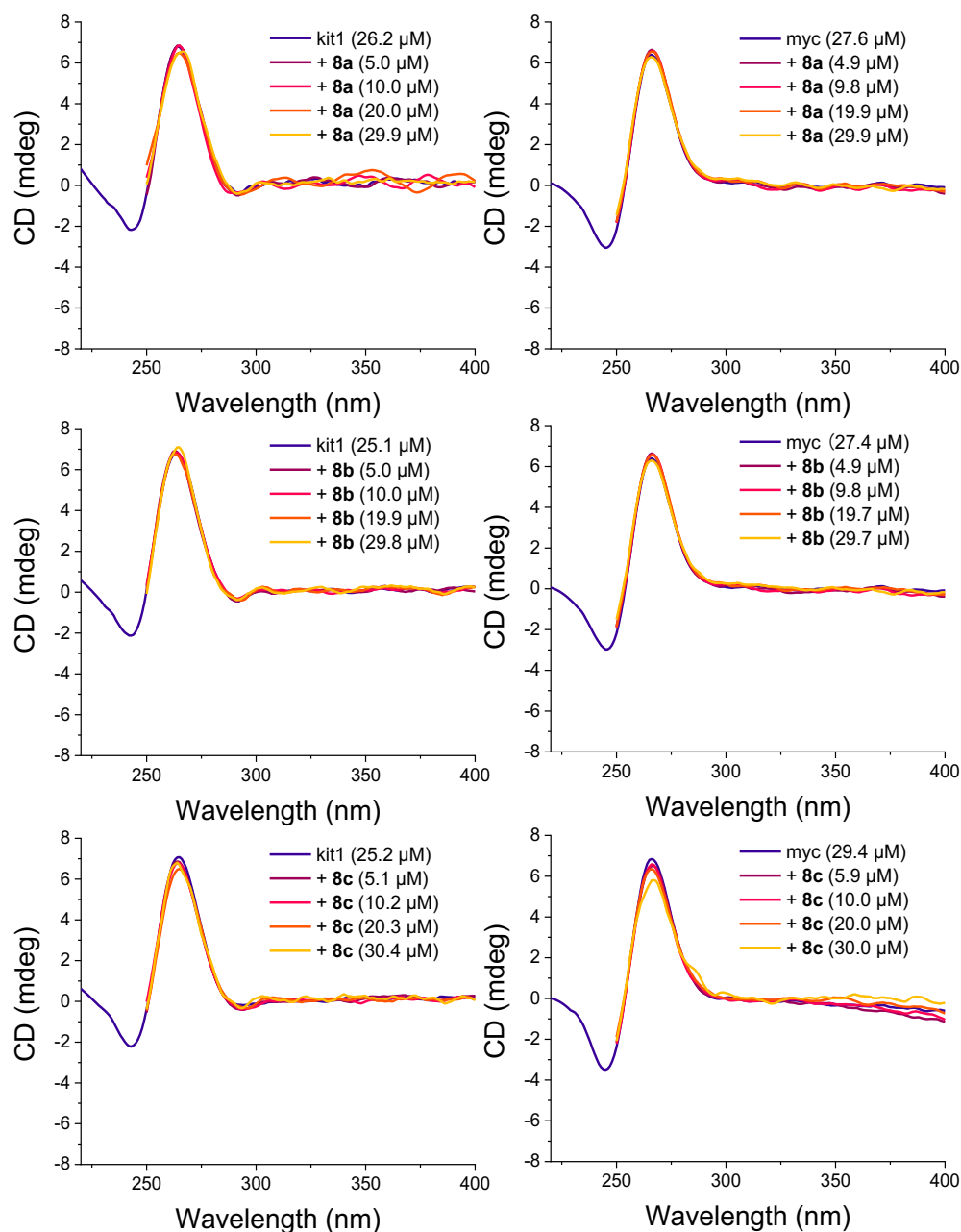


Figure 79. CD spectra of kit1 (left) and myc (right) in the presence of increasing aliquots of **8a-c** at the indicated concentrations. Concentration of the oligonucleotides is reported per bases. When the metal-based compounds are in solution, the cut-off of the spectra was set at 250 nm due to the presence of DMF as co-solvent (% <1.7%).

To compare the behavior toward canonical duplex DNA, analogous experiments were carried out using calf thymus DNA (ct-DNA) as a B-DNA model. The CD spectra showed the typical positive and negative bands at 275 and 245 nm, which were unaffected by increasing concentrations of **8a-c**. This confirms that the interaction with B-DNA does not cause significant structural alterations (Figure 80).

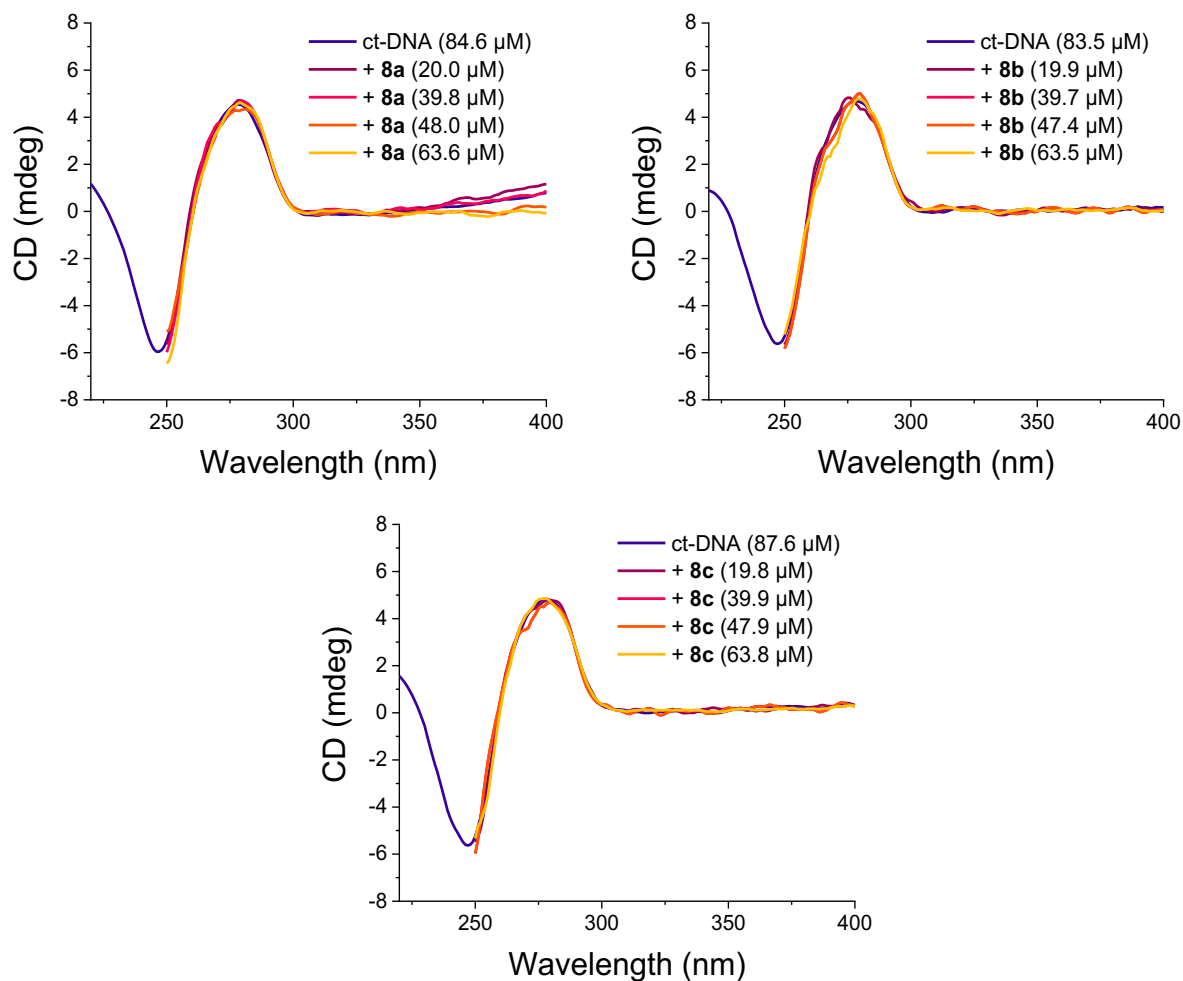


Figure 80. CD spectra of ct-DNA in the presence of increasing aliquots of **8a-c** at the indicated concentrations. Concentration of ct-DNA is reported in bases. When the metal-based compounds are in solution, the cut-off of the spectra was set at 250 nm due to the presence of DMF as co-solvent (% < 1.7%).

Mass Spectrometry

Because Ru(II)–arene compounds contain a halido leaving group capable of substitution by DNA bases after aquation, adduct formation with 9-ethylguanine was evaluated by MS. All three ruthenacycles readily formed adducts under the tested conditions (Figures 81-83).

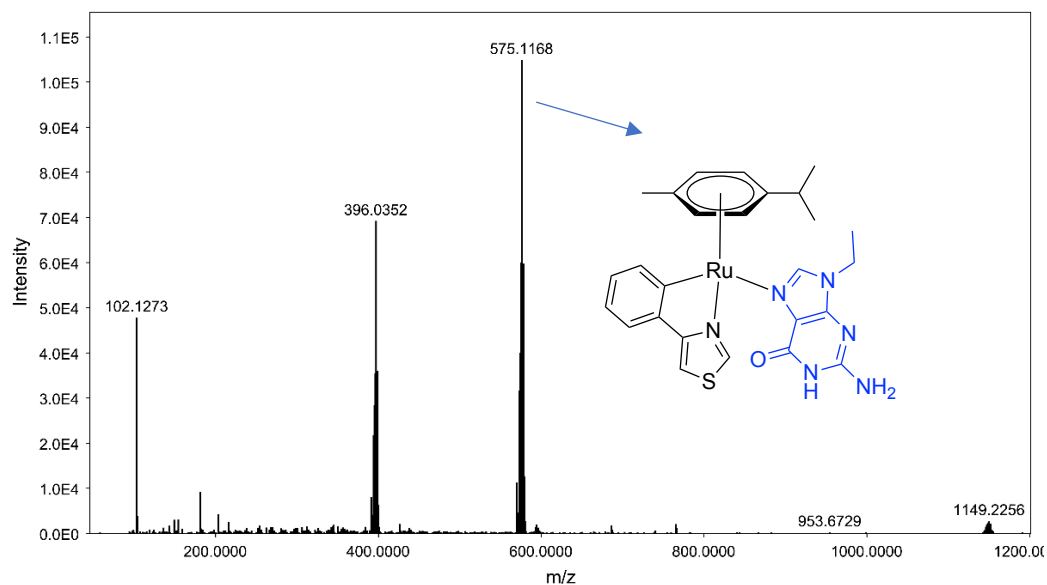


Figure 80. Mass spectrum of 9-ethylguanine incubated with **8a**. Final concentrations in MeOH/H₂O before injection were 0.2 mM for the metal compound and 0.6 mM for 9-EtG. Mass of 9-EtG-2a adduct: found 575.1168, calcd.: 575.1167.

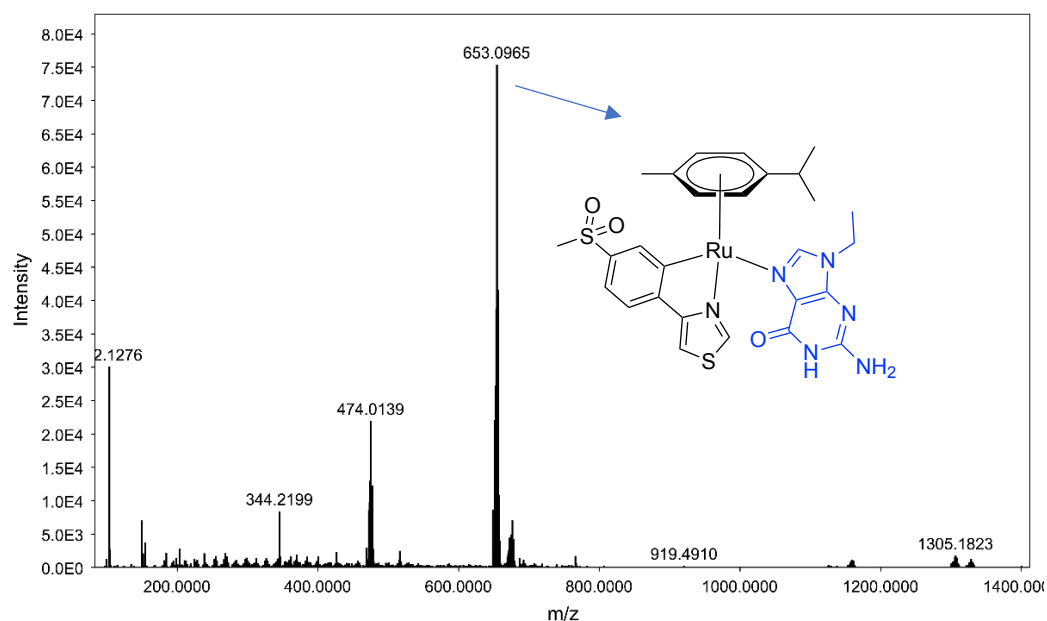


Figure 81. Mass spectrum of 9-ethylguanine incubated with **8b**. Final concentrations in MeOH/H₂O before injection were 0.2 mM for the metal compound and 0.6 mM for 9-EtG. Mass of 9-EtG-2c adduct: found 653.0995, calcd.: 653.0943.

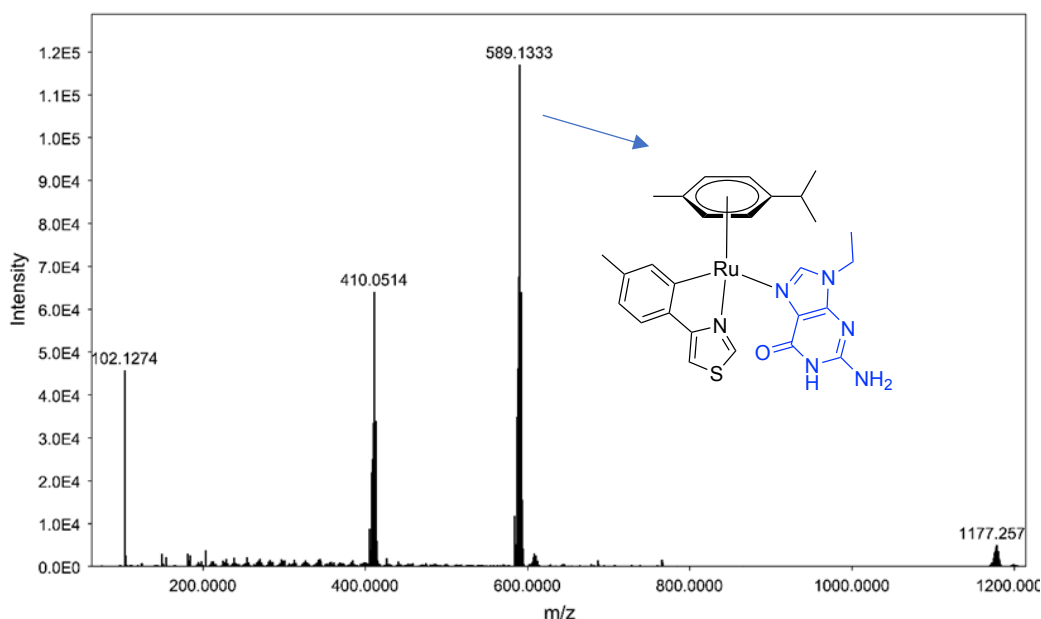


Figure 82. Mass spectrum of 9-ethylguanine incubated with **8c**. Final concentrations in MeOH/H₂O before injection were 0.2 mM for the metal compound and 0.6 mM for 9-EtG. Mass of 9-EtG-2d adduct: found 589.1333, calcd.: 589.1324.

Consistently, MS confirmed the formation of a **8b**-myc adduct, corroborating the stabilization observed in FRET experiments (Figure 83),

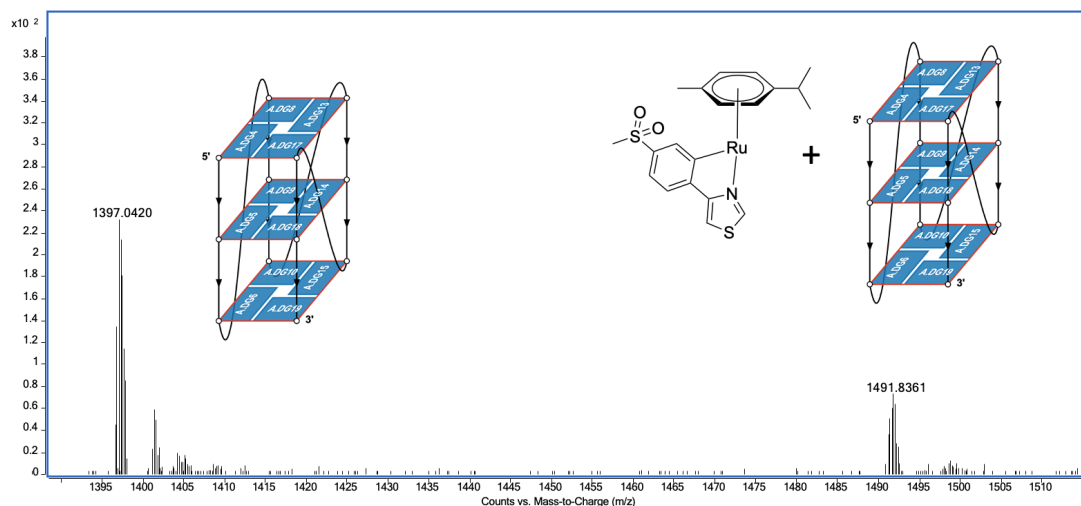


Figure 83. Mass Spectrum of **8b** incubated with myc for 2 h at room temperature. Five charged mass of c-MYC: found: 1397.2403, calcd: 1397.2318 (M-H⁺ for C₂₂₀H₂₇₀O₁₃₁N₉₅P₂₁.) Five charged mass of **8b**-c-MYC adduct: found: 1491.8361, calcd: 1491.8332 (M-H⁺ for C₂₂₀H₂₇₀ O₁₃₁ N₉₅P₂₁C₂₀H₂₁NO₂RuS₂).

Molecular docking

The interactions of compounds **8a-c** (R and S enantiomers) with selected G4 structures were explored through molecular docking calculations. The NMR-resolved structures of myc (PDB ID: 1XAV) and kit1 (PDB ID: 2O3M) were used as G4 models, while the geometries of both enantiomers of the compounds were optimized by DFT. Among 50 docking poses, the most representative from each cluster are shown in Figure 84 and Figure 85 for myc and kit1, respectively. Docking results revealed that the metallacycles preferentially bind within the DNA grooves rather than stacking on the G-tetrads, unlike classical planar G4 binders.

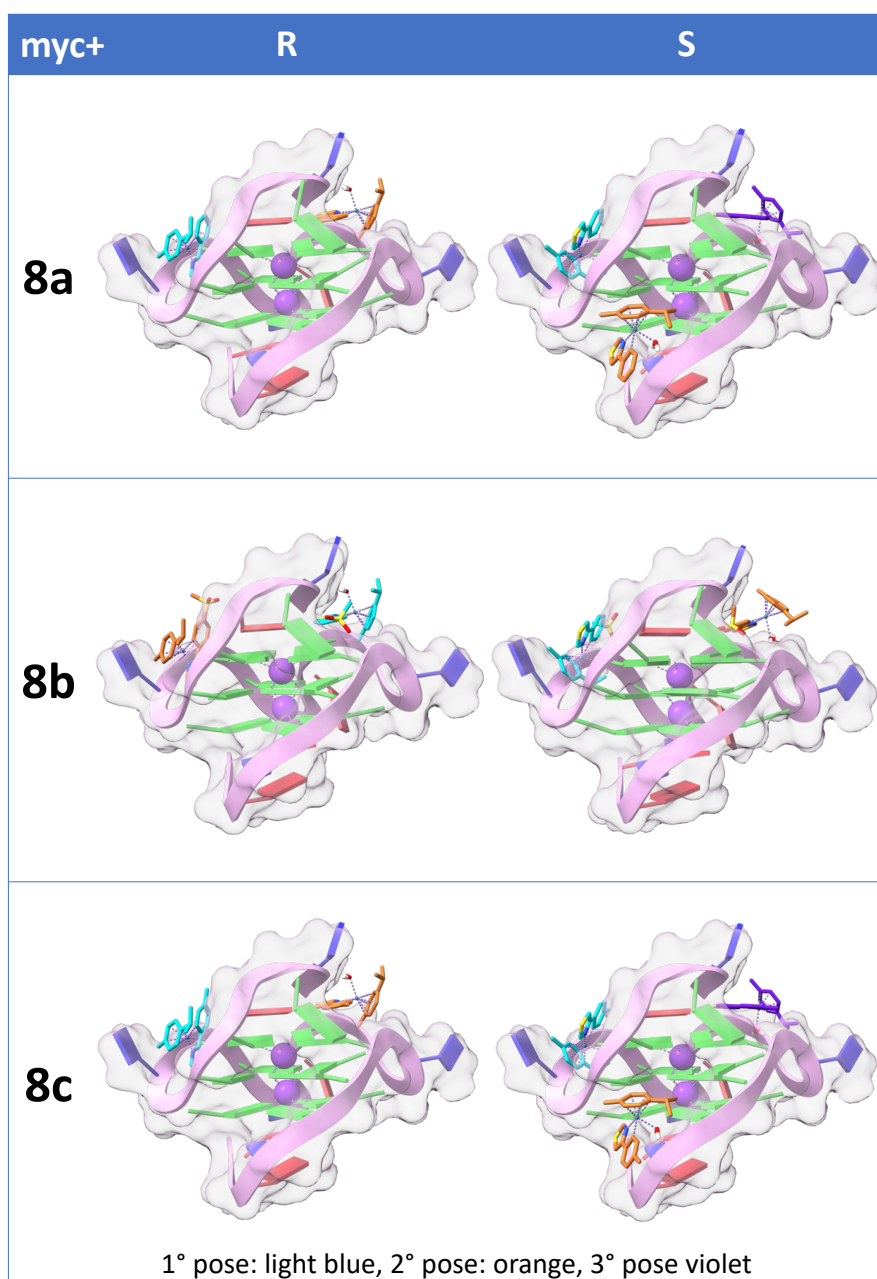


Figure 84. Cartoons showing possible binding sites of both enantiomers of **8a-c** with myc G4 (PDB id: 1XAV).

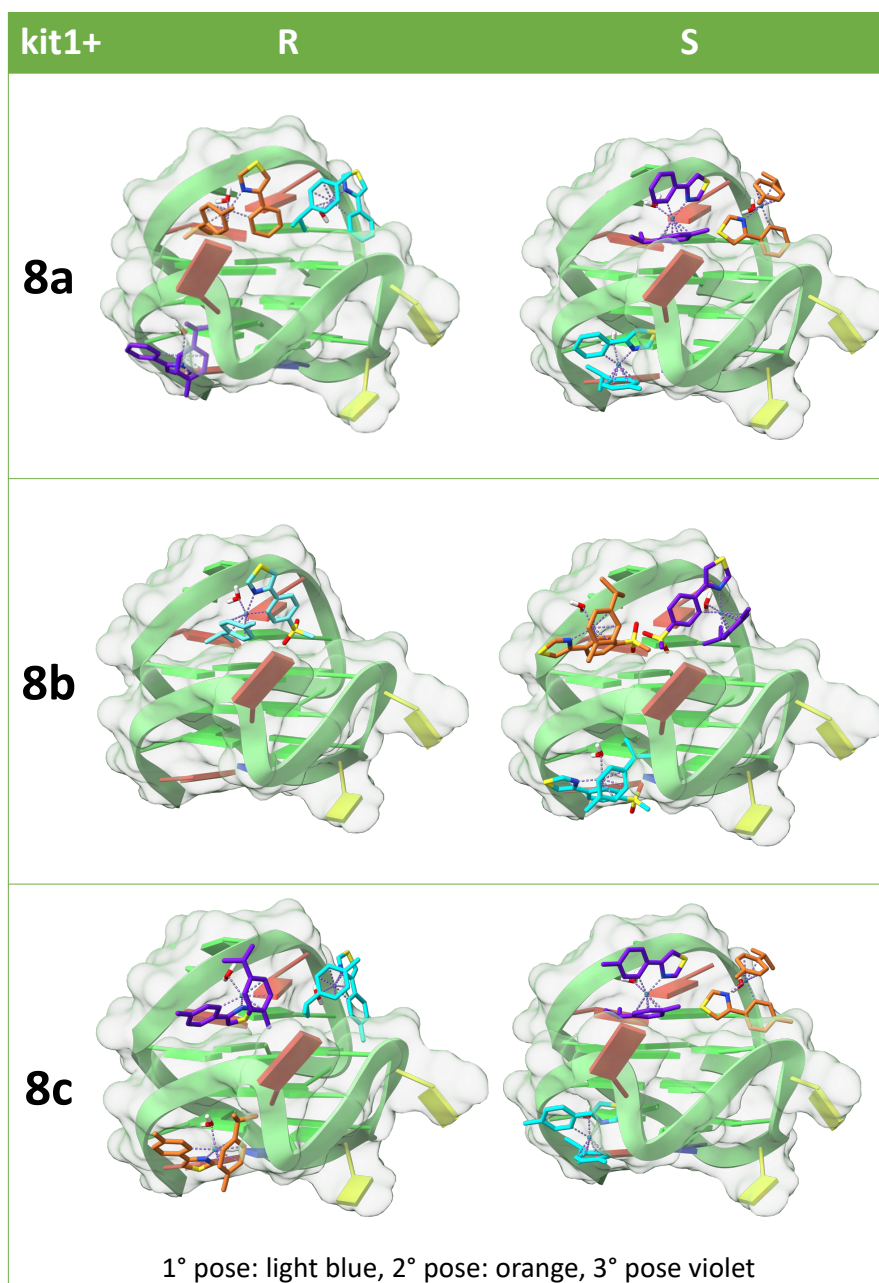


Figure 85. Cartoons showing possible binding sites of both enantiomers of **8a-c** with kit1 G4 (PDB id: 2O3M).

Binding energy values (Tables 5-6) indicated stronger non-covalent interactions for the R enantiomers with both G4s, with **8b** (R) emerging as the most effective myc binder, consistent with the FRET data. Notably, docking was performed with the aqua complexes, and the poses likely represent electrostatically driven intermediates preceding substitution of the coordinated water by a DNA base.

	Kcal/mol (1 st pose)	Kcal/mol (2 nd pose)	Kcal/mol (3 rd pose)
8a (R)	-7.48	-7.06	-7.01
8a (S)	-6.37	-5.92	-5.90
8b (R)	-8.61		
8b (S)	-7.84	-7.80	-7.53
8c (R)	-7.97	-7.20	-6.57
8c (S)	-6.73	-6.45	-6.20

Table 5. Docking free energies of binding with kit1 G4.

	Kcal/mol (1 st pose)	Kcal/mol (2 nd pose)	Kcal/mol (3 rd pose)
8a (R)	-7.77	-7.65	
8a (S)	-7.55	-6.72	-6.41
8b (R)	-9.04	-8.66	
8b (S)	-8.72	-8.20	
8c (R)	-7.97	-7.69	
8c (S)	-8.01	-7.10	-6.81

Table 6. Docking free energies of binding with myc G4.

3. Experimental

FRET Melting Assay. FRET melting experiments were performed on an Applied Biosystems QuantStudio 6 PCR cyclers in 96-well format using a FAM (6-carboxyfluorescein) filter. Oligonucleotides labeled with FAM and TAMRA (6-carboxytetramethylrhodamine) were diluted to the desired concentration in 60 mM potassium cacodylate buffer (pH 7.4). To induce proper folding into B-DNA or G-quadruplex structures, solutions were heated at 95 °C for 5 min and cooled slowly to room temperature overnight. Final oligonucleotide concentration was 0.2 μ M in a total reaction volume of 30 μ L per well. Metal complexes were dissolved in DMF to obtain 2 mM stock solutions and subsequently diluted with buffer, ensuring a final DMF content <0.05%. Mixtures of DNA and complexes were incubated for 2 h at room temperature prior to measurement. Thermal denaturation profiles were recorded from 25 to 95 °C, with a temperature ramp of 1 °C every 30 s. Each experiment was carried out in duplicate and repeated twice. FAM emission values were normalized from 0 to 1 for cross-comparison. Melting temperatures ($T_{1/2}$) were defined as the point at which normalized emission reached 0.5. DNA concentrations are expressed as single strands.

CD Spectroscopy. CD spectra were recorded on a Jasco J-715 spectropolarimeter at 25 °C. DNA was maintained at a constant concentration, while increasing aliquots of metal complex solutions were added. Measurements were acquired in the 220-400 nm range with the following settings: response time 0.5 s, scan speed 200 nm·min⁻¹, and four accumulations per spectrum. Experiments were carried out in Tris-KCl buffer (50 mM Tris-HCl, 100 mM KCl, pH 7.4). G4 oligonucleotides were folded as described for the FRET assays. DNA concentrations are reported in nucleobases.

Mass Spectrometry. For 9-ethylguanine adduct formation, metal complexes were dissolved in DMF and mixed with a stock solution of 9-ethylguanine in H₂O, then diluted to final concentrations of 0.2 mM complex and 0.6 mM 9-ethylguanine (4% DMF). After 2 h of stirring at room temperature, samples were analyzed by direct infusion into an Agilent 6540 QTOF LC/MS Mass Spectrometer. For oligonucleotide binding, sequences were dissolved in 100 mM NH₄OAc, heated to 95 °C for 5 min, and slowly cooled to room temperature to allow folding. Complexes were prepared in DMF and diluted in 100 mM NH₄OAc to 30 μM. Equimolar mixtures of complex and oligonucleotide (30 μM each) were incubated for 2 h under constant stirring prior to MS analysis. Spectra were recorded by direct infusion on an Agilent 6540 QTOF LC/MS Mass Spectrometer.

Molecular docking. The structures of the metal complexes underwent optimization through Density Functional Theory (DFT) calculations within the Gaussian16 program package.¹⁷⁸ Calculations employed the M06L¹⁷⁹ functional with the LANL2TZ (f) pseudopotential basis set for metal atoms¹⁸⁰ and the 6-311G (d,p) basis set for all other atoms.¹⁸¹ For docking studies, pdbqt files of kit1 (PDB ID: 2O3M), myc (PDB ID: 1XAV), and the selected complexes (**8a-c**) were generated with AutoDock Tools 1.5.6.¹⁸² Blind docking was performed with AutoDock 4.2,⁶¹ using a grid box of 80 × 80 × 80 Å with 0.375 Å spacing, centered at (−0.062, 1.272, 0.974) for myc and (−1.029, 4.110, −1.269) for kit1. The Lamarckian Genetic Algorithm was applied to explore binding poses and predicted free binding energies are reported in kcal·mol⁻¹. Figures were prepared with ChimeraX.¹⁸³

4. Conclusion

In this collaborative work, the DNA binding potential of a family of 4-phenylthiazole-derived Ru(II) arene metallacycles and related derivatives was studied. The methyl-imidazole complexes (**9a-c**) did not show any binding properties with either B- or G4 DNA. Conversely, for the chlorido complexes (**8a-c**), the data revealed a clear preference for G4 over duplex binding, especially for complex (**8b**) with myc G4. FRET-melting showed concentration-dependent stabilization of oncogenic G4 models,

while producing negligible effects on B-DNA under comparable conditions. CD spectroscopy indicated that the canonical parallel G4 signatures were largely retained, consistent with non-disruptive recognition. ESI-MS established adduct formation with a guanine mimic (9-ethylguanine) and, for the most active member (**8b**), with the myc oligonucleotide, supporting the feasibility of covalent engagement after aquation. Molecular docking showed that the preferred binding poses locate the metallacycles in G4 grooves rather than on terminal tetrads. Finally, **8b** R enantiomers displayed more favorable binding energies.

Chapter V: Alloxazine-Based Ruthenium Complexes as G-Quadruplex Binders

1. Introduction

In collaboration with Prof. Luca Salassa of the Donostia International Physics Center (DIPC, Spain), the DNA-binding properties of Ru(II) complexes incorporating phenanthroline–alloxazine hybrid ligands were investigated, within a broader investigation primarily focused on their redox behavior and photochemical properties. This collaborative work resulted in the scientific publication “Alloxazine-Based Ligands and Their Ruthenium Complexes as NADH Oxidation Catalysts and G4 Binders” (*Inorganic Chemistry*, 2024).¹⁸⁴

Flavins are versatile biological cofactors that mediate single- and multi-electron redox processes, playing central roles in metabolism and energy transfer. Their isoalloxazine core enables efficient electron shuttling, while interactions with metal ions can modulate redox properties, electron transfer rates, and excited-state reactivity. Despite this potential, metal–flavin complexes remain relatively underexplored. Alloxazines structural isomers of flavins with distinct tautomeric and redox features have recently emerged as promising chelating ligands, with applications ranging from catalysis to redox-flow batteries.

In this context, phenanthroline–alloxazine hybrid ligands were synthesized and coordinated to prototypical Ru(II) scaffolds, namely **10** and **11**, (Figure 86) and their photophysical and catalytic properties were evaluated. My contribution focused on examining their interactions with DNA, given the structural analogy to Ru-dppz complexes, well-established DNA intercalators. Specifically, the DNA-binding behavior of complexes **10** and **11** was assessed through FRET melting assays, circular dichroism spectroscopy, and fluorescence spectroscopy.

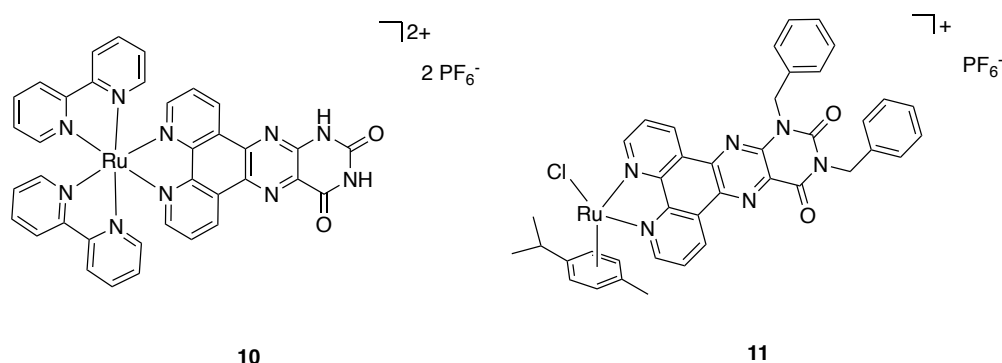


Figure 86. Chemical structure of compounds **10** and **11**, studied in this collaborative work.

2. Results and Discussion

FRET melting assay

In FRET assays, **10** and **11** were incubated with the G4-forming oligonucleotides htelo (hybrid topology) and kit1 (parallel topology), alongside duplex dsDNA as a B-DNA model. These sequences were chosen for their biological relevance: htelo is a telomeric G-rich sequence whose stabilization inhibits telomerase, whereas kit1 derives from the *KIT* oncogene promoter, a validated therapeutic target.^{31,151,185}

Stabilization was quantified as the increase in melting temperature ($\Delta T_{1/2}$). At lower complex/oligonucleotide ratios (5:1), no relevant stabilization was observed, but at higher ratios, concentration-dependent effects emerged with both **10** and **11**. Compound **11** produced the strongest stabilization, with $\Delta T_{1/2}$ values of 29.7 °C (kit1) and 24.7 °C (htelo) at a 20:1 ratio, whereas dsDNA was unaffected up to 10:1. Only at the highest ratio **11** also stabilize B-DNA, indicating a preference for G4s (Table 7 and Figure 87).

complex	[complex]/[oligo]	dsDNA	htelo	kit1
10	5	2.3	2.6	5.6
	10	4.4	4.2	7.8
	20	8.5	11.0	12.8
11	5	0	2.3	1.2
	10	1.0	7.7	4.3
	20	17.9	24.7	29.7

Table 7. $\Delta T_{1/2}$ Values of 0.2 μ M dsDNA, h-Telo and Kit1 at the indicated [Complex]/[Oligo] Ratios with **10** and **11**.

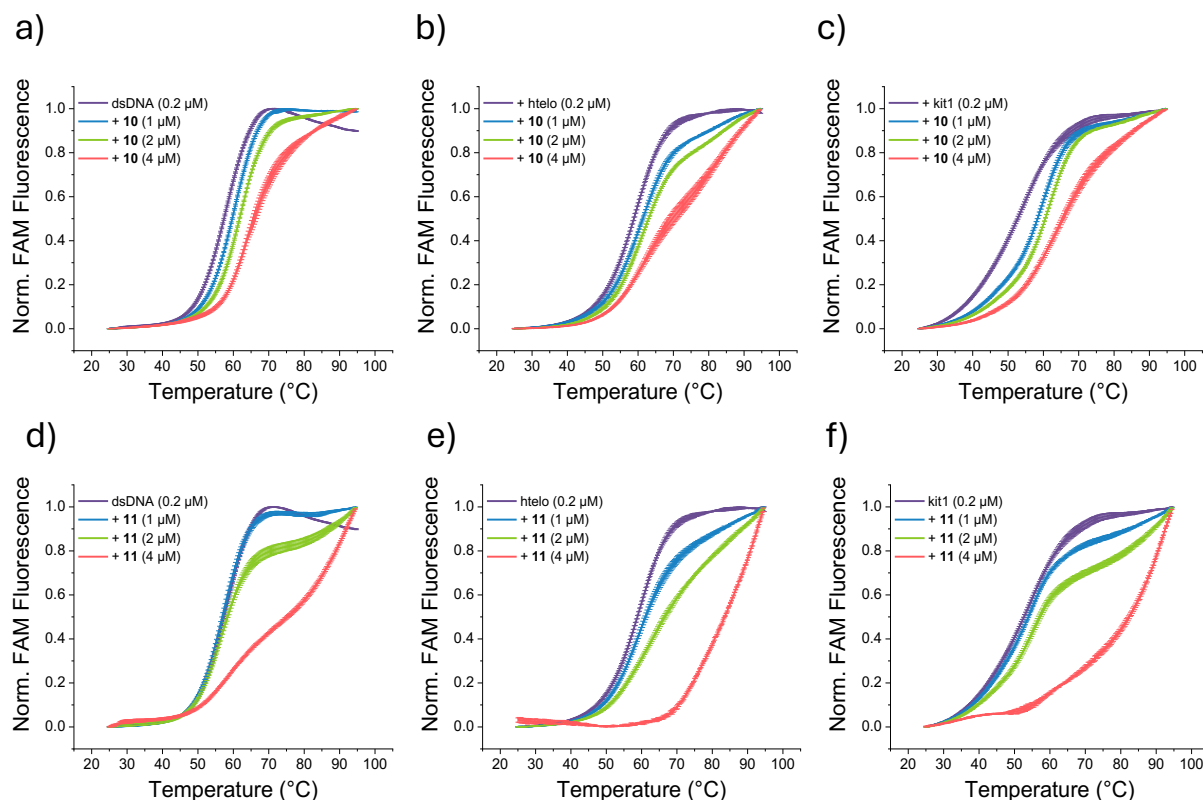


Figure 87. FRET melting profiles of dsDNA, h-Telo and Kit1 upon interaction with a-c) **10** and d-f) **11** at the indicated concentrations. Buffer: 60 mM potassium cacodylate, pH 7.4. ODN concentrations are reported per strands.

CD spectroscopy

CD titrations with htelo revealed that **10** induced marked spectral changes, including enhancement at 295 nm and decreased intensity at 265 nm, consistent with a conformational shift toward an antiparallel topology (Figure 88a). In contrast, **11**, despite stronger FRET stabilization, produced only minor CD alterations, likely due to steric hindrance from its benzyl substituents preventing close interaction of the alloxazine core with the G-tetrads (Figure 88b).

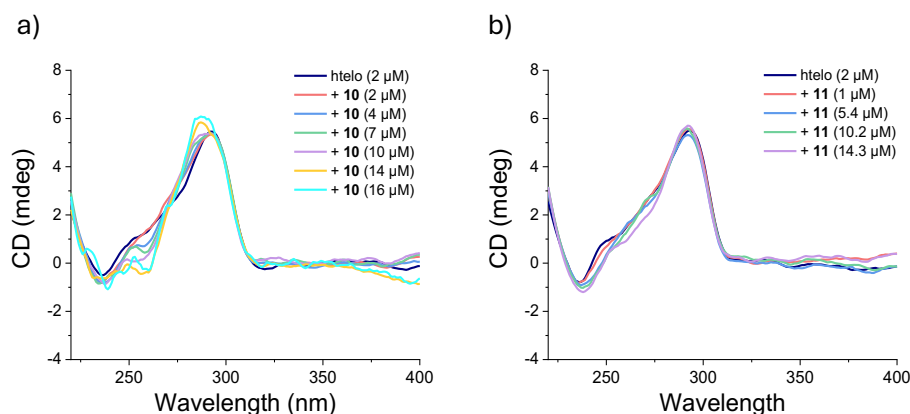


Figure 88. CD spectra of htelo upon titration with increasing concentrations of (a) **10** and (b) **11**. Measurements were carried out in Tris-HCl buffer (50 mM, 100 mM KCl, pH 7.4). DNA concentration is expressed in strands.

Emission spectroscopy

Fluorescence studies further highlighted contrasting behaviors. Whereas **11** showed no emission enhancement upon DNA addition, **10** exhibited a clear “switch-on” response with both ct-DNA and htelo (Figure 89). Binding constants were determined as 9.5×10^3 for h-Telo and 3.3×10^4 for ct-DNA, indicating comparable affinity under these conditions. This effect is consistent with shielding of the ligand’s nitrogen atoms upon intercalation, which prevents quenching through solvent interactions, as described for Ru-dppz complexes.¹⁸⁶

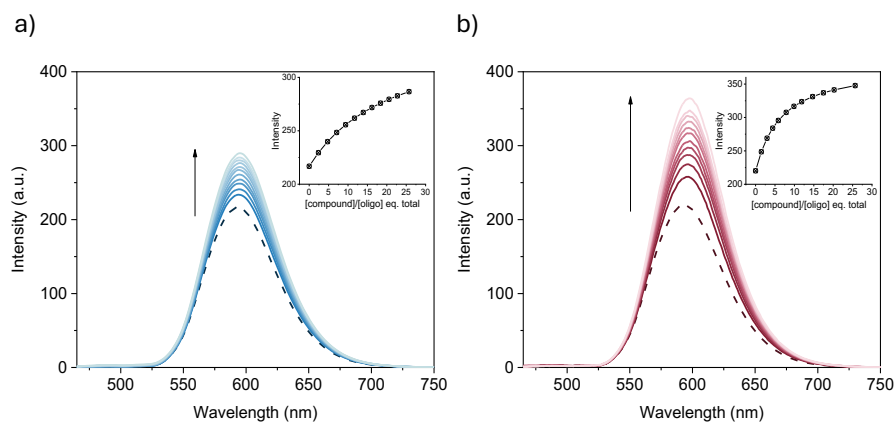


Figure 89. Emission spectra of **10** recorded upon titration with increasing amounts of (a) htelo and (b) ct-DNA in Tris-HCl buffer (50 mM, 100 mM KCl). Arrows indicate spectral changes upon DNA addition. Insets show the corresponding variation in emission intensity at 593 nm. [**10**] = 5.1 μM.

3. Experimental

FRET Melting Assay. FRET melting experiments were conducted in 96-well format using an Applied Biosystems QuantStudio 6 PCR cyclor equipped with a FAM (6-carboxyfluorescein) filter. Oligonucleotides labeled with FAM and TAMRA (6-carboxytetramethylrhodamine) were diluted to

the required concentration in 60 mM potassium cacodylate buffer (pH 7.4). Samples were heated at 95 °C for 5 min and slowly cooled to room temperature overnight to promote folding into B-DNA or G4 topologies. The final oligonucleotide concentration was 0.2 μ M in a total volume of 30 μ L per well. Metal complexes were dissolved in DMSO (2 mM stock) and diluted in buffer, ensuring the final DMSO content did not exceed 0.3%. Fluorescence data were collected in duplicate over the 25-95 °C range with a ramp of 1 °C per 30 s. Emission values were normalized between 0 and 1 for comparison across experiments. The melting temperature ($T_{1/2}$) was defined as the temperature at which normalized FAM emission equals 0.5.

Circular Dichroism. CD spectra were recorded on a Jasco J-715 spectropolarimeter at 25 °C by titrating aliquots of metal complex solutions into DNA samples at fixed concentration. Stock solutions of the complexes were prepared in acetonitrile. Experimental settings were wavelength range 220-400 nm, response time 0.5 s, four accumulations, and scan speed 200 nm/min. Titrations were performed in Tris-KCl buffer (50 mM Tris-HCl, 100 mM KCl, pH 7.4). G4 oligonucleotides were folded as described for FRET experiments.

DNA Binding by Emission Spectroscopy. Fluorescence titrations were carried out on a Jasco FP-8300 spectrofluorometer using 1 cm quartz cuvettes. Parameters were set as follows: excitation bandwidth 5 nm, emission bandwidth 10 nm, response 0.1 s, medium sensitivity, and scan speed 1000 nm/min. Stock solutions of the complexes were prepared in acetonitrile and diluted in Tris-KCl buffer (50 mM Tris-HCl, 100 mM KCl, pH 7.4). DNA aliquots were added incrementally, allowing ~30 s equilibration after each addition. Emission intensities were corrected for dilution effects. Binding constants were determined using the Thordarson equation across the 550-650 nm emission range.

4. Conclusions

In conclusion, this work showed that phenanthroline-alloxazine ligands can be integrated into Ru(II) scaffolds to yield nucleic-acid-active complexes whose behavior can be tuned by ligand design. Overall, the extended aromatic surfaces of ligands of **10** and **11** enable their ruthenium complexes to interact with both B-DNA and G4 structures. Complex **11** exhibited a clear preference for G4s, strongly stabilizing the kit1 and htelo sequences. In contrast, the distinct scaffold of **10** imparts switchable luminescence upon binding to B-DNA and htelo G4, while also promoting conformational changes in the htelo quadruplex.

3. Conclusions

This doctoral project aimed to develop and characterize novel Ru(II) complexes specifically functionalized for mitochondrial targeting and capable of interacting with G-quadruplex (G4) structures, with the ultimate goal of exploring their potential as anticancer agents. This thesis combined synthetic chemistry, DNA-binding studies, and cellular evaluation to provide a comprehensive understanding of the antiproliferative activity of different families of Ru(II) complexes.

In the first two chapters, two distinct classes of Ru(II) complexes bearing triphenylphosphonium (TPP) units were synthesized to promote mitochondrial accumulation and engage mitochondrial G4s. These derivatives demonstrated effective G4 recognition/stabilization and exhibited moderate cytotoxicity that translated into a reduction of long-term growth in colony and spheroid assays. Mechanistically, they disrupted mitochondrial respiration, ATP production, and mitochondrial gene expression, consistent with the targeting strategy, and displayed synergy with metabolic inhibitors such as BAY-876 and metformin. Overall, this part establishes mitochondria-targeted G4-binding Ru(II) complexes as a versatile platform for dual-action anticancer agents, capable of coupling DNA recognition with mitochondrial dysfunction. Through future advances, Ru(II) polypyridyl complexes may pave the way for next-generation metallodrugs that combine precise molecular targeting with organelle-level selectivity.

In the third chapter, a new family of Ru(II) polypyridyl-oxadiazole complexes was developed to examine how heteroaromatic ligands modulate DNA recognition and cellular effects. Under the tested conditions, these compounds showed limited and non-specific G4/B-DNA stabilization yet retained antiproliferative activity in different cell lines, while ICP-MS uptake proved the intracellular accumulation.

In the last two chapters, the focus shifted toward the study of different families of newly designed Ru(II) complexes, explored for their ability to interact with G4 structures in nuclear DNA. These investigations, carried out in different collaborative contexts, aimed to broaden the understanding of Ru(II)-G4 recognition beyond the mitochondrial framework. Through these studies, distinct coordination environments and ligand architectures were examined, ranging from cyclometalated and arene-based systems to redox-active hybrid ligands, and providing complementary insights into how structural and electronic factors govern G4 binding selectivity and stabilization.

Appendix A. NMR Spectra

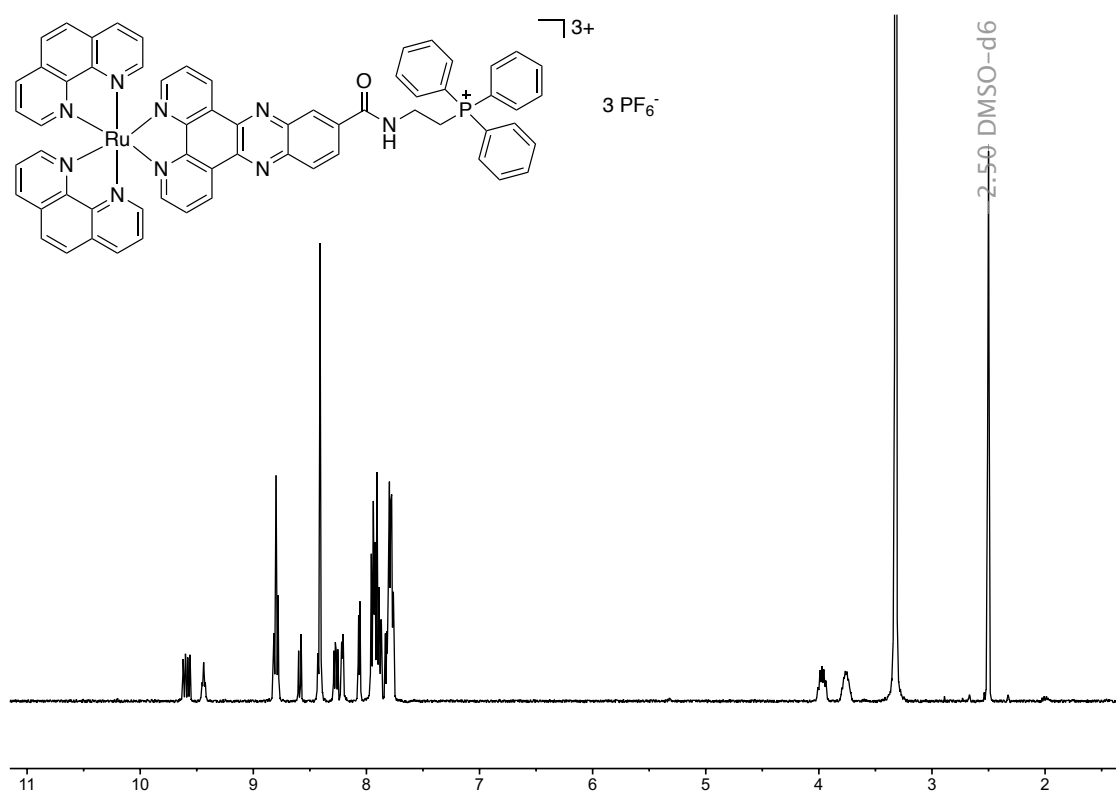


Figure A.1. ¹H NMR of complex 1 in DMSO-d₆.

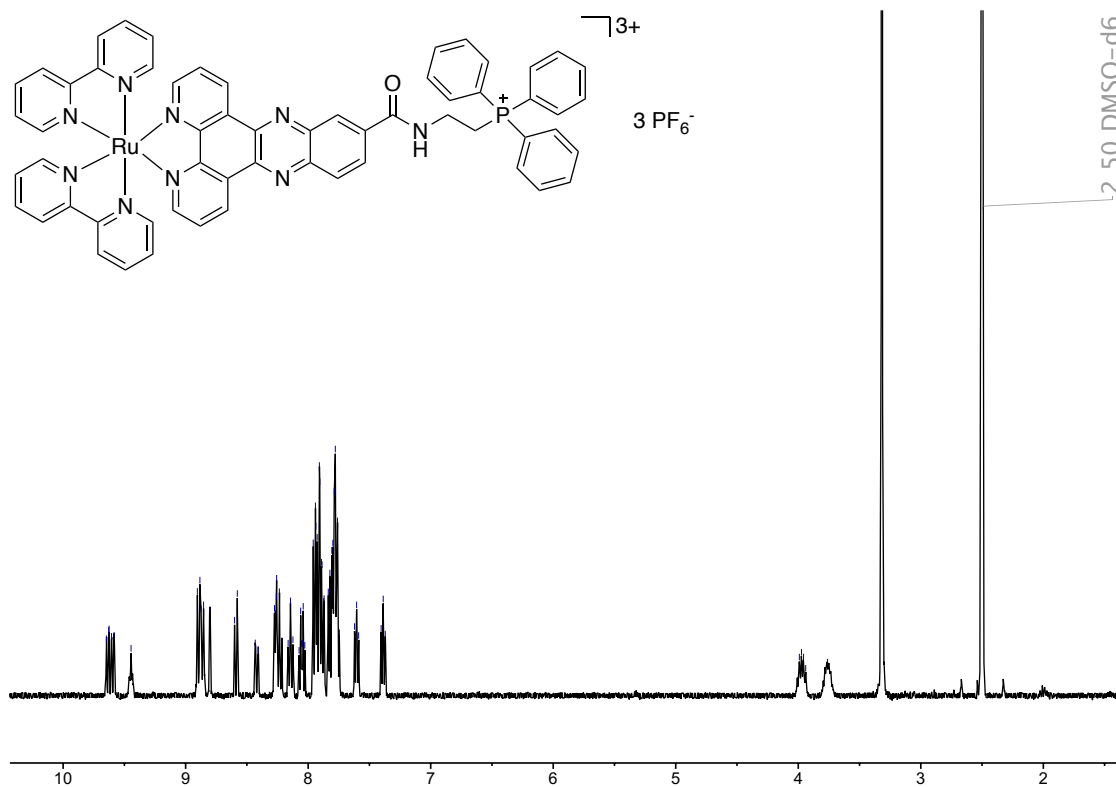


Figure A.2. ¹H NMR of complex 2 in DMSO-d₆.

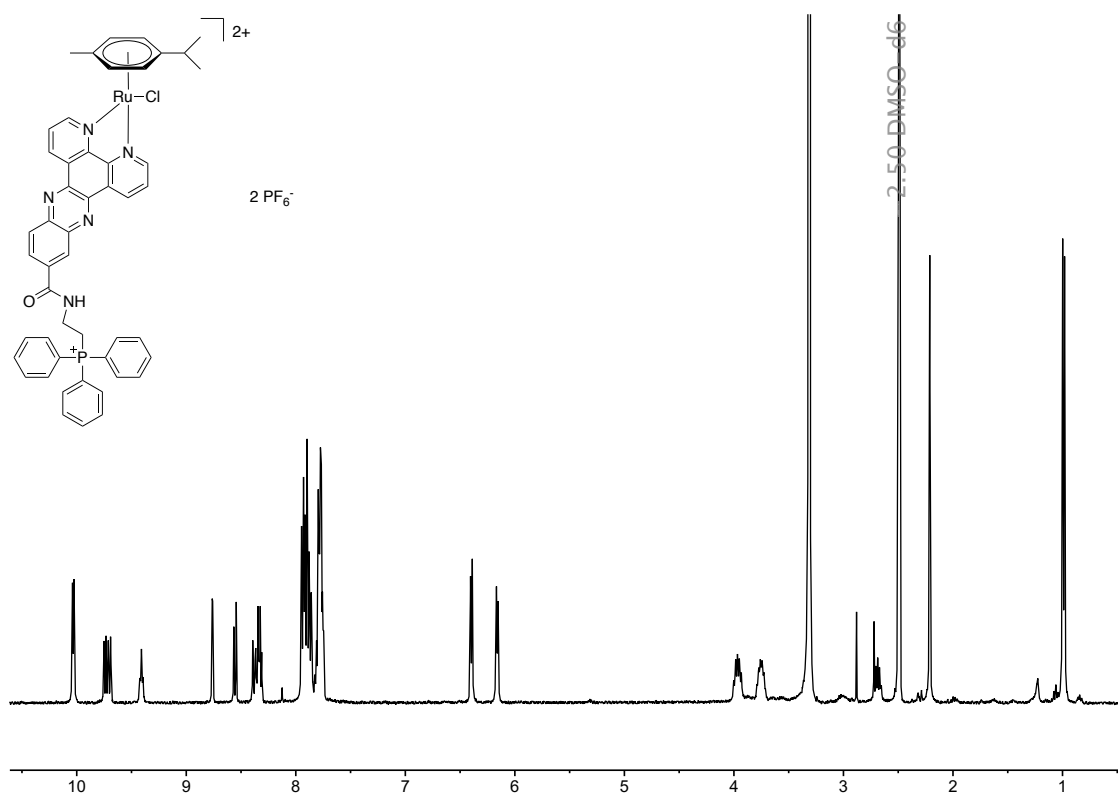


Figure A.3. 1H NMR of complex 3 in DMSO- d_6 .

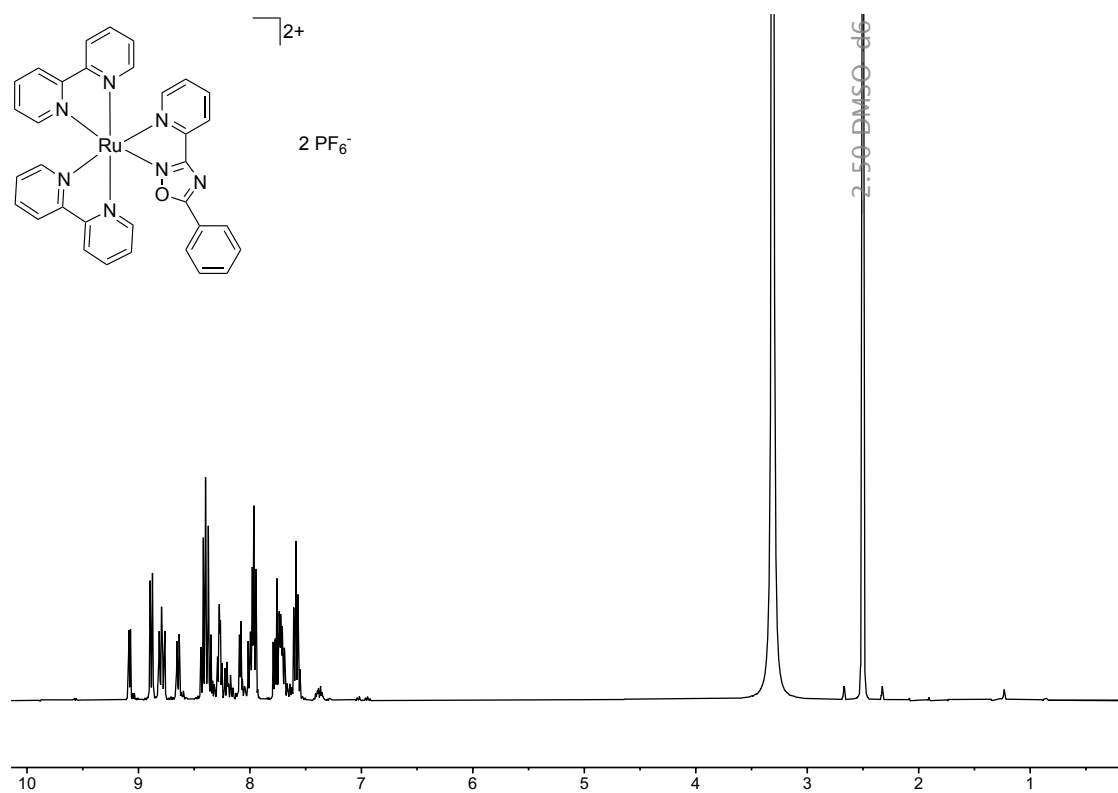


Figure A.4. 1H NMR of complex 4 in DMSO- d_6 .

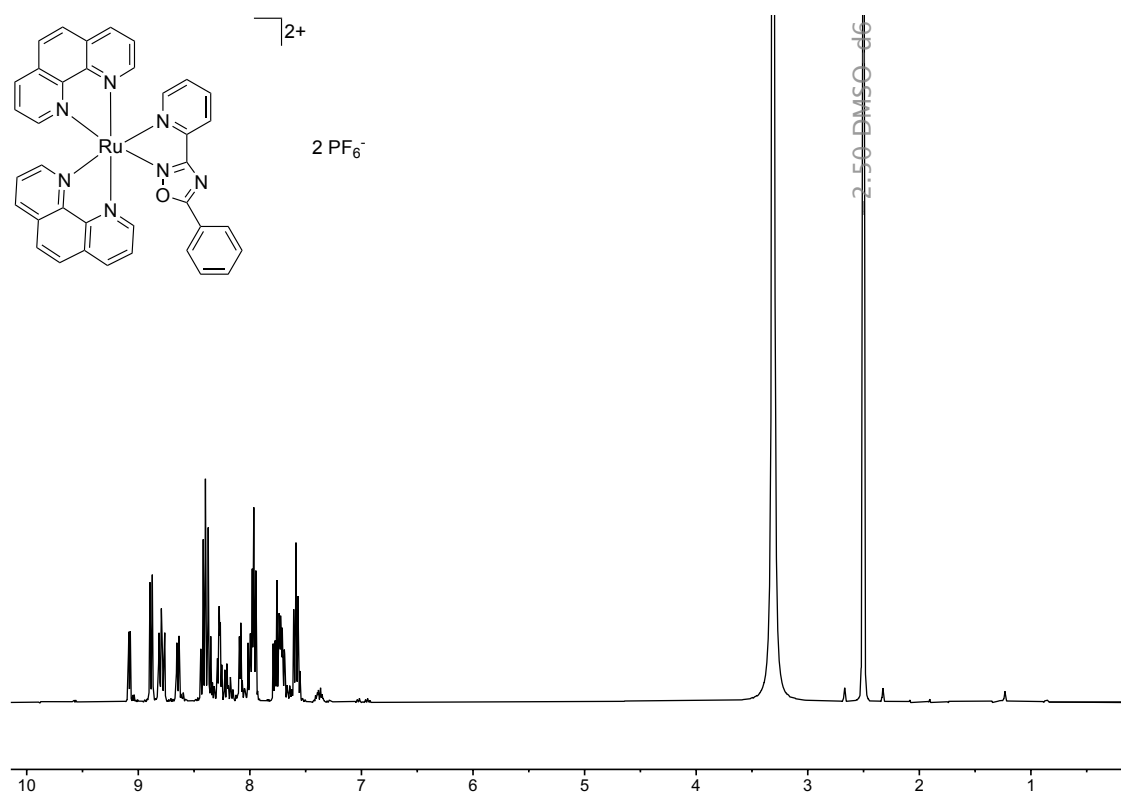


Figure A.5. ^1H NMR of complex **5** in DMSO-d_6 .

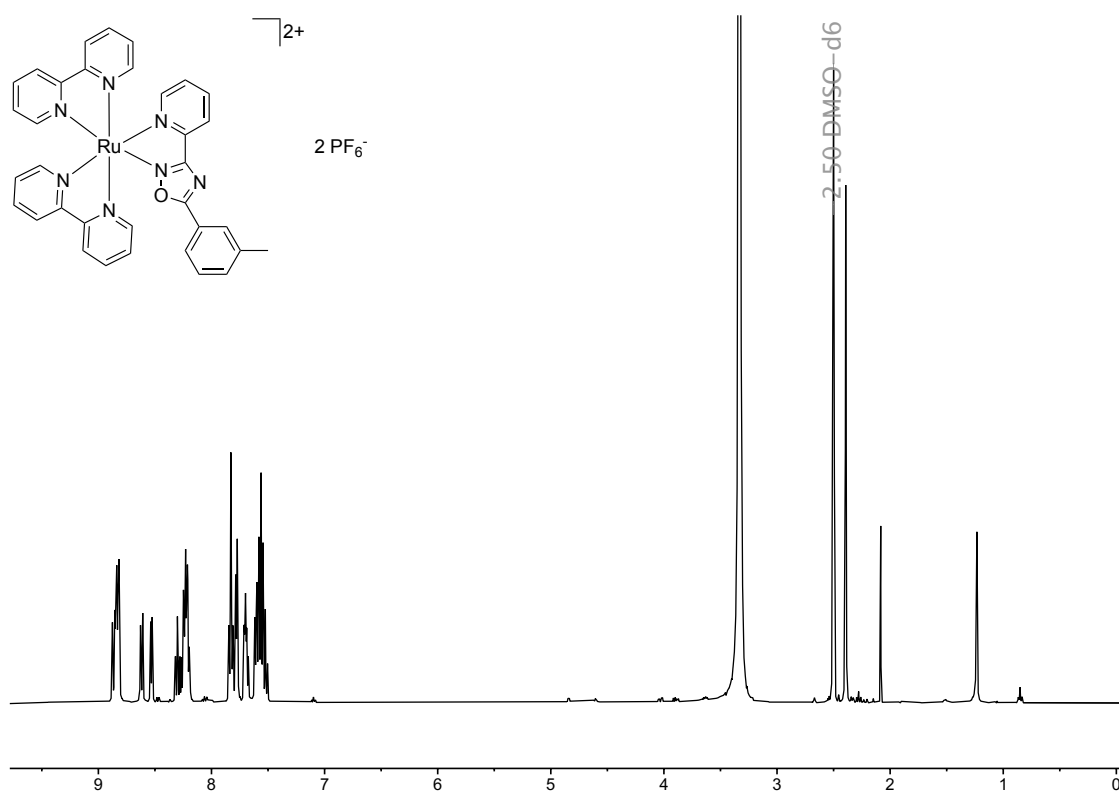


Figure A.6. ^1H NMR of complex **6** in DMSO-d_6 .

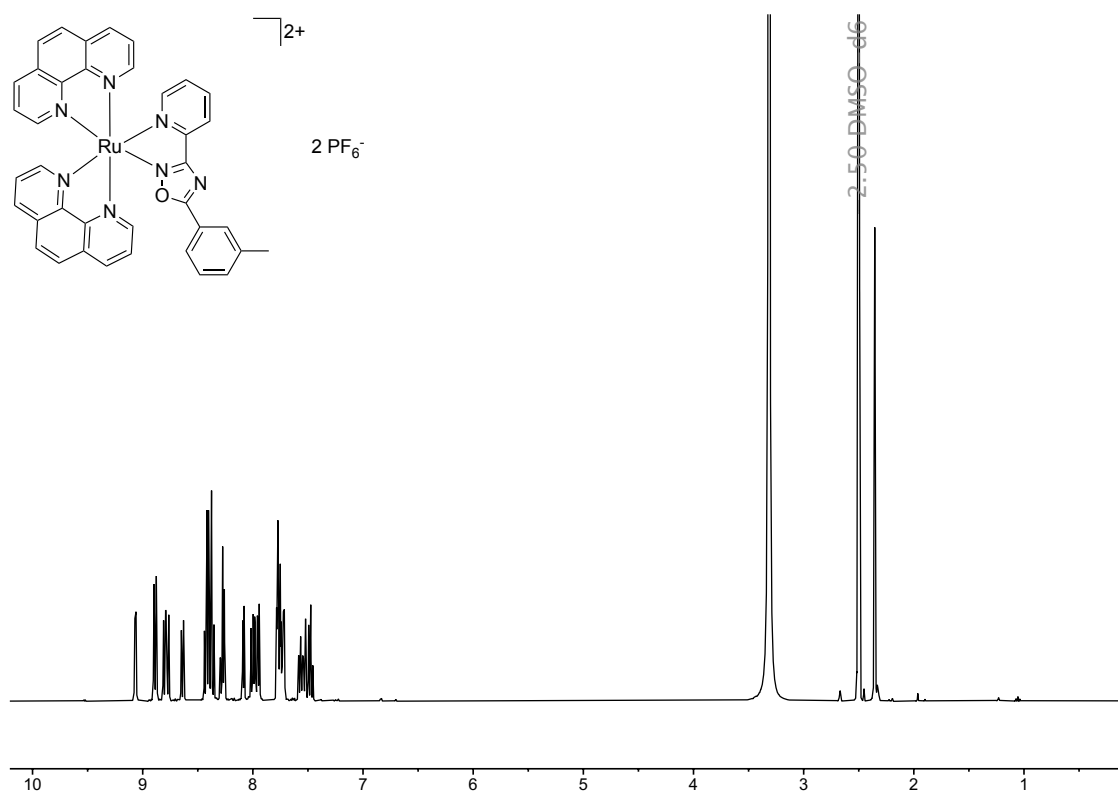


Figure A.7. 1H NMR of complex **7** in $DMSO-d_6$.

Appendix B. Mass Spectra

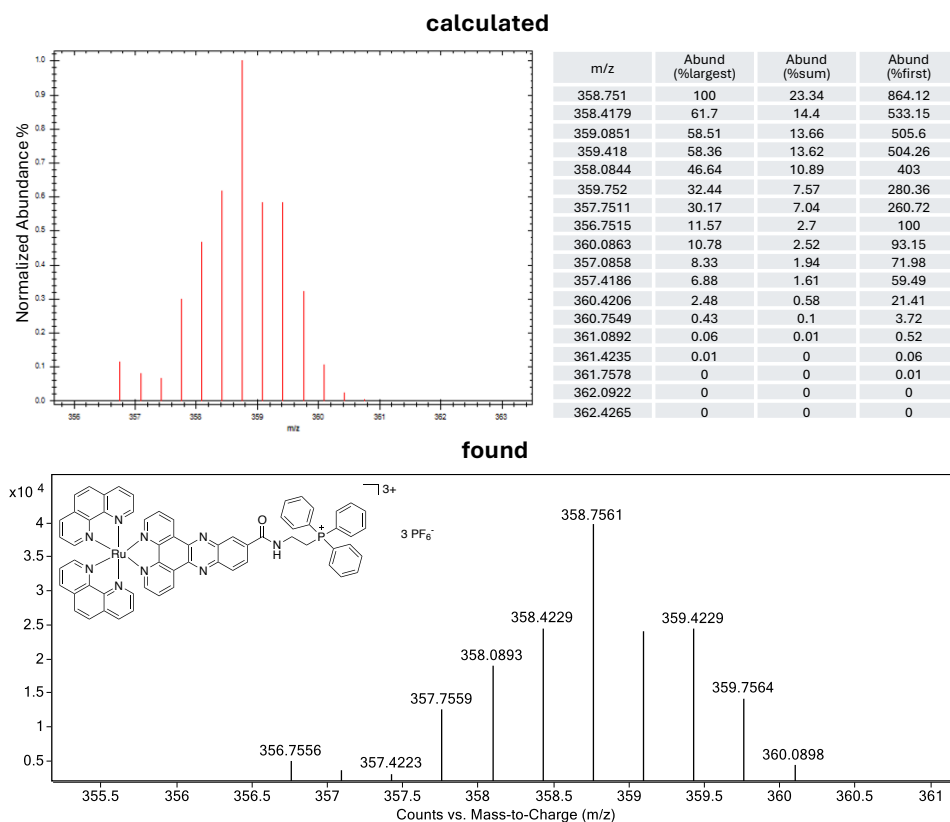


Figure B.1. Calculated and found ESI-MS of complex 1.

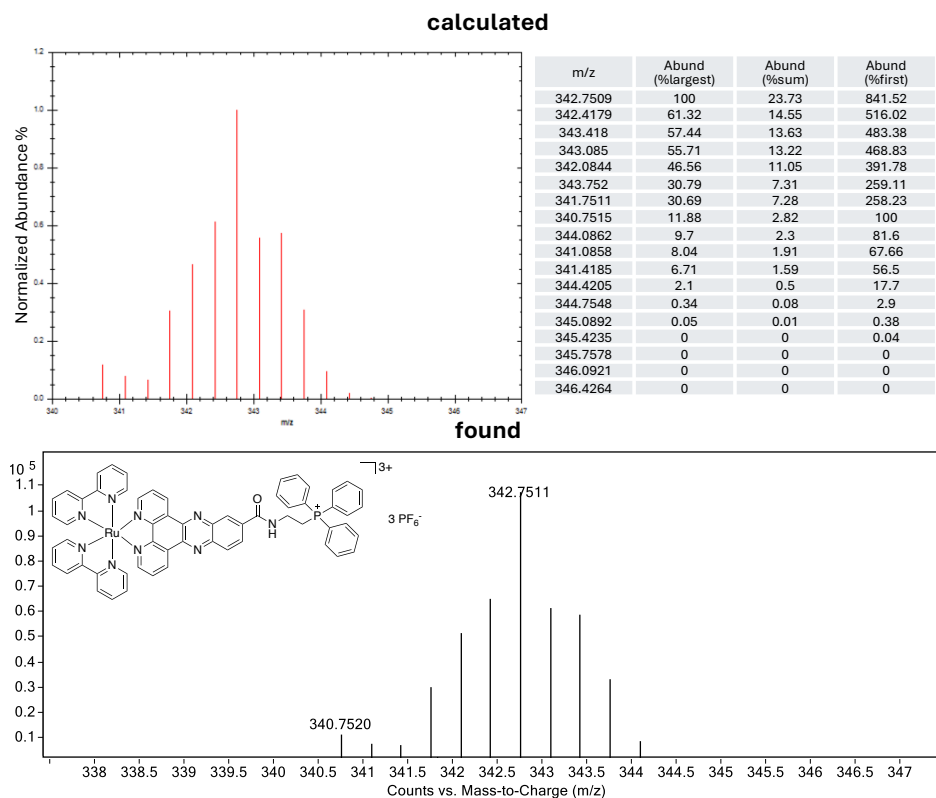


Figure B.2. Calculated and found ESI-MS of complex 2.

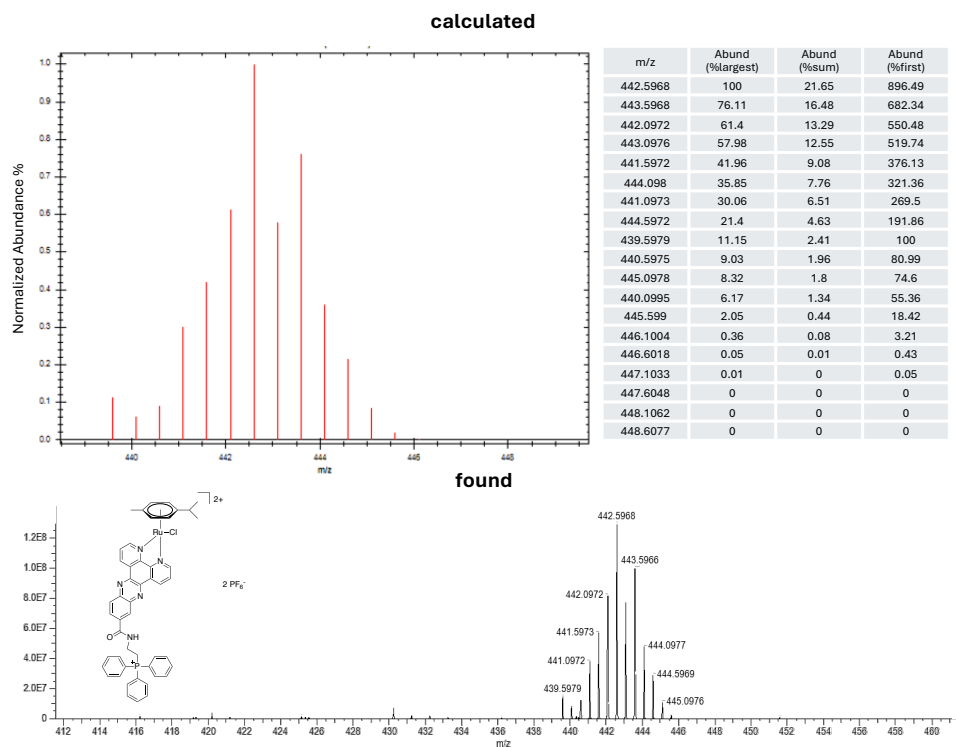


Figure B.3. ESI-MS of complex **3**.

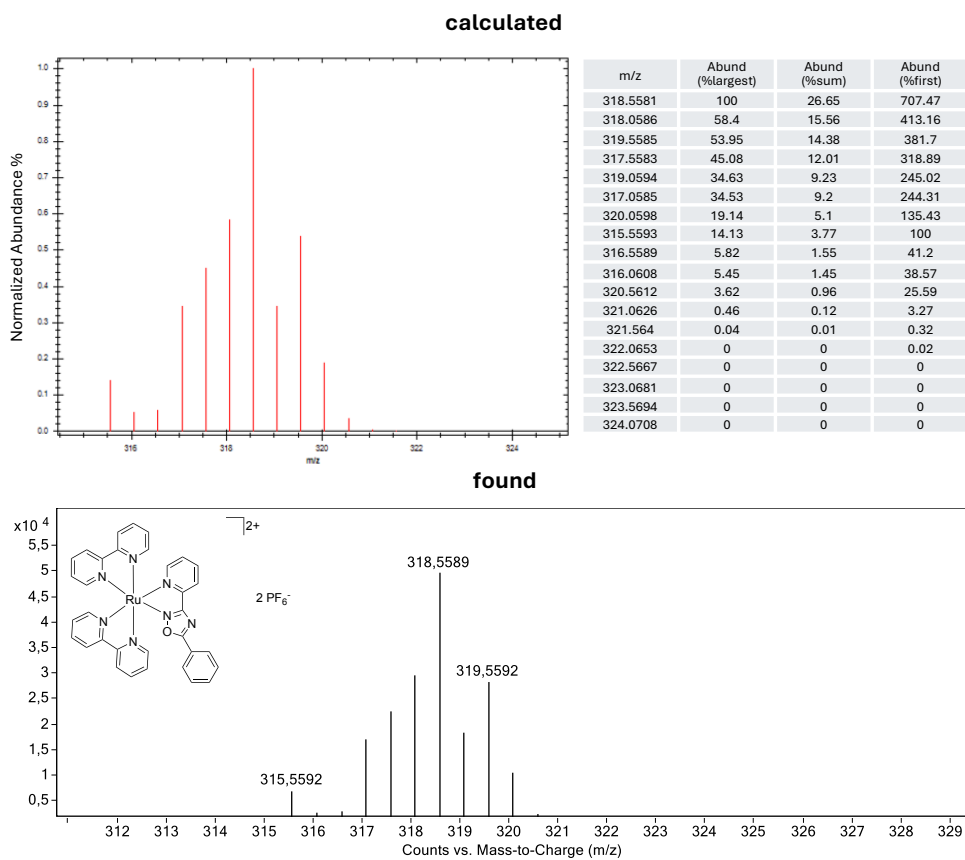


Figure B.4. ESI-MS of complex **4**.

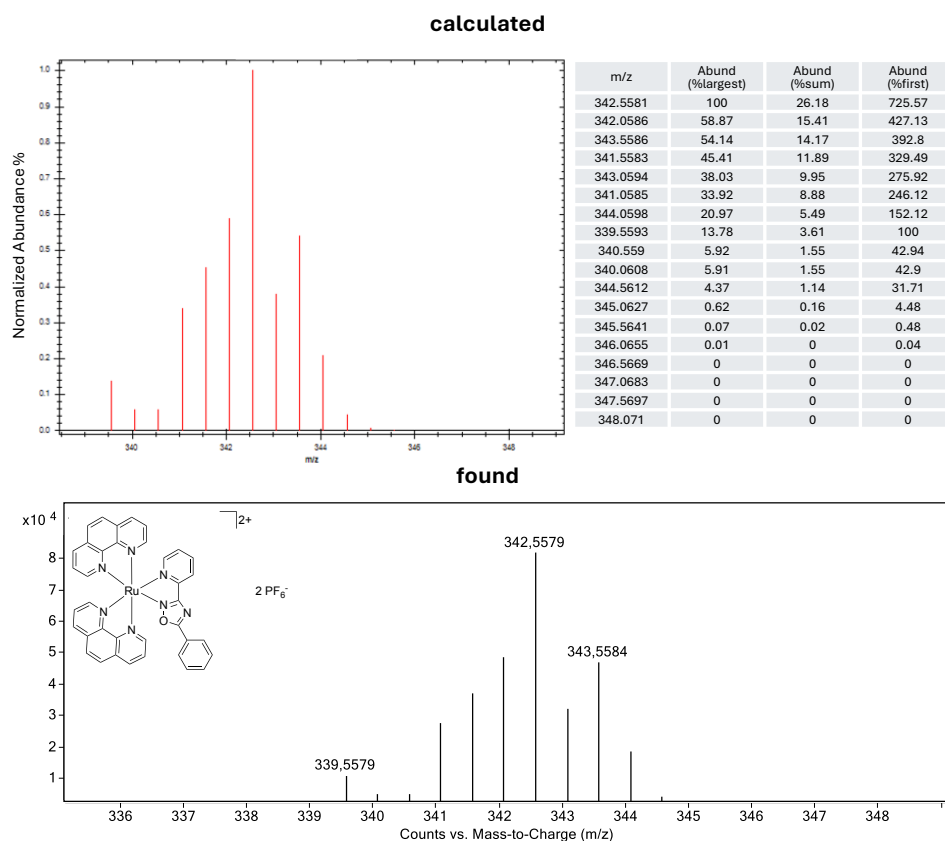


Figure B.5. ESI-MS of complex **5**.

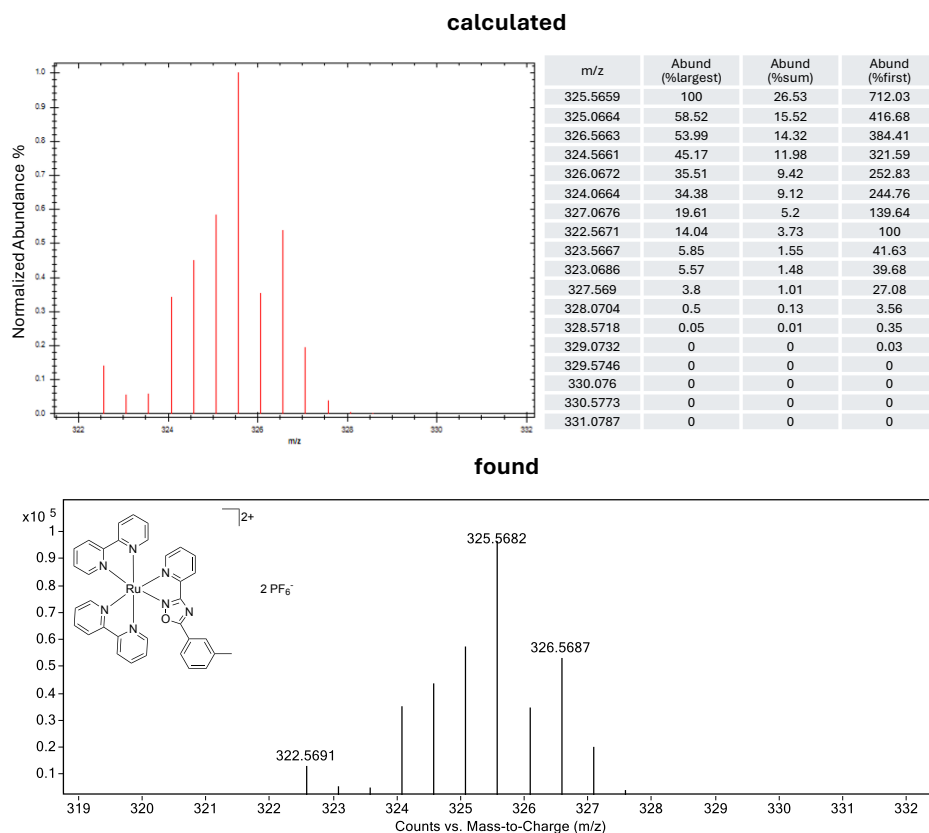


Figure B.6. ESI-MS of complex **6**.

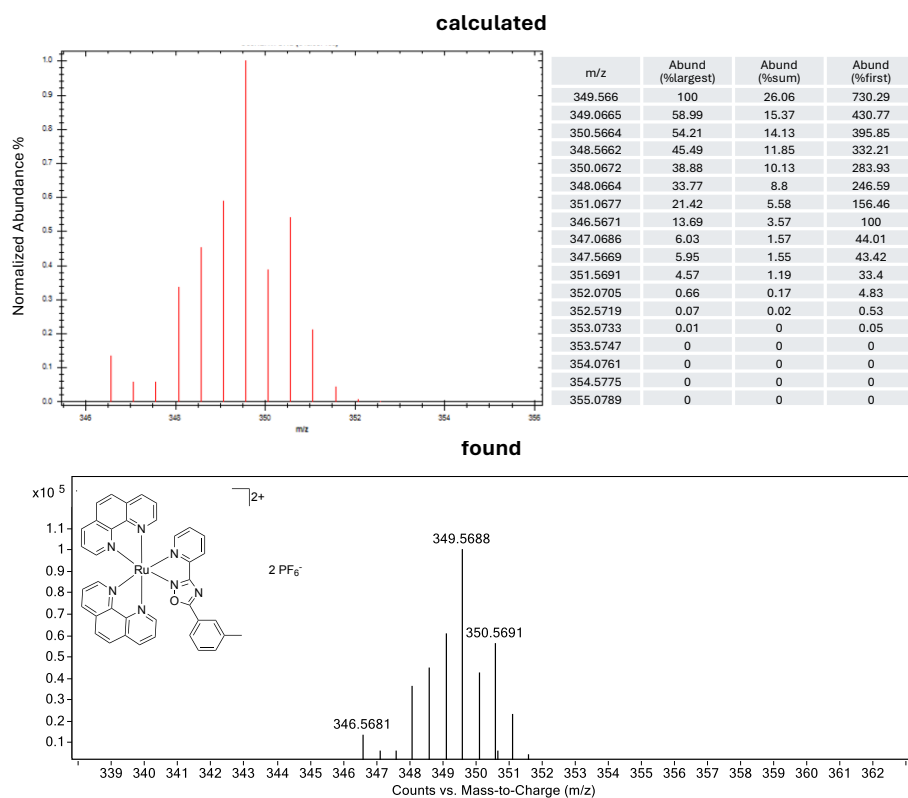


Figure B.7. ESI-MS of complex **7**.

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