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„Ruthenium(II) and Rhodium(I) arene complexes for
G-Quadruplex Recognition“

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ABSTRACT

Cancer is a burden on society that continues to engage research efforts for improved treatment strategies. Despite the large progress made over the past decades in the development of antitumor drugs, cancer patients still suffer in great extent from side effects and a lack of response arising from drug resistance. A strategy to tackle those issues remains in the development of targeted drugs; that is, drugs interacting selectively with a molecular target that is overrepresented in tumor cells or the tumor microenvironment.

Within the vast array of anticancer drugs that have been developed and investigated over the years, metal-based anticancer drugs such as cisplatin remain a mainstay in treatment regimens. In light of the dose-limiting toxicity of platinum and acquired or intrinsic resistance, other metal centers continue to be explored as selective cytotoxic entities that can be functionalised and derivatised in a multitude of ways. Several ruthenium compounds in particular were found to possess favourable qualities such as a lessened systemic toxicity and activity against select platinum-resistant cancers.

G-quadruplexes are secondary DNA structures in which four guanine bases form planar, cyclic arrangements (G-quartets) that can stack atop one another. G-quadruplexes represent an attractive cellular target on the genome-level, since these structural motifs are overrepresented in the human telomeric sequence and several prominent oncogene promoters. Inability to resolve these structures will impede cell cycle progression and with it, terminate cell viability.

Many organic compounds with extended planar aromatic ring systems have been found to stabilise G-quadruplex structures or interact with duplex DNA *via* intercalation. Combining these non-covalent interactions with a metal complex' capabilities of coordinating to DNA bases is considered a viable strategy to maximise the detrimental effects of these compounds on DNA replication and/or transcription. The aim of this thesis was to evaluate two classes of compounds designed to possess such dual binding properties.

The first class of compounds examined in this thesis presents four ruthenium(II) arene complexes with 1,3-dioxindan-2-carboxamide ligands (in themselves topoisomerase II inhibitors) bearing a pendant naphthalene moiety, specifically designed to bind G-quadruplexes by both stacking and coordinating interactions. Replacing the chlorido leaving ligand for a pyridine improved the hydrolytic stability of these compounds and in turn greatly enhanced their G4 stabilising interaction as well as their activity against ovarian cancer cells.

The second class of compounds comprise a set of metal (Ru^{II} , Rh^{I}) arene complexes with *N*-heterocyclic carbene ligands bearing 4-ethylthio-1,8-naphthalimide sidechains. In addition to the complexes' antiproliferative properties, the fluorescent naphthalimide ligand confers useful photophysical properties that can be utilised in cellular imaging studies. Besides a coordinative binding mode (to 9-ethylguanine), the complexes studied herein were confirmed to effectively bind B-DNA by intercalation, as well as binding to G-quadruplexes in a top-stacking mechanism. While rhodium compounds tended to be more effective than ruthenium in their binding interactions with DNA, rhodium complexes had a disadvantageous tendency to precipitate from solution.

KURZFASSUNG

Krebs ist eine Belastung für die Gesellschaft, welche weiterhin die Anstrengungen der Forschung für die Suche nach verbesserten Behandlungsstrategien vorantreibt. Trotz der großen Fortschritte, die über die letzten Jahrzehnte bei der Entwicklung von Krebsmedikamenten erzielt wurden, leiden Krebspatienten weiterhin in hohem Maße an Nebenwirkungen und mangelnder Wirkung aufgrund von Arzneimittelresistenzen. Eine Strategie zur Bewältigung dieser Probleme besteht nach wie vor in der Entwicklung gezielter Therapien: Arzneimittel, welche selektiv mit einem molekularen Ziel interagieren, welches im Tumorgewebe oder in der Tumormikroumgebung überrepräsentiert ist.

Unter der Vielzahl an Krebsmedikamenten, welche im Laufe der Jahre entwickelt und getestet wurden, bleiben Antitumormedikamente auf Metallbasis wie Cisplatin eine Hauptstütze diverser Behandlungsschemata. Angesichts der dosislimitierenden Toxizität von Platin sowie erworbener oder intrinsischer Resistenzen werden weiterhin andere Metallzentren als selektive zytotoxische Einheiten untersucht, welche auf verschiedenste Weise funktionalisiert und derivatisiert werden können. Insbesondere bei mehreren Rutheniumverbindungen wurden günstige Eigenschaften gefunden, wie beispielsweise eine verminderte systemische Toxizität und Aktivität auch gegen platinresistente Krebsarten.

G-Quadruplexe sind sekundäre DNA-Strukturen, in welchen vier Guaninbasen planare, zyklische Anordnungen einnehmen, welche übereinandergestapelt vorkommen. G-Quadruplexe stellen ein attraktives zelluläres Ziel auf Genomebene dar, da diese Strukturelemente in der menschlichen Telomersequenz sowie mehreren prominenten Onkogenpromotoren überrepräsentiert sind. Verminderte Fähigkeit, diese Strukturen aufzulösen, behindert das Fortschreiten des Zellzyklus und damit die Lebensfähigkeit der Zellen.

Viele organische Verbindungen mit erweiterten planaren Ringsystemen sind in der Lage, G-Quadruplexe zu stabilisieren oder über Interkalation mit DNA zu interagieren. Die Kombination solcher nonkovalenten Wechselwirkungen mit der Fähigkeit eines Metallkomplexes, an DNA-Basen zu koordinieren, wird als praktikable Strategie angesehen, um die schädlichen Auswirkungen dieser Verbindungen auf die DNA-Replikation und/oder Transkription zu maximieren. Das Ziel dieser Arbeit war es, zwei Klassen von Verbindungen hinsichtlich ihrer *dual binding* Eigenschaften zu bewerten.

Die erste der im Rahmen dieser Arbeit untersuchten Klasse von Verbindungen präsentiert vier Ruthenium(II)-Aren-Komplexe mit 1,3-Dioxindan-2-carboxamid Liganden (an sich genommen Topoisomerase II Inhibitoren), welche eine anhängende Naphthalin-Einheit tragen, die speziell für die Bindung an G-Quadruplexe eingeführt wurde. Das Ersetzen des Chlorido-Liganden durch Pyridin verbesserte die Hydrolysestabilität, was sich wiederum maßgeblich positiv auf die G-Quadruplex stabilisierenden Eigenschaften sowie die Aktivität gegen Eierstockkrebszellen auswirkte.

Die zweite Gruppe von Verbindungen umfasst eine Reihe von Metall(Ru^{II} , Rh^{I})-Aren-Komplexen mit *N*-heterozyklischen Carbenliganden mit 4-Ethylthio-1,8-naphthalimid Seitenketten. Zusätzlich zu ihren antiproliferativen Eigenschaften verleiht der fluoreszierende Ligand den Komplexen nützliche photophysikalische Eigenschaften, welche in zellulären Bildgebungsstudien genutzt werden können. Neben koordinativer Bindung (an 9-Ethylguanin) wurde bestätigt, dass die hier untersuchten Komplexe DNA durch Interkalation sowie an

G-Quadruplexe in einem *top stacking* Mechanismus binden. Während Rhodiumverbindungen tendenziell wirksamer als Ruthenium in ihren Bindungswechselwirkungen mit DNA waren, neigten die untersuchten Rhodiumkomplexe nachteilig dazu, aus der Lösung auszufallen.

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ABBREVIATIONS

5'-GMP	5'-guanine monophosphate
5'-UTR	5'-untranslated region
9-EtG	9-Ethylguanine
ACN	Acetonitrile
BCL-2	B-cell lymphoma 2 apoptosis regulator
c-KIT	KIT proto-oncogene receptor tyrosine kinase
c-MYC	Avian Myelocytomatosis Viral Oncogene Homolog transcription factor
CAM	Cell-cell adherence molecules
CD	Circular dichroism
COD	cyclooctadiene
Cp*	pentamethylcyclopentadienyl ligand
ctDNA	calf thymus DNA
cym	<i>para</i> -cymene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	double-stranded DNA
<i>E. coli</i>	Escherichia coli
EGF-R	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EPR	Enhanced permeability and retention effect
ER	Estrogen receptor
ESI-MS	Electrospray ionisation mass spectrometry
FAM	6-carboxyfluorescein
FDA	US Food and Drug Administration
FRET	Förster resonance energy transfer
G4	G-Quadruplex
GSH	Glutathione
HPLC	High performance liquid chromatography
hPOT1	Human protection of telomeres protein 1
HSA	Human serum albumin
hTelo	human telomeric G4
hTERT	human telomerase reverse transcriptase
IC50	half maximal inhibitory concentration
IT-139	sodium trans-[tetrachlorobis(1H-indazole)ruthenate(III)], formerly NKP-1339
KP1019	indazolium trans-[tetrachlorobis(1H-indazole)ruthenate(III)]
KRAS	Kirsten rat sarcoma virus oncogene GTPase
MeOH	Methanol
MES	2-(N-morpholino)ethanesulfonic acid
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAMI-A	imidazolium trans-[tetrachloro(1H-imidazole)(S-dimethylsulfoxide)ruthenate(III)]
NHC	N-heterocyclic carbene
NKP-1339	sodium trans-[tetrachlorobis(1H-indazole)ruthenate(III)]
NMR	Nuclear magnetic resonance
ODN	Oligodeoxynucleotide
PACT	Photoactivated chemotherapy
PCR	Polymerase chain reaction
PDT	Photodynamic therapy
PET effect	Photoinduced electron transfer

pH	<i>pondus hydrogenii</i>
pta	1,3,5-triaza-7-phosphatricyclo-[3.3.1.1]decane, phosphadamantane
pyr	pyridine
RAED	Ruthenium arene ethylenediamine
RAPTA	Ruthenium arene pta
Rb	Retinoblastoma associated tumor suppressor protein
RNA	Ribonucleic acid
TAMRA	tetramethylrhodamin
TERRA	Telomeric repeat-containing RNA
TMPyP4	<i>meso</i> -tetrakis(N-methyl-4-pyridyl)porphine
TP53, p53	Tumor protein 53
TRAP	Telomere repeat amplification protocol
TRIS-HCl	tris(hydroxymethyl)aminomethane hydrochloride
TSS	Transcription start site
UV-Vis	Ultraviolet-Visible Spectroscopy
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

1.1. Cancer: General Considerations

Death due to non-communicable disease accounts for the majority of all cases of death worldwide, death from cancer (22%) ranking second only to cardiovascular diseases (44%).^[1] Over 9.5 million deaths globally were attributable to cancer in 2018.^[2] Cancer has the highest incidence rates in prosperous, industrialised regions (North America, Western Europe, Australia and New Zealand, Japan and South Korea); however, cancer mortality rates tend to be higher in less economically developed regions as illustrated in Fig. 1. The geographic heterogeneity of cancer goes beyond general incidence, as the cancer burden in developing countries shifts from cancers associated with poverty and infection (cancers of the stomach, liver, cervix, and oesophagus) towards cancers associated with more affluent lifestyles (colorectal cancer, lung cancer, female breast cancer, prostate cancer).^[3]

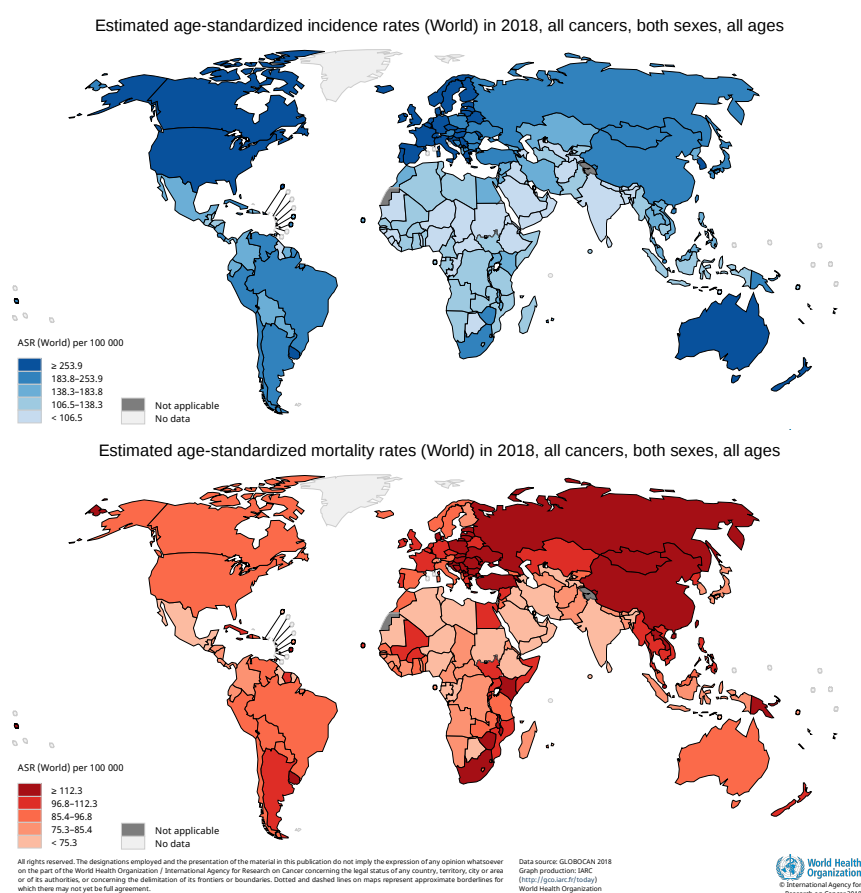


Fig. 1. Cancer incidence (top) and mortality (bottom) rates worldwide.^[2]

In Austria, cancer is the second most common cause of death after cardiovascular disease and is responsible for about a quarter of all recorded cases of death each year. Cancer of the breast, lung, prostate, and colorectum were highest in incidence in 2015 (Fig. 2). However, the most frequently diagnosed cancers in men (prostate, 23%)^[4] and women (breast, 29%)^[4] have a generally good prognosis and therefore a relatively low mortality rate compared to *e.g.* lung cancer, which is estimated to be the leading cause of death from cancer for both men and women in 2018.^[5]

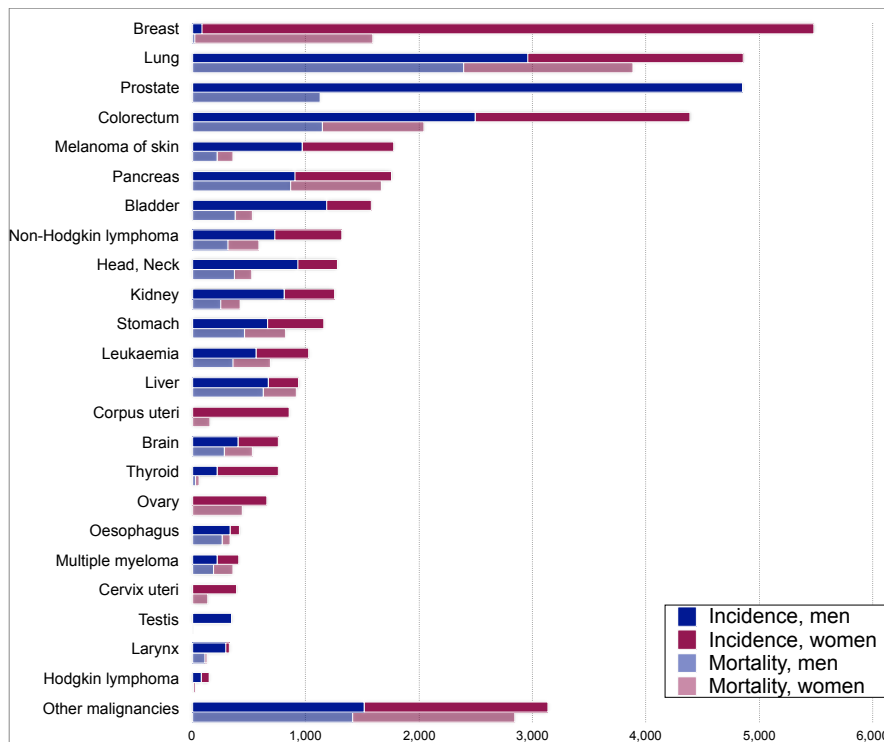


Fig. 2. Cancer incidence and mortality of men and women by primary site in Austria in 2015.^[6]

1.2. Carcinogenesis and Tumor Biology

Cancer as a medicinal term describes a vast number of diseases defined by abnormal and uncontrolled cell growth. Cancer can afflict any part of the body, carcinomas originating from epithelial cells, sarcomas from connective tissue (mesenchymal) cells, and tumors of the hematopoietic and lymphoid tissues affecting the blood, bone marrow, and lymphatic system.^[7]

Cells subjected to external stress (carcinogens, mutagenic agents) can suffer mutations of the DNA through the substitution, insertion, or deletion of nucleotides in the genomic sequence (point mutation) as well as large scale chromosomal changes involving gene translocation, amplification, homozygous deletions, inversions, and breakage of genes.^[8] Mutations affecting cell fate determination, cell survival, and genome maintenance can provide the cell with a selective growth advantage and initiate carcinogenesis. Carcinogenesis—the evolution of cancerous cells from healthy tissue—is not a singular event, but rather an accumulation of alterations in proto-oncogenes (by activation) and tumor suppressor genes (inactivation, loss) allowing the cell to devolve into malignant derivatives that can grow and divide indefinitely.

As a cell acquires critical mutations, cellular repair mechanisms, regulatory circuits, or apoptosis triggers are no longer effective. Cancer cells share characteristics that were identified by Hanahan and Weinberg in 2000 as the six »hallmarks of cancer«^[9]: in order to advance through the stages of tumorigenesis, cancer cells must (1) sustain proliferative signalling, (2) evade growth-suppressors, (3) resist cell death, (4) possess limitless replicative potential, (5) induce angiogenesis, and ultimately (6) gain the ability to invade neighbouring tissue and metastasise. These core characteristics of cancer were later expanded by the authors to contain (7) the avoidance of immunogenic destruction, and (8) the deregulation of cellular metabolism. Enabling the acquisition of these traits is the (9) genomic instability exhibited by cancer cells and (10) the tumor-promoting inflammatory response.^[10]

The underlying genetic changes of these hallmarks are highly variable and can be put into effect in innumerable ways.^[7] Each trait inherent to cancer cells can however offer a therapeutic target by distinguishing pathological cancer cells from healthy tissue. Thus, understanding the pathways governing the life cycle of cancer is crucial to drug development. Several triggers of the hallmark capabilities are exemplified below, as many of them are linked to the proposed modes of action enabling G-quadruplex targeting anticancer drugs as discussed in following sections.

(1) The growth-and-division cycle of cells is naturally regulated to maintain homeostasis in the tissue. Cancer cells however can perpetuate growth signalling in several ways, either by autocrine production of growth factors, stimulating other cells to produce growth factors, modifying growth factor receptors into ligand-independent firing, or by overexpression of cell surface receptors. Growth factor receptors such as **EGF-R** carry intracellular tyrosine-kinase activated domains that initiate a circuitry of downstream pathways.

(2) In cellular regulatory programs, growth-inhibiting circuits will counteract positively acting growth signals. These pathways rely on the action of tumor suppressor genes, the two most prominent being the *Rb* (retinoblastoma-associated) and *TP53* genes, the most frequently mutated genes in human cancer.

(3) The upregulation of certain oncogenes (*e.g.* *MYC*) or excessive DNA damage will normally trigger apoptosis. Tumor cells can evade apoptosis by *e.g.* loss of function of the **p53** tumor suppressor protein, which reacts to substantial DNA damage. Another example of apoptotic regulators is the **BCL-2** family of proteins, comprised of both pro- and antiapoptotic acting proteins that are frequently misregulated in cancer cells.

(4) Normal cells will undergo only a limited number of successive growth-and-division cycles after which they either enter a viable, nonproliferative state called senescence, or proceed to a stage of crisis leading to cell death. Telomeres, repeating sequences protecting the ends of DNA strands, shorten with each cell generation. In most immortalised cells this process is counteracted by the action of **telomerase** which adds telomere repeat segments to the DNA.^[11]

(5) In most solid tumors an angiogenic switch is triggered to ensure the sprouting of new blood vessels to supply the tumor with nutrients and oxygen. An important angiogenesis inducing agent is the vascular endothelial growth factor-A (**VEGF-A**). *VEGF* gene expression can be upregulated both by hypoxia and oncogene expression.

(6) The invasion-metastasis cascade involves multiple steps. Factors aiding the invasion of tissue will frequently diminish cell-to-cell adherence (cell-cell adherence molecules CAMs, cadherins) or supply matrix-degrading enzymes. Several pathways have been identified to induce an epithelial-mesenchymal transition (EMT) that turns cells into multipotent stromal cells.

(7) The immune response naturally eliminates aberrant immunogenic cells and acts as a barrier to tumorigenesis. The secretion of immunosuppressive factors allows tumor cells to evade immunological destruction by T and B lymphocytes, macrophages, and natural killer cells.

(8) Cancer cells tend to favour glycolysis—even under aerob conditions—over oxidative phosphorylation, counteracting the less efficient metabolic pathway by increased uptake of glucose. The altered energy metabolism of cancer cells has been linked to the *MYC* and *KRAS* oncogenes, mutant tumor suppressor gene *TP53*, and the general physiological response to hypoxia.^[12]

(9) The acquisition of hallmark capabilities in cancer cells is further aided by disrupting the genome maintenance and repair systems operating in the cell. A cell that has acquired critical mutations in certain tumor suppressor genes, a prominent example being *TP53*, is more liable to undergo further mutation.

(10) The inflammatory response is the immune system's attempt to restore normal tissue structure and function after injury or infection. Immune cells migrate to the inflamed tissue and release various inflammatory mediators implicated in tumor initiation, promotion, and progression. Neoplastic tissue is infiltrated by immune cells that create inflammatory conditions in the so-called tumor microenvironment.^[13] Inflammation can supply tumor-promoting bioactive molecules (growth and survival factors, proangiogenic factors, extracellular matrix-modifying enzymes) as well as mutagenic chemicals such as reactive oxygen species.

1.3. Cancer Therapy

The great challenge in the treatment of cancer lies in the heterogeneity present in tumors, not only from patient to patient, but also within clonal subpopulations and the various cell types present in the tumor microenvironment.^[7] The genetic heterogeneity of tumor cells as well as acquired drug resistance necessitates the targeting of different pathways in cancer therapy, which in almost all cases involves a combination of drugs and strategies.^[14]

Surgical removal of the primary tumor is generally the first treatment option for solid malignancies. Supplementing the surgical treatment of cancer will often be radiotherapy, by which ionising radiation is applied to locally damage cells at the tumor site. Radiotherapy as well as chemotherapy may be included in treatment schemes to reduce tumor size prior to resection (neoadjuvant) or eliminate micrometastases and tumor residue *post* operation (adjuvant).

Unlike surgery and radiotherapy, systemic approaches of treatment—i.e. chemotherapy^[15], immunotherapy^[16], hormonal therapy—can reach distant micrometastatic sites of the disease. Most established anticancer agents target the DNA and/or impair vital steps of the cell cycle in both cancer and normal cells. The therapeutic agents accumulate in cancer cells through the increased demand of nutrients and cell matter. However, the systemic damage to all fast-dividing tissue (*i.a.* bone marrow, lining of the digestive system, hair follicles) can lead to side-effects such as nausea, anaemia, gastrointestinal irritation, hair loss, *etc.*

Drug response often diminishes over time with prolonged drug exposure, as the first round of treatment leaves cell populations with intrinsic resistance to the initially applied agent (monodrug resistance) and related agents of the same class of drugs (cross-resistance). Late-stage cancers often showcase higher genomic instability and subsequently have a higher risk of genetic alterations favouring cell survival. Cells may acquire drug resistance through decreased influx/increased efflux, altered drug metabolism, enhanced repair of drug-induced damage, alteration or amplification of the target, or cellular inactivation. Multi-drug resistance is most often linked to decreased drug accumulation through increased energy-dependent drug efflux.^[7]

The key to more successful treatment options is the development of selective drugs for suitably sensitive targets. An overview of established antiproliferative drugs, utilising a wide variety of approaches, is given below.^[7]

1.3.1. Chemotherapeutic Drugs

Since most cancer cells are characteristically fast-replicating, DNA integrity and synthesis are naturally targets of major interest in cancer therapy. Alkylating agents, such as nitrogen mustards, are among the earliest and most widely applied anticancer drugs.^[15] Their bifunctional structure allows for the formation of cross-links between DNA strands, thereby inhibiting replication. Other DNA targeting agents act by intercalation (*e.g.* anthracyclines such as doxorubicin, Fig. 3), in which a planar ring structure is inserted in-between nucleotide bases. Both covalent and non-covalent binding (alongside groove binding and electrostatic interactions) distorts the shape of the double-helix and inhibits DNA and RNA synthesis.^[7]

Many drugs do not exclusively attack the DNA directly, but rather interfere with enzymatic activity in different stages of the cell cycle. Antimetabolites inhibit enzyme function by mimicking natural substrates, for example nucleobase analogues like 5-fluorouracil (Fig. 3). Drugs with biological analogues often add the advantage of active transport into the cell. Folate antagonists (antifolates like methotrexate, Fig. 3) can compete with folates for cell-uptake to then inhibit the function of folate coenzymes dihydrofolate reductase and thymidylate synthase. Another enzymatic target of great interest is the class of topoisomerases, which are vital for DNA replication and transcription. Topoisomerase inhibitors will be discussed further in Chapter II.

Protein kinases, including EGFR and VEGF, govern a wide range of signal transduction pathways through the phosphorylation of proteins on sites of threonine/serine and/or tyrosine. Inhibition of protein kinases then interferes with the action of cellular targets, either acting on multiple targets (sunitinib) or by selective action on *e.g.* the epidermal growth factor receptor EGFR (gefitinib, erlotinib).^[17] Such selective drug–target interaction allows in some cases a more disease-specific treatment, for example with the drug imatinib (Fig. 3) that acts on a tyrosine kinase present in 90% of all cases of chronic myelogenous leukaemia.^[15]

Another viable strategy to disrupt cell-cycle progression is by targeting the microtubule spindle structure (antimitotics), either by inhibiting the assembly of microtubules (vinca alkaloids) or by stabilising the microtubule structure (taxanes, *e.g.* paclitaxel, a natural plant alkaloid). Natural products and their derivatives play a major role in the treatment of cancer. Roughly half of 175 small molecules approved in cancer medicine until 2014 were either natural products or derivatives thereof.^[18]

Aside from classical chemotherapeutic drugs, cancer medicine employs strategies as varied as biological agents (hormone-analogues, monoclonal antibodies)^[19], photodynamic and photoactivated therapy, and nanochemistry^[20]. Linking cytotoxic drugs to nanoparticles seeks to exploit the enhanced permeability and retention (EPR effect) observed in tumor tissue, owed to rogue angiogenesis and erratic vascularisation. Photodynamic therapy encompasses the application of photosensitising agents that lead to the release of reactive oxygen species when irradiated at a specified wavelength.^[21] Photoactivation refers to the administration of non-cytotoxic compounds which are only locally activated *in vivo* by irradiation.^[22] The emergence of targeted therapies—for proteins or receptors that are specifically overexpressed in the phenotype of certain types of cancer cells—is a pathway exploited by monoclonal antibodies like the drug Bevacizumab, which binds and blocks the vascular endothelial growth factor VEGF.^[19]

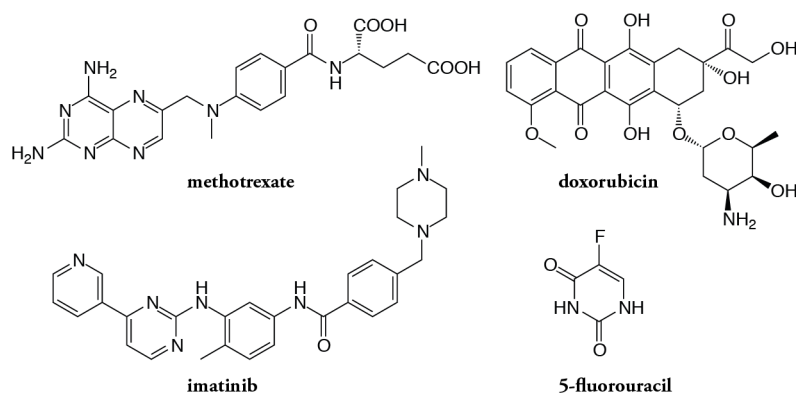


Fig. 3. Select examples of major classes of classical chemotherapeutic drugs.

1.3.2. Metal-Based Anticancer Drugs

Augmenting the arsenal of chemotherapeutic medication is the ever-expanding field of cytotoxic, antiproliferative metal-coordination compounds.^{[23], [24]} Since the FDA approval of cisplatin in 1978,^[15] wide-ranging research effort looks into the development of novel substances that balance drug toxicity, selectivity, bioavailability, cost-effectiveness, and possible adverse effects.

The model compound of clinically applied metal-based anticancer drugs is the complex *cis*-diamminedichloridoplatinum(II), or cisplatin. While the substance itself was known since 1845, the serendipitous discovery of its anticancer capabilities was made by Barnett Rosenberg, who studied the effect of electromagnetic fields on the growth of *E. coli* bacteria in the 1960s.^[25] Today, cisplatin is still one of the most widely applied chemotherapeutics, being part of roughly half of all treatment regimens, in particular for testicular cancer, small cell lung cancer, ovarian cancer, cervical cancer, bladder cancer, carcinomas of the head and neck and osteosarcoma.

The main cellular target of cisplatin has been established to be DNA.^[26] Cisplatin is administered intravenously and enters the cell as a neutral complex primarily *via* passive diffusion, although transport via Cu transporter proteins has also been investigated. Subjected to the reduced chloride concentration of the cytosol (4-20 mM; extracellular level 104 mM), cisplatin undergoes aquation and accumulates in the cell. The highly reactive aqua-species reacts with nucleophilic sites of the DNA to form covalent platinum-DNA adducts; most frequently observed are intrastrand guanine adducts, with hydrogen bonding adding stability owed to the non-leaving *N*-donor ligands. Adduct formation leads to a loss of conformation in the DNA, in turn leading to the inhibition of DNA replication and transcription. Resistance to platinum agents has been linked to reactions with other biological nucleophiles (*e.g.* elevated levels of glutathione), transporter-mediated altered drug influx/efflux, and specialised mechanisms that bypass or repair the impediment posed by platinum-DNA adducts.^[27]

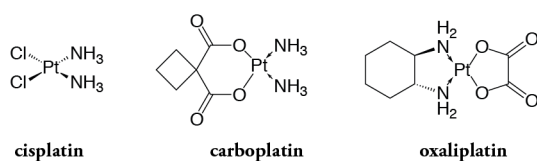


Fig. 4. Platinum based anticancer drugs in worldwide clinical use.

Dose-limiting in the application of cisplatin is its considerable nephrotoxicity, neurotoxicity, and ototoxicity; side effects of the drug also include bone marrow suppression and gastrointestinal irritation. Two derivatives of cisplatin have gained worldwide approval in the clinical setting. Carboplatin (FDA approved in 1989), bearing a more stable leaving group than cisplatin's chlorido ligands, shows both less severe side effects and better retention in the body, while oxaliplatin (2002) is less myelosuppressive than carboplatin and in addition effective in the treatment of colorectal cancer (cancers that do not respond to cisplatin) in combination with 5-fluorouracil.^[27]

The cancer selectivity of classical cancer drugs through accumulation relies on the high metabolic demand of cancer cells, but simultaneously poses the problem of how to limit the inflicted cell damage to the tumor and spare healthy, albeit fast-replicating tissue. A promising strategy in drug design is the application of the drug in a form that will only be activated upon reaching the target site *in vivo*. The high metabolic activity and inadequate vascularisation of many cancers result in hypoxic cells and an altered microenvironment suitable for distinguishing the cancer site. In the case of platinum drugs, this so-called prodrug strategy focuses on the development of chemically more inert octahedral platinum(IV) compounds from the significantly more cytotoxic square-planar platinum(II) analogues. Pt^{IV} prodrugs are expected to be reduced to Pt^{II} in the reductive environment of the tumor. The ligand sphere of Pt^{IV} prodrugs can be designed to contain non-leaving nitrogen donors, leaving groups to allow for activation by aquation, as well as bioactive ligands and/or targeting moieties in axial positions.^[28]

While cisplatin-derived anticancer drugs remain a staple of cancer therapy, the limitations of the drug, namely both its dose-limiting toxicity and resistance issues, continue to urge the development of new and improved drugs based on other cytotoxic metal centers. The success of new metallodrugs depends on a variety of factors and needs to take into account *i.e.* the method and timeframe of drug delivery, its bioavailability after administration, mechanism of cellular uptake and drug efflux, the rate of ligand exchange and adduct formation, as well as metabolism of the drug. The ideal metal-based drug exhibits lower systemic toxicity while maintaining high antineoplastic efficacy comparable to or exceeding that of established anticancer drugs.

Virtually every option the inorganic »metal toolkit« has to offer has been explored to some degree or another, with particular research interest remaining on platinum, ruthenium, gallium, titanium, and gold; other transition metals under preclinical investigation include palladium, rhodium, osmium, and iridium.^[29] A staggering amount of compounds has been investigated as to their cytotoxicity, but limiting factors are encountered in, among others, (1) stability in physiological condition in order to reach the target site before inactivation occurs (drug delivery), (2) solubility to allow for maximally effective dosage, and (3) lipophilicity and/or transporter-protein interaction that affect passive/active transport into the cell.

1.3.2.1. Ruthenium

Transition metal centers can act as scaffolds for drug design that allow for tunable biochemical, electrochemical, and photoactivable properties. The preclinical and clinical investigational successes of ruthenium(III) complexes has spurred on the research effort to fully explore their efficacy against cisplatin-resistant cell lines.^[30]

Two distinct leading ruthenium(III) compounds in anticancer research have reached clinical trials. KP1019, and its later modification IT-139 (formerly known as NKP-1339) by exchange of

the indazolium counterion for sodium, is less toxic than cisplatin (allowing higher tolerable dosage) and is modest in acquired resistance. KP1019/IT-139, in contrast to NAMI-A (*u.i.*), is cytotoxic, although less so than cisplatin. While the mechanism of action remains to be elucidated in minutest detail, its cytotoxic effects have been linked to oxidative stress inducing apoptosis by the intrinsic mitochondrial pathway.^[31] NAMI-A, while showing conspicuously low cytotoxic activity *in vitro*, interferes with metastatic progression while showing no effect on the primary tumor. Its main target seems not to be on a cellular level, but rather interaction with membrane proteins responsible for cell motility and adhesion.^[31]

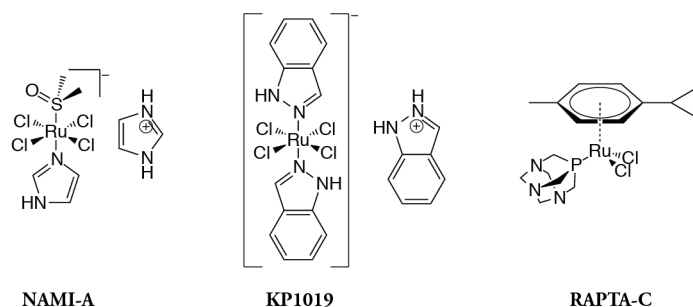


Fig. 5. Most extensively studied ruthenium anticancer drug candidates NAMI-A (left) and KP1019 (middle). Ruthenium arene compound RAPTA-C (right).

While the main mechanism of action of platinum anticancer drugs is widely accepted to be the interaction with DNA, no such ubiquitous main biological target could be identified for ruthenium agents.^[32] Ruthenium complexes can coordinate to DNA—with the preferred binding site being the guanine N7 position—the resulting adducts are however less stable than for platinum and the loss of conformation is less severe.^[33] Seeing as many cancers suffer from iron deficiency and in consequence overexpress transferrin receptors has led to hopes early on that ruthenium would mimic iron *in vivo* and compete with it for cellular uptake.^[34] KP1019 indeed binds to the human iron trafficking protein apo-transferrin, and binding preserves the axial indazole ligands and halts the degradation otherwise observed for KP1019. Binding to the more abundant serum protein HSA (human serum albumin) leads to a loss of the original ligand sphere; however, HSA might still act as a drug transfer for toxic ruthenium ions *via* the EPR effect.^[35]

Ruthenium can attain several oxidation states in physiological conditions (+II, +III, and +IV being most common for ruthenium)^[36] and is thus suitable for the development of prodrug strategies according to the activation-by-reduction hypothesis.^[31] Solid tumors exhibit both an overabundance of cellular reducing agents in hypoxic cells (GSH, ascorbic acid) and a pH gradient (7.4 for blood, down to ~6.7 in hypoxic areas) due to accumulation of carbon dioxide from cellular respiration and metabolism. Ligand exchange kinetics in different media can drastically change drug behaviour, and thus should be considered in drug development.

Aside from ruthenium(III) compounds acting as prodrugs for the more active ruthenium(II) analogues, ruthenium(II) arene complexes have been investigated as anticancer agents that are activated by ligand exchange.^[37] The chemical reactivity of organometallic ruthenium compounds has been studied extensively for their role in organic catalysis. Their antitumor activity has attracted research effort owing to their stability, opportunity for functionalisation, accessibility in synthesis, and the facilitated cellular uptake due to the more lipophilic aromatic ligand.^[38] The

arene ligand occupies three of the octahedral coordination sites—the η^6 -coordination stabilising the +II oxidation state—thus leaving three facial binding sites for ligands to complete the three-legged »piano stool« configuration.

Ruthenium arene compounds that have been developed as promising antiproliferative agents to an advanced preclinical stage include RAED (ruthenium arene 1,2-ethylenediamine complexes) and RAPTA type complexes with an amphiphilic phosphadamantane ligand (pta) to confer desirable physiological properties. The prototypical compound of the latter class, RAPTA-C (Fig. 5), was found to act antimetastatic and antiangiogenic with only marginal toxicity.^[39]

While activation by reduction/pH and activation by tumor-associated proteins are two proposed prodrug strategies, even better spatial and temporal control is desirable. One such way to physically limit toxicity is the application of drugs that only realise their cytotoxic potential after a photosensitive switch has been triggered. Irradiation by a light source leads to a short-lived excited state of the molecule, which can dissipate energy in the form of light or heat, undergo chemical modification, or transfer energy to another species. Particularly suitable to this approach are transition metal complexes with d^3 or d^6 electronic configurations, as their excited states are often easily accessible using visible and UV light.^[22]

Photodynamic therapy (PDT) puts the aforementioned approach into practice by administration of a photosensitiser that upon irradiation with a light source will induce the formation of singlet oxygen or reactive oxygen species. A design staple of these compounds are macrocyclic ring structures (porphyrin-like sensitisers, *e.g.* Photofrin), which can easily be coordinated to a metal center. Ruthenium-compounds in particular are appealing in this regard as the intersystem crossing to the triplet metal-to-ligand charge transfer excited state is very efficient.^[40] However, since PDT approaches require molecular oxygen, this approach is somewhat hindered in a hypoxic environment.

Photoactivated chemotherapy (PACT) instead applies compounds that release cell-damaging products upon irradiation, either through conversion to a cytotoxic form of the complex (*e.g.* Pt^{IV} azido complexes) or by photoinduced ligand release.^[22] While purely organic compounds (*e.g.* psoralen, coumarin) are also of interest in both approaches, the rich photochemical reactivity of metal compounds expands the field considerably.^[23] Photosubstitution reactions of ruthenium complexes with polypyridyl ligands in the visible light spectrum—more favourable for biological applications than UV—have been reported, mostly at blue or green wavelengths. The therapeutic window however is considered to be about 600–900 nm, wavelengths that enable optimal penetration of living tissue.^[41]

1.3.2.2. Rhodium

Complexes of rhodium have been investigated for their antiproliferative activity for decades now, in particular dirhodium(II) tetracarboxylate complexes having roused early research interest.^[42] Taylor *et al.* noted the anticancer activity of rhodium(III) chloride trihydrate twelve years before Rosenberg's discovery of cisplatin as an anticancer agent.^[43] The general cytotoxicity, inertness, and low solubility in aqueous media however turn rhodium compounds into poor candidates for drug development. Nevertheless, rhodium compounds with high affinity for DNA base pair mismatches (associated with certain types of cancer) have been reported.^[44] Such rhodium metalloinsertors can recognise and bind to thermodynamically destabilised base pair

mismatches; the mismatched nucleobases are ejected from the double helix while the aromatic ligand acts as a π -stacking nucleobase replacement. Metalloinsertion can then be studied by photocleavage directed by the metal complex to the base mismatch site.^[44]

Aqua complexes of rhodium(III) and iridium(III) have a rather slow water exchange rate, orders of magnitude below that of platinum.^[43] The coordination of a facial arene ligand increases the water exchange rate considerably (*e.g.* 14 orders of magnitude for Cp*); the labilising effect is however less severe in the case of neutral arene ligands such as benzene or *p*-cymene. More inert rhodium complexes have potential for noncovalent interactions with proteins and DNA, independent of ligand exchange, and rhodium(III) polypyridyl compounds have been investigated for their capabilities as metallointercalators.^[45] In addition, rhodium complexes with bidentate *N,N*-donor ligands (such as 1,10-phenantroline) may undergo photosubstitution reactions that allow the formation of covalent DNA adducts even when the same complex cannot bind DNA in the dark.^[45]

1.3.3. Biologically Active Ligands

Due to the limitations on classical chemotherapeutic drugs, and as acting modes other than covalent DNA interactions continue to be explored, small molecule drugs with known inherent biological activity offer enticing new possibilities to enhance metal-based cancer drugs. The design of such multi-target pharmacophores seeks to transfer (and ideally, amplify) advantageous properties from the metal complex to the ligand and *vice versa*, as well as benefit from any possible synergistic effects.^[46]

Ligands of a defined structure that are recognised by cell-surface receptors can be employed to improve drug delivery. Roughly two thirds of breast cancer cases are ER-positive, *i.e.* express the estrogen receptor and are hormone dependent; a potent drug for the disease is the natural product tamoxifen. Ferrocene or ruthenocene analogues with a tethered moiety of tamoxifen have been shown to possess cytotoxic activity equal or superior to that of tamoxifen.^[47] Several more drug conjugates of hormones, sugars peptides, antibiotics, and even vitamins with metallodrugs have been developed with a particular focus on drug delivery.^[34]

Complexes of Co(III), Cu(II), and Fe(III)—essential metals in their own right—are far less toxic to the body than other transition metal complexes; however, complexes of these metals can act as drug »chaperones« by releasing bioactive molecules upon reduction in hypoxic cells.^[23]

The nature of the donor atoms attached to the metal influences solution stability, ligand exchange kinetics, and overall mode of action significantly. Bioactive *O,O*-chelating ligands such as flavonoids, lapachol, paullones, and quinolone-based antibiotics have all been investigated for their anticancer activity and synergistic effects in metallodrugs.^{[30], [48]}

1.4. G-quadruplex DNA

1.4.1. Secondary DNA Structures

Double-stranded DNA natively occurs in bulk in a right-handed double-helical conformation known as B-form DNA, the two strands bonded by the characteristic Watson-Crick base pairings. DNA can, based on its base sequence motifs and external conditions, fold into variant secondary structures such as Z-form DNA (left-handed helix), triplexes, hairpins, cruciforms, and the extensively studied G-quadruplex structures.^[49]

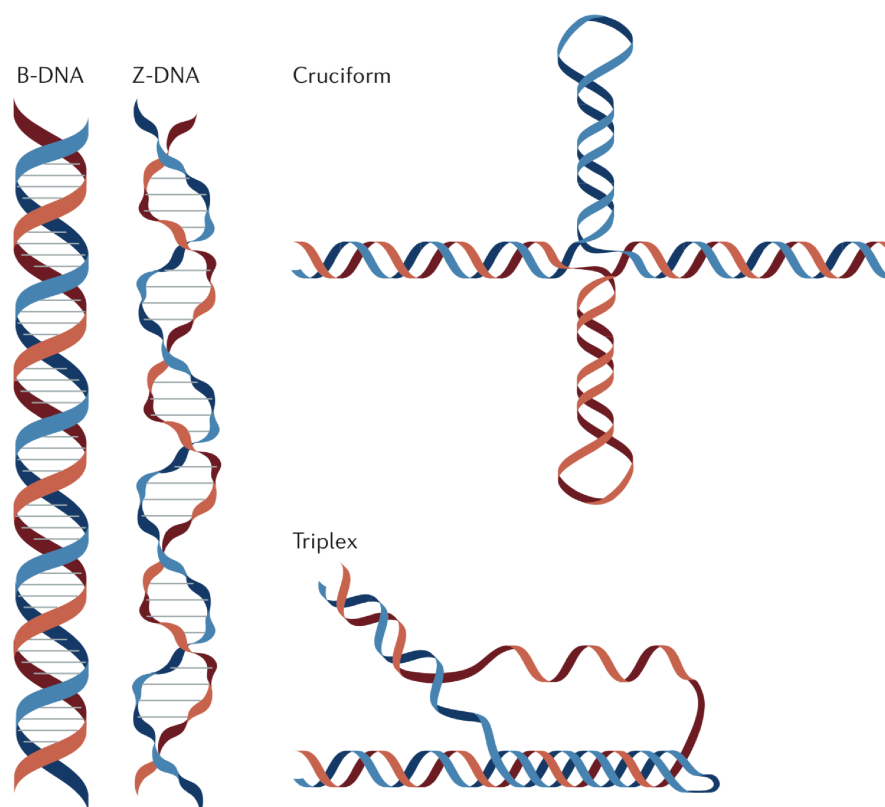


Fig. 6. Several secondary structures of DNA other than G4s.^[49]

The formation of a gelatinous substance in solutions of guanylic acid has been reported as early as 1910,^[50] and in 1962 Gellert *et al.* identified a helical supramolecular structure comprised of hydrogen-bonded tetramers of 5'-guanosine monophosphate (5'-GMP).^[51] By 1987, the formation of these non-canonical guanine base-pairings was reported in DNA, in human telomeric oligonucleotides.^[52] In 1990 followed a review on the subject of »4-stranded nucleic acid structures«,^[53] henceforth referred to as quadruplexes or tetraplexes.

A G-tetrad or G-quartet—terms are used interchangeably—is a square planar arrangement of four guanosyl moieties hydrogen-bonded between the Watson-Crick edge of one guanine and the Hoogsteen edge of another. Multiple G-quartets can stack atop one another, arranged and stabilized by a centered monovalent cation between quartets, forming the G-quadruplex (G4) structure.^[54] In DNA, G4s can form in guanine-rich sequences, with the nucleotides separating the G-tracts (runs) in a G4 motif extruding as single stranded loops of varying length. G4 motifs can be generalised as sequences of four tracts of three or more guanines separated by loops of other nucleotides ($G_{\geq 3}N_xG_{\geq 3}N_xG_{\geq 3}N_xG_{\geq 3}$, with x generally ranging from 1-7). Several arrangements in topology of intramolecular and intermolecular G4s are illustrated in Fig. 7.^[55]

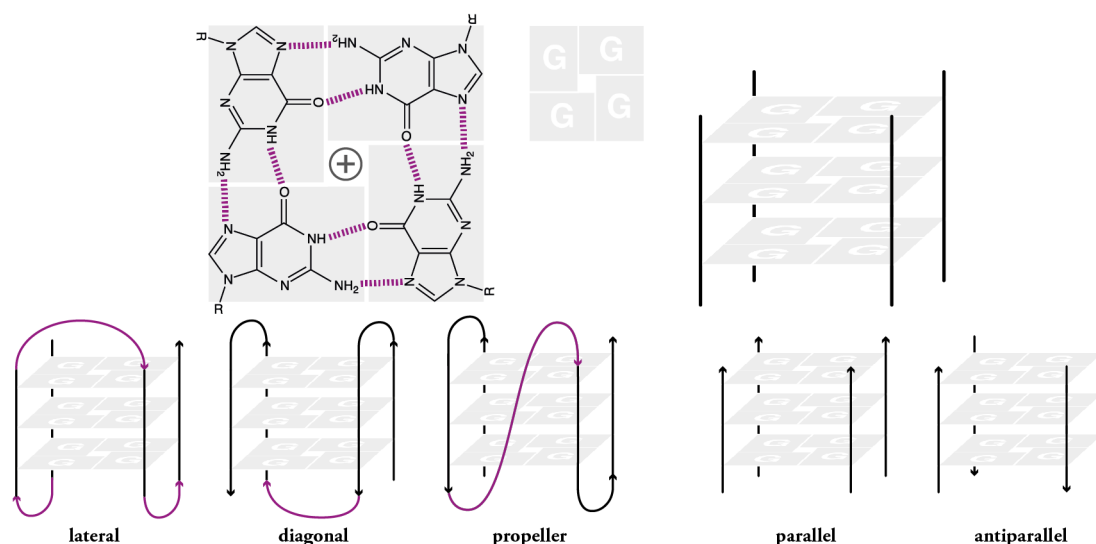


Fig. 7. Schematic representation of different G4 topologies.^[49] Top left: arrangement of four guanines in a G-tetrad around a monovalent ion. Top right: three stacked G-tetrads. Bottom left: three possible intramolecular G4 arrangements with different loop sections highlighted. Bottom right: parallel and antiparallel intermolecular G4s.

1.4.2. G-quadruplex Prevalence in the Human Genome

Computational analyses of the human genome have postulated more than 375,000 putative G4 motifs,^[56] although more recent structure elucidations (allowing for longer loops and unconventional motifs) suggest the number to be even higher.^[57] Moreover, G4 motifs are not randomly distributed in the genomic sequence, but rather overrepresented in certain areas such as transcription factor binding sites, nuclease-hypersensitive gene promoter regions, preferred double-strand break sites, in the ribosomal DNA, and in the telomeric sequence.^[58] The evolutionary conservation of G4 motifs across species^[59] suggests an underlying selective pressure and thus a biological role for G4s that remains to be fully understood.^[60]

Since a prerequisite for G4 formation is single-stranded DNA, G4s *in vivo* will likely be formed transiently during replication and transcription, or in the telomeric single-stranded overhang.^[61] Formation of G4s also occurs in RNA, most notably the telomeric RNA transcript TERRA.^[62] Protein interactions with G4 have been investigated, and the potential recruitment of proteins to G4 sites can vary as transcriptional enhancing or suppressing factors. Several helicases have been found capable of resolving G4s *in vitro*; deficiency in certain helicases can lead to diseases linked to genomic instability and cancer predisposition.^[63] The high thermal stability and ready self-assembly observed for G4s *in vitro* strongly suggest their existence *in vivo*. Indirect evidence for the *in vivo* formation of G4 has been obtained by mutational disruption of selected G4 motifs or the inhibition of transcription by G4 binders. Direct evidence of G4 formation in human chromatin has been provided by high-affinity antibodies and immunoprecipitation studies.^[64]

The human telomeres are several kb-long, guanine-rich repeating hexanucleotide sequences of d(TTAGGG), ending in a single-stranded 3'-overhang of 100-200 nucleobases. Telomere length is maintained by the reverse transcriptase holoenzyme telomerase, an enzyme that is downregulated in most somatic cells. This in turn results in a shortening of telomeres with each cell generation, which is characteristic of cellular ageing. In most solid tumors on the other hand, telomerase is upregulated, turning it into an attractive target for antiproliferative drugs. G4

formation in the guanine-rich telomeres blocks the action of telomerase, prompting the search for agents that selectively stabilise telomeric G4 structures.^[65] Quadruplex formation in the 3'-telomere ends is thought to cause displacement of telomere-bound proteins such as hPOT1 (protection of telomeres protein 1), which triggers a damage-response signal in the cell.

Aside from telomeres, G4 motifs are found predominantly in gene regulatory regions; Nearly 70% of proto-oncogenes contain putative G4 motifs, whereas tumor suppressor genes and housekeeping genes show a particularly low G4 motif count.^[58] Interestingly, while G4s are enriched in the first intron region downstream of gene promoters, 85% of the exons of human genes do not carry G4 motifs. Upstream of transcription start sites (TSS), G4 motifs are distributed evenly between template and nontemplate strand, while G4 motifs downstream of the TSS occur enriched in the nontemplate strand of intron regions. G4 formation in the template strand of DNA will negatively affect transcription as it inhibits the action of polymerase; G4 formation in the nontemplate or lagging strand (downstream and near to TSS) can instead have a positive effect by keeping the strands single-stranded.^[66]

1.4.3. Methodology Concerning G-quadruplex Folding

Structural assignment of G4 topologies can be achieved by X-ray crystallography and high-field NMR studies. Circular dichroism (CD) spectroscopy can also be employed to distinguish between parallel and antiparallel strand topology in G4s, owing to the presence of both *anti*- and *syn*-glycosidic torsion angles in antiparallel arrangements.^[67] CD spectra of parallel G4 structures generally have a positive band at ~260 nm and a negative band at ~240 nm, antiparallel geometries show a positive band about ~290 nm and a negative band at ~260 nm.^[55] Although immensely valuable from for studying drug interaction, CD techniques are also sensitive to matrix composition and buffer conditions. G4 topology is in some cases affected by the nature of the stabilising cation; *e.g.* the human telomeric sequence forms predominantly antiparallel G4 (1 diagonal and 2 lateral loops) in sodium salt solutions, but folds into parallel arrangements in potassium-rich solutions.^[68]

Genome-wide mapping of G4s has been achieved by sequencing experiments in which G4 dependent stalling of polymerase activity is tracked.^[60] First, isolated DNA is sequenced in conditions unfavourable to G4 formation, followed by a re-sequencing step in the presence of K⁺ or G4 stabilisers. Immunoprecipitation and sequencing experiments on chromatin have revealed enriched G4 content in promoter regions and 5'-UTR of cancer-related genes.

Cellular G4 formation can be visualised by employing molecular probes, either inherently fluorescent small molecule G4-binders or G4-specific antibodies.^[69] Metal-based optical probes are especially suitably for this application, since the fluorescence of organic ligands is often quenched in aqueous solution. Binding to DNA by intercalation in turn makes the ligand less accessible to water, and an increased luminescence is observed. With this method, cellular uptake of a drug can be visualised by confocal fluorescence microscopy, and high-affinity G4 binders can even be employed *i.a.* to visualise differences in G4 prevalence in certain cancer cell lines.^[60]

Binding affinity of small molecule G4 binders is most readily assessed by estimating an enhancement of G4 stability. Screening of compounds can be performed by means of fluorescence displacement assays or by comparing the melting profiles of native G4 and drug-treated G4.^{[70], [71]} The latter employs the method of fluorescence resonance energy transfer (FRET), in which a G4 oligonucleotide is labelled at both ends with a donor-acceptor pair of

fluorophores, such as FAM and TAMRA. Melting of the quadruplex structure leads to an increased distance of the fluorophores and thus to an increase in emission signal. The thermal transition can be recorded upon heating samples in a PCR cycler, with the midpoint of the transition recorded as the melting temperature $T_{1/2}$ ($\Delta T_{1/2}$ being the difference of melting temperatures of the oligonucleotide in the absence and presence of the ligand). Assessing G4 stability can be used as an indicator of G4 affinity before determining quantitative binding parameters by *e.g.* surface plasmon resonance techniques or isothermal titration calorimetry.^[72]

Binding constants to certain structural motifs or DNA models can also be determined by optical methods in titration style experiments.^[73] The UV-Vis absorption or fluorescence emission profile of the metal complex changes with the incremental addition of DNA, often a red (bathochromic) shift accompanied by a decrease in absorption intensity (hypochromicity). The binding constant of the complex to DNA can then be gathered from the non-linear plot-fitting of hypochromicity *vs.* concentration ratio (see Chp. 3). Information on the binding mode of the small molecule under investigation (top-stacking, groove binding, intercalation or insertion) can be gathered from CD titrations, by X-ray structure analysis, NMR spectroscopy, or by measuring changes in the viscosity of DNA-drug solutions. A G4 binding drug's ability to stabilise or induce G4 formation can be further assessed *in vitro* by PCR stop assays or TRAP methods (telomere repeat amplification protocol).

1.4.4. G-quadruplex Recognition in Cancer Therapy

Stabilisation of the G4 structure has a detrimental effect on telomerase activity and can alter the gene expression of oncogenes, making G4s an attractive target in anticancer drug development. Treating cells with highly selective G4 binders can measurably alter the mRNA transcript levels of genes with suitable G4 sequences in their promoter regions, as has been shown in proto-oncogenes like *c-MYC* and several cancer cell lines.^[74] An impediment to resolving non-B-form DNA will also turn the affected sites into hotspots for genomic instability.

High affinity to G4 structures over B-DNA is to be desired in the development of new compounds; however, the planar structures found to be particularly good G4 ligands often prove to be strong DNA intercalators as well, and many compounds classified as intercalators have been evaluated as G4 binders, *e.g.* antibiotic anthracyclines like daunomycin, which has been shown to inhibit telomerase activity. Structural selectivity of G4 drugs to discriminate between certain G4 motifs will be dependent on the unique shape of sequence-dependent grooves and loops of the G4 in question.^[75]

A large number of purely organic small molecule G4 binders are polyaromatic heterocyclic ring systems, often bearing cationic side-chain substituents to increase water solubility and DNA affinity.^[76] Following the discovery that bisamidoanthraquinones could inhibit telomerase activity based on their quadruplex interaction^[77], many structural groups have been assessed for their therapeutic potential: porphyrins (TMPyP4, well-studied but unselective), acridine derivatives (BRACO-19, good G4 binding preference), perylene diimides (PIPER), atypically acyclic compounds like pyridostatin, and many more.^[76] Fluoroquinolone derivative quarfloxin progressed to phase II clinical trials as a first-in-class G4 binding drug before being withdrawn for bioavailability issues, with a second G4 drug having entered clinical trials more recently.^{[78], [79]}

The tetracationic porphyrin-derived macrocycle TMPyP4 exhibits high G4 affinity and strongly stabilises G4.^[80] TMPyP4 is known to effectively inhibit telomerase and downregulate

the expression of several oncogenes such as *c-MYC*. Its preference for G4 over duplex DNA is however fairly poor, the stabilising effect it confers to G-quadruplexes nevertheless make it a suitable control compound in melting assays and other G4 evaluations. It is also known to convert antiparallel topology to parallel quadruplexes, another indication of its strong interaction with G4.^[76]

The macrocyclic natural product telomestatin^[81] possesses nanomolar affinity for the human telomeric G-quadruplex in addition to having very low duplex DNA affinity due to its neutral charge and slightly out-of-plane geometry. It is a potent inhibitor of telomerase and causes telomere shortage in cells, but is highly insoluble in aqueous media.^[49]

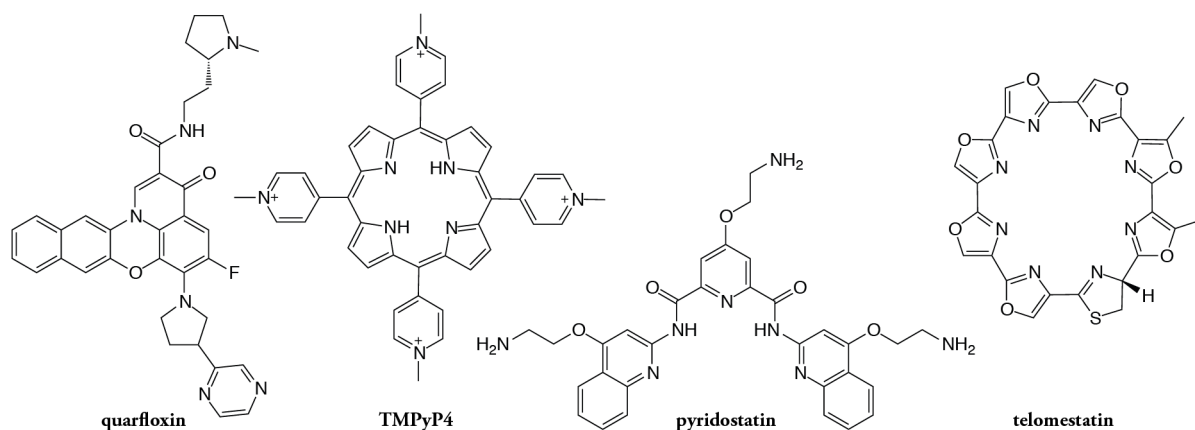


Fig. 8. Examples for organic G4 binders: natural product telomestatin, porphyrin-type macrocycle TMPyP4, and acyclic compound pyridostatin. Quarfloxin progressed to phase II clinical trials.

The transcription factor *MYC*, although overexpressed in up to 80% of human cancers, is an unsuitable target on the protein level.^[82] On a genetic level, transcription of this important cellular signalling factor is controlled upstream of the TSS by a nuclease hypersensitive element (NHE IIIi), which contains a G4 motif in the non-coding strand.^[66] The *c-Myc* G-quadruplex is one of the most extensively studied to date. Other cancer-related genetic targets with G4 motifs include those exemplified in Tab. 1. Treatment with G4 stabilising agents have been shown to decrease gene expression of said targets, although certain motifs like *k-RAS* can be activated instead. Effects on specific targets generally have to be evaluated on both mRNA and protein level.

The proto-oncogene *c-KIT* encodes a transmembrane tyrosine kinase glycoprotein that acts as a stem cell factor receptor. Two distinct G4-forming sequences (termed *c-KIT1* and *c-KIT2*) are found in the core promoter region of the receptor tyrosine kinase *KIT*, a clinically validated drug target for gastrointestinal stromal cancer.^[83] *c-KIT1* occurs between –87 and –109 base pairs upstream of the TSS, *c-KIT2* between –140 and –160 base pairs, respectively. *KIT* expression can be effectively downregulated by G4 binders, even in cells resistant to standard treatment with imatinib. Isoalloxazines as specific *c-KIT* G4 binders show up to 14-fold preference of *c-KIT* over human telomeric G4.^[84] Other oncogene promoter G4s occur in sequences with more than four G-tracts and are thus conformationally more complex; *c-MYC* contains five short tracts and both quadruplexes can be formed; the G4 sequence of *BCL-2* contains six G-tracts and the *hTERT* quadruplex sequence even 12, making the G4 topology on a cellular level harder to determine and on-target effects harder to validate.^[79]

Tab. 1. Selected proto-oncogenes and the (primary) function of their translational products alongside frequently encountered modes of activation in cancer cells.^[7] Exemplified genes contain G4 motifs in their regulatory regions.

proto-oncogene	protein function	mechanism of activation
<i>myc</i>	transcription factor	deregulated activity, amplification, translocation
<i>ras</i>	GTPase	point mutation
<i>kit</i>	stem cell factor receptor	constitutive activation/point mutation
<i>bcl2</i>	anti-apoptotic protein	constitutive activity
<i>VEGF</i>	proangiogenic growth factor	
<i>hTERT</i>	human telomerase reverse transcriptase	

1.4.5. Metal-based G4 Drug Design

While purely organic G4 binders continue to be investigated as G4 binders, the coordination to a metal center offers attractive possibilities as the structural template of the metal offers a specific geometry to build upon. Planar coordinated ligands retain their end-stacking capabilities, while the metal center draws electron density from the π -system and potentially enhances electrostatic interaction with the G-tetrad and the central cation channel. The downside of many planar organic compounds is their insufficient selectivity over dsDNA, which they can bind by intercalation. Metal-based compounds on the other hand possess potentially more diverse modes of action, allowing for groove/loop interactions as well as direct coordination and electrostatic interactions.^[85]

Complexes of organic G4 binders have been synthesised with various transition and main group metal centers; earliest reported were complexes of porphyrin structures like TMPyP4 with Ni^{II}, Cu^{II}, and Mn^{III}, wherein the metal center was found to determine affinity, specificity, and telomerase inhibition.^[85] Metalloporphyrins mainly interact non-covalently with the target, much in the manner of free porphyrins such as TMPyP4, the cationic meso-substituents enhancing their G4 binding properties *via* proposed interactions with the negatively charged DNA backbone.^[85] Two further porphyrin-derived G4 ligand classes are metallophthalocyanines and metallocorroles. Metallophthalocyanines (especially Zn^{II}, Ni^{II}) offer an even larger aromatic surface for end-stacking and more sites for functionalisation to introduce cationic sidearms. These exhibit better G4 affinity and higher selectivity than their metalloporphyrin counterparts.^[85] Metallocorroles offer an atypical saddle-like geometry and have been reported to be potent telomerase inhibitors as well as effective G4 binders.^[85]

Other than conventional macrocyclic compounds, metal complexes with nonmacrocyclic polydentate ligands have proven to be effective G4 binders as well, even relatively simple ones like metal terpyridine complexes (prominently among them Pt^{II}, Cu^{II}, Zn^{II}, Pd^{II}) showing good selectivity and affinity. A growing body of work handles metal salphen and salen complexes as G4 binders, attractive for their relative ease in synthesis and structural flexibility (Pt^{II}, Cu^{II}, Ni^{II}, among others).^[86] Several metal phenantroline (Pt^{II}, Ru^{III}) complexes and their derivatives, besides being efficient G4 binders, also possess interesting photophysical properties.^[85]

Despite avid research interest in ruthenium compounds as outstanding anticancer drugs, ruthenium G4 binders became a topic of interest rather more recently. While the majority of G4 binders show square-planar geometries or at least one planar face, metal complexes with octahedral ligand geometry (Ru^{II}, Ir^{III}, Fe^{III}) have also been synthesised and several proved to be potent G4 stabilisers. Because of their octahedral geometry, these complexes are less likely to interact with G4s by end-stacking or intercalation, and could potentially increase selectivity among specific G4s by groove binding or loop interactions. Among these complexes are a growing number of ruthenium polypyridyl complexes^[87], many also possessing attractive »light-switch« effects (see also Chp. 3). Less extensively explored than polypyridyl complexes are ruthenium(II) arene compounds, an example being Ru^{II} arene complexes with phenantroimidazole ligands which were found to stabilise the *c-MYC* quadruplex *via* a groove-binding mechanism.^[88]

Multinuclear assemblies such as supramolecular Pt^{II} quadrangular »boxes« strongly bind to G4 DNA in a size-dependent fashion, and are capable of telomerase inhibition and oncogene downregulation.^[89] Because telomere attrition and downregulation of gene expression are processes requiring relatively long drug exposure to take full (antiproliferative) effect, DNA cleaving agents present another anticancer strategy; a number of multinuclear metal-based G4 ligands have been reported to direct such damage selectively to G4 structures.^[85]

Exemplary chemotypes given above bind G4s through noncovalent interaction; however, the presence of labile ligands at the metal center also offers the possibility of inflicting cell damage by metallation of DNA, spatially directed to the site of the G4 by G4-specific interaction. Cisplatin-type G4 binders were among the first to be conceived to act in a combined covalent/non-covalent binding of G4 DNA, a first example being achieved in linking cisplatin to a quinacridine moiety via a hydrophilic linker spanning the length of the G-stack.^[90]

1.5. Aim of the Thesis

In designing G4 binding metal drugs one must bear in mind that ruthenium's octahedral ligand geometry does not naturally lend itself to G4 interaction, which favours large, planar systems capable of π -stacking interactions. Although many out of the number of ruthenium G4 binders described in the literature bear flat heterocyclic ligands, another promising approach is affixing the end-stacking planar moiety *via* a flexible linking chain to the metal center. The ligand sphere of ruthenium-based drugs should be fine tuned to optimally combine binding strategies and improve properties in solution.

This thesis describes two different approaches along the same structural design principles (Fig. 9): the ligand sphere of the metal ion (Ru^{II} and Rh^I) has been designed to contain (1) a chelating ligand to maintain specific functionality until the drug reaches its target, this bearing (A) a flexible spacer and (B) a pendant planar aromatic moiety for G4 stacking interactions, (2) a facial arene ligand to increase lipophilicity and facilitate cellular uptake, and (3) one or more leaving group ligands that leave coordination sites for possible covalent DNA interactions.

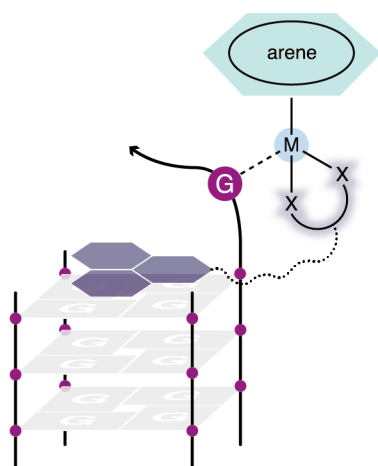


Fig. 9. Schematic representation of the dual binding strategy pursued in this thesis: metal-arene compounds that can simultaneously bind covalently to guanine bases of the DNA and stack a planar ligand moiety on top of a G-quadruplex.

The first class of compounds examined in this thesis presents four ruthenium(II) arene complexes with 1,3-dioxoindan-2-carboxamide ligands (in themselves topoisomerase II inhibitors) bearing a pendant naphthalene moiety, specifically designed to bind G-quadruplexes by both stacking and coordinating interactions. The hydrolytic stability of these compounds was studied extensively *via* UV-Vis and NMR spectroscopy; their G4 interaction was studied in FRET melting assays against four G4 model oligodeoxynucleotides (human telomeric G4 *hTelo*, *c-KIT1*, *BCL-2*, *hTERT*). Replacing the chlorido leaving ligand for pyridine improved the hydrolytic stability of these compounds and in turn greatly enhanced their G4 stabilising interaction as well as their activity against ovarian cancer cells.

The second class of compounds comprise a set of metal (Ru^{II} , Rh^{I}) arene complexes with *N*-heterocyclic carbene ligands bearing 4-ethylthio-1,8-naphthalimide sidechains. In addition to the complexes' antiproliferative properties, the fluorescent naphthalimide ligand confers useful photophysical properties that can be utilised in cellular imaging studies. B-DNA and G4 interactions were studied *via* CD, UV-Vis, and fluorescence spectroscopy titrations as well as FRET melting assays. Besides a coordinative binding mode (to 9-ethylguanine, studied *via* mass spectroscopy), the complexes studied herein were confirmed to effectively bind B-DNA by intercalation, as well as bind to G-quadruplexes in a top-stacking mechanism. While rhodium compounds tended to be more effective than ruthenium in their binding interactions with DNA, rhodium complexes had a disadvantageous tendency to precipitate from solution.

2. RUTHENIUM(II) ARENE COMPLEXES WITH NAPHTHYL-SUBSTITUTED 1,3-DIOXOINDAN-2-CARBOXAMIDE LIGANDS

2.1. Background

Several potent anticancer drugs target the enzyme family of topoisomerases,^[91] which govern DNA supercoiling by controlling the cleavage and religation of DNA; single-strand DNA breaks are induced by topoisomerase isoform I or double-strand breaks by topoisomerase isoform II. Overexpression of topoisomerase correlates to genomic instability and tumorigenesis. The human genome encodes five known topoisomerases (topoisomerase I, topoisomerase II α and II β , topoisomerase III α and III β), of which topoisomerase II α plays an important role in mitosis and is found to be overexpressed in proliferating cells. Inhibition of topoisomerase II α leads to cell cycle arrest in the phase and has become a target in the development of anticancer drugs.^[7]

Drugs that interfere with topoisomerase activity may either »poison« the enzyme (stabilise the enzyme-DNA complex) or inhibit the enzyme (forming stable enzyme-ligand complexes). A number of drugs that form DNA-adducts may act as topoisomerase poisons, an example of topoisomerase I poisons being the class of camptothecins, used in the treatment of several tumor phenotypes (Fig. 10).^[91] Microbially derived anthracyclines like doxorubicin and its stereoisomer epirubicin bind DNA by intercalation and act as an inhibitor of topoisomerase II.^[92]

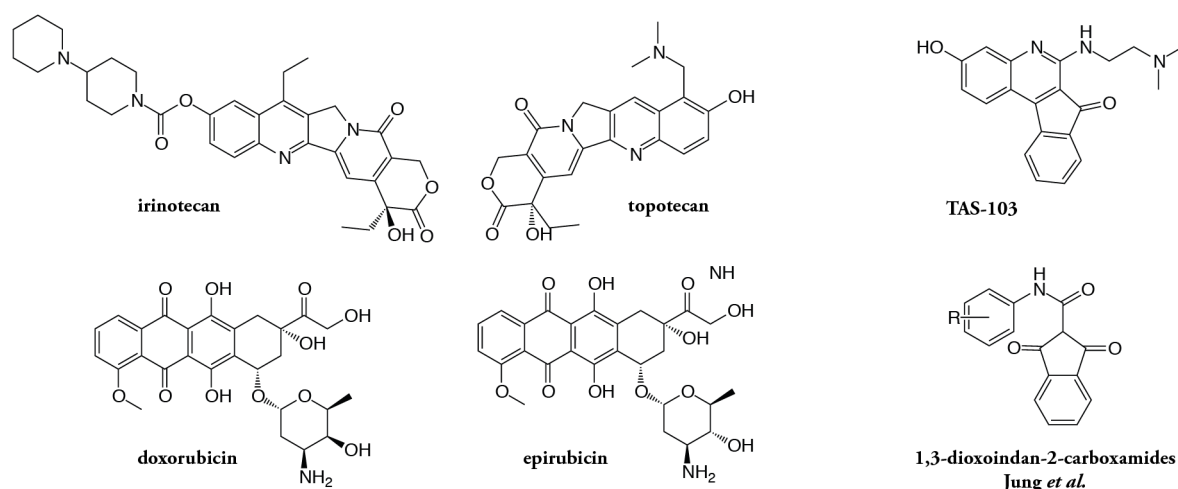


Fig. 10. Left: Topoisomerase poisons applied in clinical settings: camptothecins irinotecan and topotecan; microbially derived anthracyclines doxorubicin and epirubicin. Right: mixed topoisomerase I/II inhibitor TAS-103 and the structurally derived 1,3-dioxoindan-2-carboxamides devised by Jung *et al.*^[93]

Since the inhibition of topoisomerase I can be partially circumvented by topoisomerase II, mixed topoisomerase I/II inhibitors have been developed, such as TAS-103 (Fig. 10).^[94] Structurally similar to TAS-103, 1,3-dioxoindan-2-carboxamides have been found by Jung *et al.* to possess potent cytotoxic activity on four different cancer cell lines, albeit with a 100-fold higher IC₅₀ value than doxorubicin.^[93] These compounds are stabilised in their enolic form and in solvents of low polarity exist purely as enols of the amide-side carbonyl group.

1,3-dioxoindan-2-carboxamides have previously been investigated by Stephan Mokesch as bioactive chelating ligand scaffolds to yield organometallic half-sandwich complexes.^[95] Synthesis of these ligands involves a zwitterionic precursor molecule that is then reacted with a primary amine. By varying the primary amine (methylamine, ethylamine, propylamine, benzylamine), the effect of the side chain shows an increase of cytotoxicity with lipophilicity in the order Me < Et < Pr < Bn. The ruthenium and rhodium based complexes with benzyl sidechains were able to inhibit topoisomerase II α activity, whereas the corresponding free ligand could not.

The organometallic ruthenium arene complex combined with the bioactive ligand scaffold of 1,3-dioxoindan-2-carboxamides yields a structural design with inherent anticancer capabilities that can be further adapted by varying the side chain introduced via the amide bond. Expanding the π -system of the pendant moiety from a benzyl group to a naphthyl group introduces a DNA binding mode to target G-quadruplex structures.

Conceptualised by Stephan Mokesch and Alessio Terenzi, a series of complexes was synthesised to investigate the G4 binding abilities of ruthenium 1,3-dioxoindan-2-carboxamide complexes.^[96] Aiming at a dual binding mode, these compounds have been designed to combine the advantages of (1) a facial arene ligand, (2) a chelating ligand derived from topoisomerase II inhibitors with (i) a flexible side-chain bearing a moiety capable of stacking onto G-quartets, and (ii) a labile ligand bond on the (3) cytotoxic metal center capable of covalently binding DNA nucleobases.

Four novel ruthenium(II) *p*-cymene complexes (**1a/b**, **2a/b**) derived from two 1,3-dioxoindan-2-carboxamide ligands (**L1**, **L2**) became the subject of this thesis (Fig. 11). The *O,O*-chelating ligand scaffold attaches a pendant naphthyl group to the metal complex *via* a flexible alkyl linking chain. The effect of the flexibility of this chain was investigated by varying the length of the linker, a methyl chain linking the naphthyl group in the case of complexes **1a/b** and an ethyl chain in the case of **2a/b**. Since the release of the leaving group via hydrolysis is considered to be a key step in activating metal-based anticancer drugs, the effect of the leaving group on the efficacy of our compounds was investigated. As ruthenium-nitrogen bonds are known to be cleaved by light exposure^[41], the chloride leaving group ligand (**1a**, **2a**) was substituted for pyridine (**1b**, **2b**), which is also known to affect hydrolytic stability.

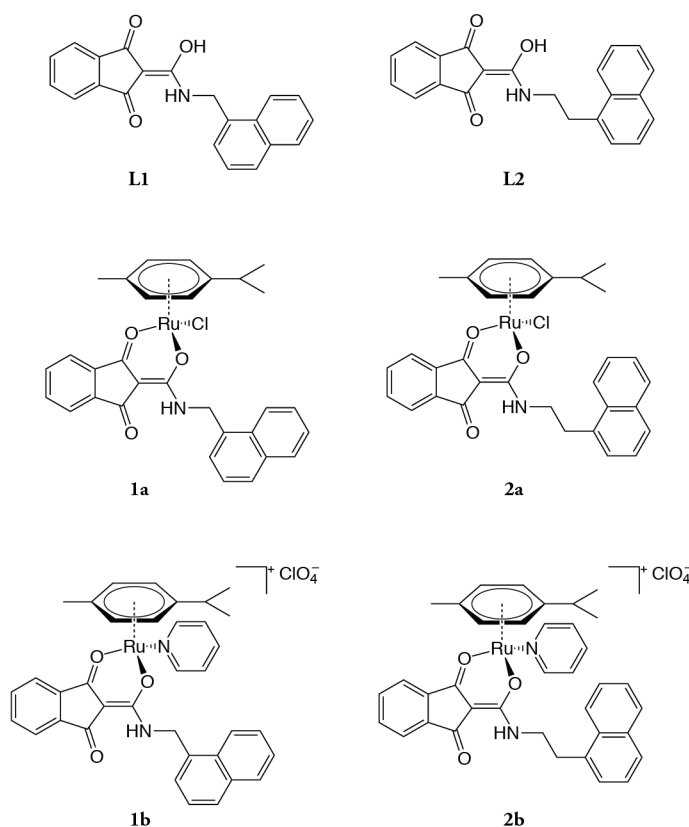


Fig. 11. First set of compounds studied in this thesis: ruthenium-arene complexes with 1,3-dioxindan-2-carboxamide ligands (**L1** and **L2**). Metal compounds differ by (i) length of the linking alkyl chain and (ii) leaving group, chlorido for **1a/2a** or pyridine for **1b/2b**.

2.2. Results and Discussion

2.2.1. Stability in Solution

Preliminary studies by Mokesch *et al.* looked into the stability of this class of compounds *via* IT-ESI-MS, and found the complexes to be stable in aqueous solution (1% DMSO/ H_2O , 50 μM) over the course of 48 h. To study the interaction of our compounds with DNA at a physiological pH and in cell culture media, the stability of compounds **1a/b** and **2a/b** in buffered solutions had to be ascertained. Stock solutions of compounds were prepared in either DMSO or acetonitrile; both chlorido and pyridine complexes (exemplified by **1a** and **1b**) are stable over 24 h in these solvents as shown *via* time-dependent ^1H -NMR.

2.2.1.1. UV-VIS Spectroscopy

FRET melting assays (see below) have to be conducted at a pH slightly above pH 7. Also, in order to study the interaction with G4, potassium ions have to be introduced to the solution. Therefore initial UV-Vis spectra were measured in a TRIS-KCl buffered solution (pH 7.8, 50 mM KCl) and recorded over the course of 24 h.

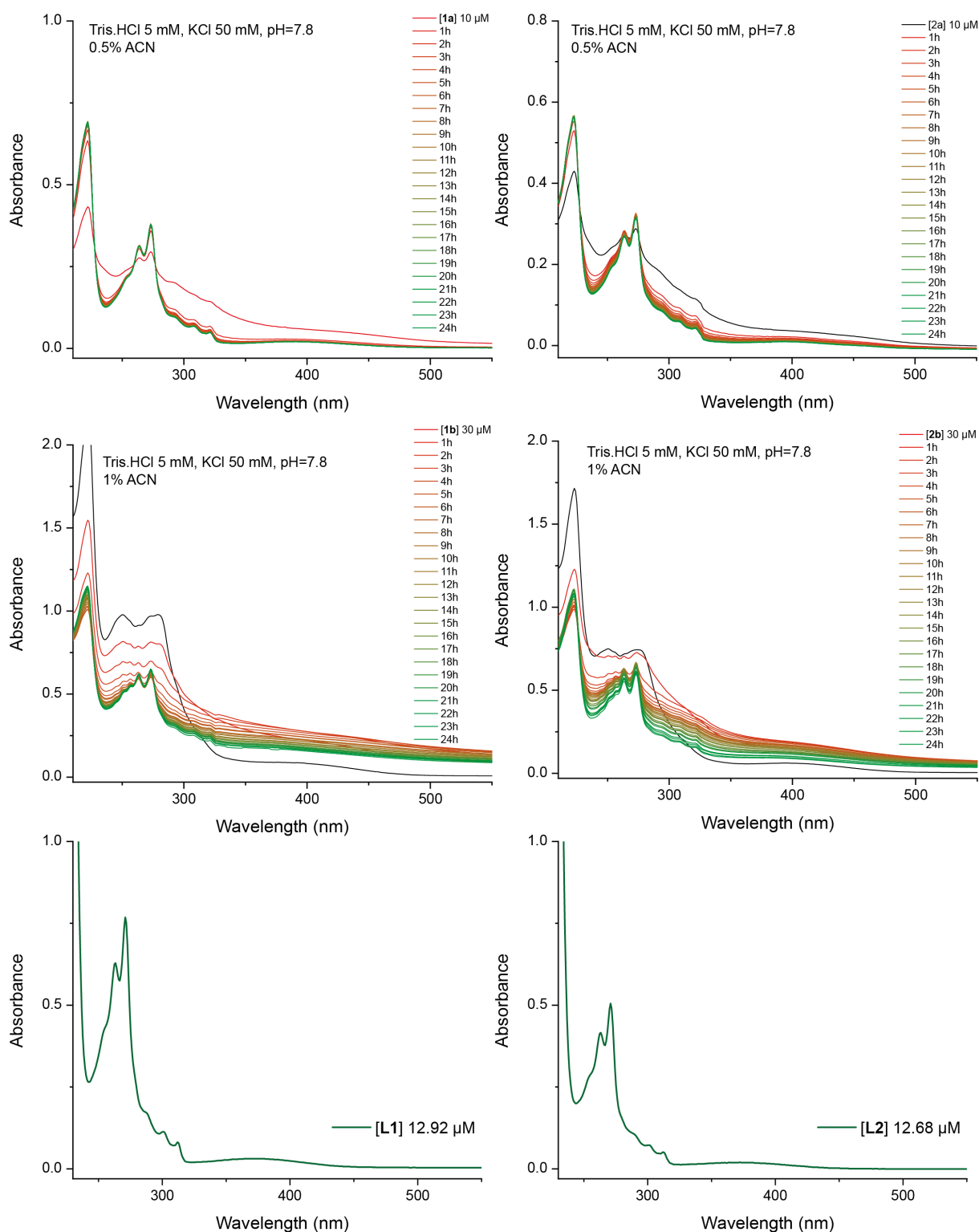


Fig. 12. Time-dependent UV-Vis spectra of metal compounds **1a/2a** (10 μM) and **1b/2b** (30 μM) in TRIS-KCl buffered solution (TRIS-HCl 5 mM, KCl 50 mM, pH 7.8) as well as reference spectra of the metal-free ligands **L1** and **L2**.

In these conditions, complexes **1a/b** and **2a/b** tend to release the *O,O*-chelating ligand over time, with the reaction being slower for pyridine complexes **1b** and **2b** (ligand release after 3–4h) than for chlorido complexes **1a** and **2a** (<1 h). Spectra of **1a** in TRIS-KCl buffer in particular showed a rapid increase of absorption bands of the free ligand **L1** (273 nm, 264 nm), in addition to noticeable precipitation after 24 h. Pyridine analogues tended to be more stable toward release of the ligand but long-term results were similar to those seen for chlorido complexes.

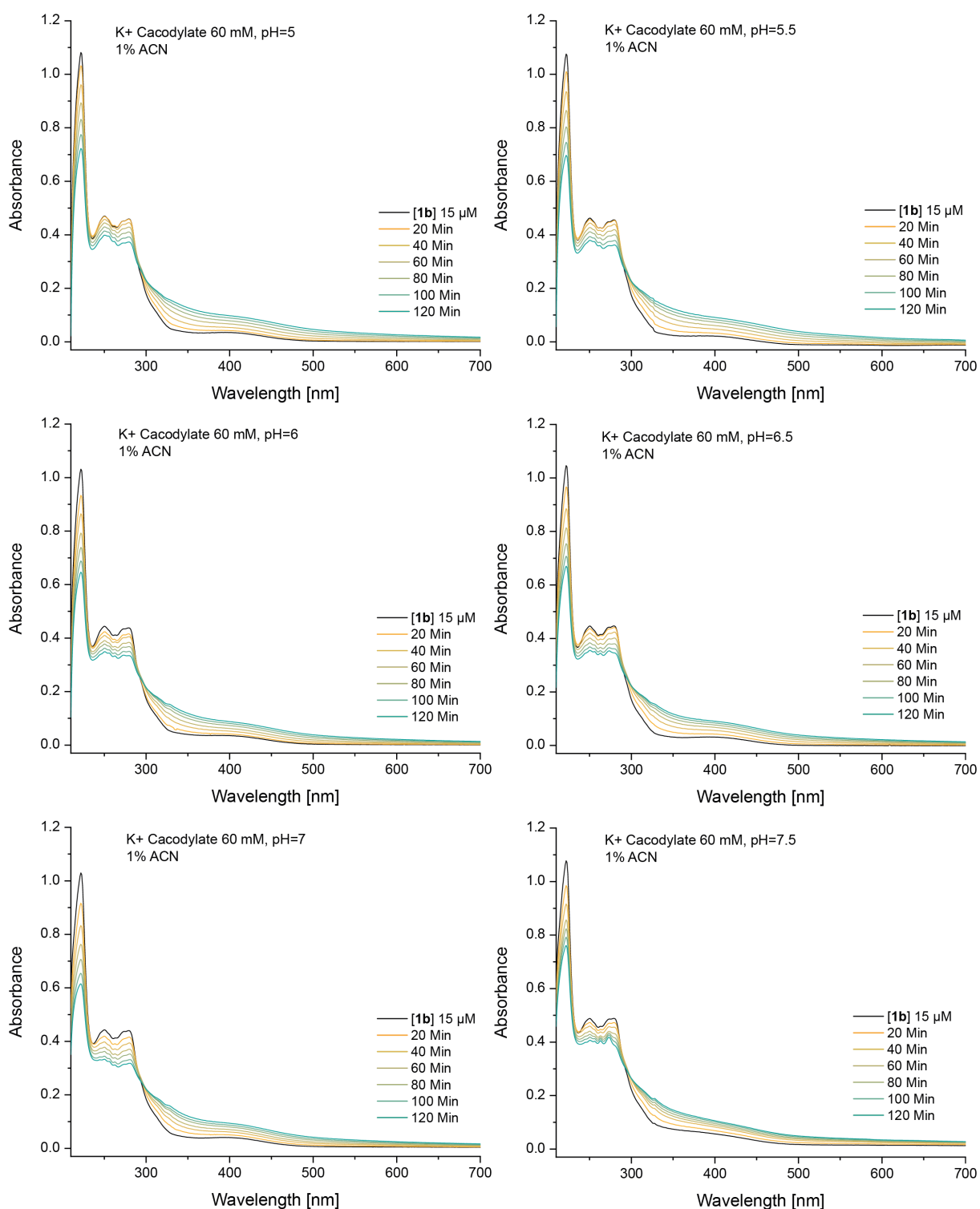


Fig. 13. Spectra of **1b** (15 μM) in potassium cacodylate buffer at different pH values.

In order to further study these compounds and their interaction with G-quadruplexes, stable conditions needed to be found for at least an experimental time-frame of three hours. Varying the pH in potassium cacodylate buffer (used in subsequent FRET experiments) reveals that a beginning release of the ligand (observable through a rise of absorption bands centered at 273 nm and 264 nm) is only noticeably visible at a pH above 7.

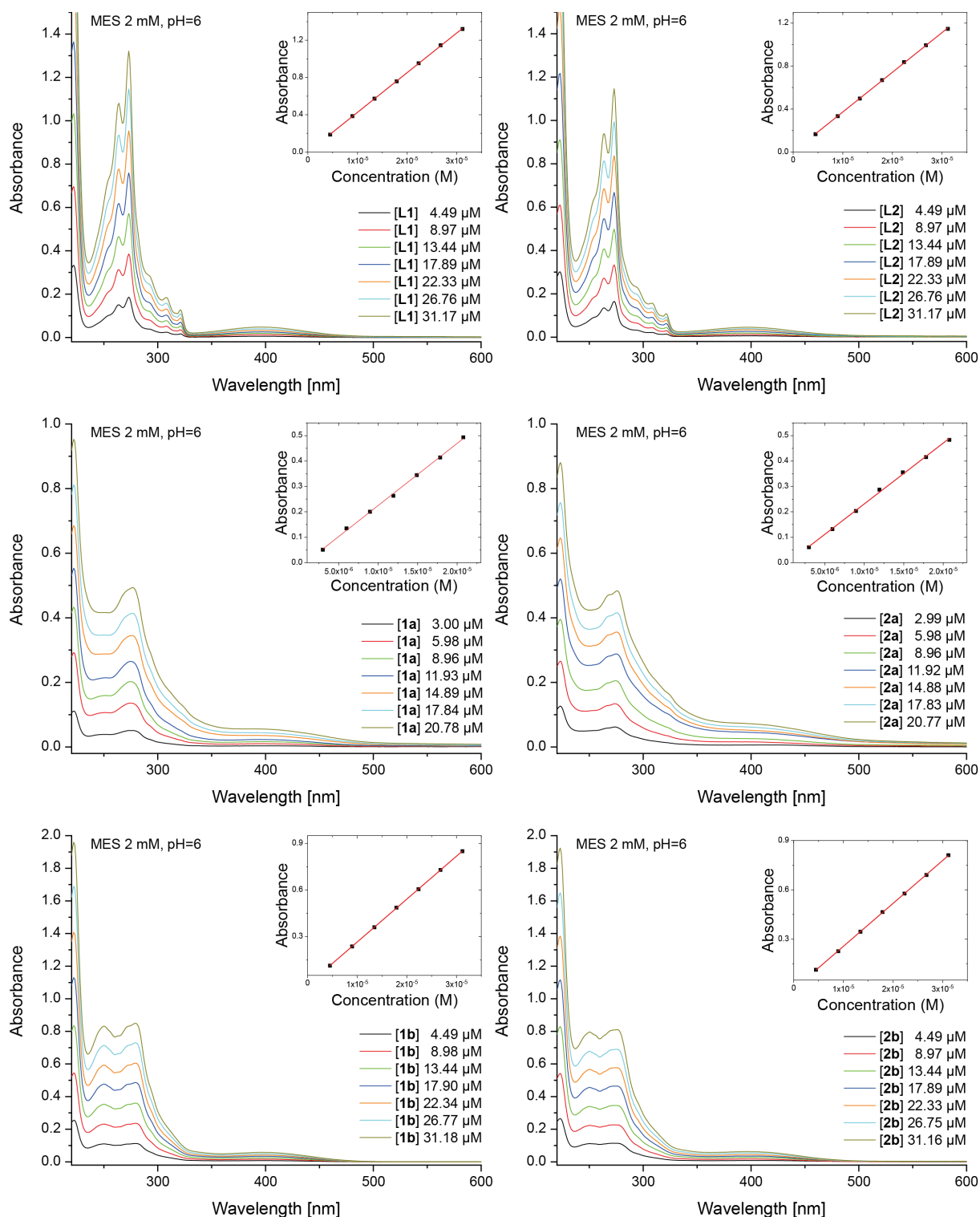


Fig. 14. UV-Vis spectra of compounds at increasing concentrations in MES buffered solution at pH 6. Extinction *vs.* concentration follows a linear trend (insets) from which Lambert-Beer extinction coefficients were calculated.

Decreasing the pH and switching the buffer system to MES (pH 6, 2mM MES), afforded stable conditions in which the metal complexes retain the ligand over 24 h (see NMR and experimental sections). However, over a period of 24 h the overall absorption is decreasing, indicating slight precipitation. Lambert-Beer extinction coefficients at the indicated wavelengths are given in Table 2.

Tab. 2. Lambert-Beer extinction coefficients for metal complexes **1a/b** and **2a/b** as well as metal free ligands **L1** and **L2**.

	ϵ_{λ} ($M^{-1} cm^{-1}$)	λ
1a	24460 ± 387	277 nm
2a	23974 ± 424	276 nm
1b	27665 ± 66	279 nm
2b	26127 ± 109	276 nm
L1	42652 ± 223	273 nm
L2	36978 ± 258	273 nm

Whether the loss of the chelating ligand was owed to the presence of chloride ions was studied by adding potassium chloride to the MES buffer. Addition of increasing concentrations of potassium chloride (2 mM, 5 mM, 10 mM, Fig. 15) to 2 mM MES buffer did not lead to release of the ligand, but produced a time-dependent shift in the spectrum over time, an effect that could be ameliorated by using 5 mM potassium sulfate instead.

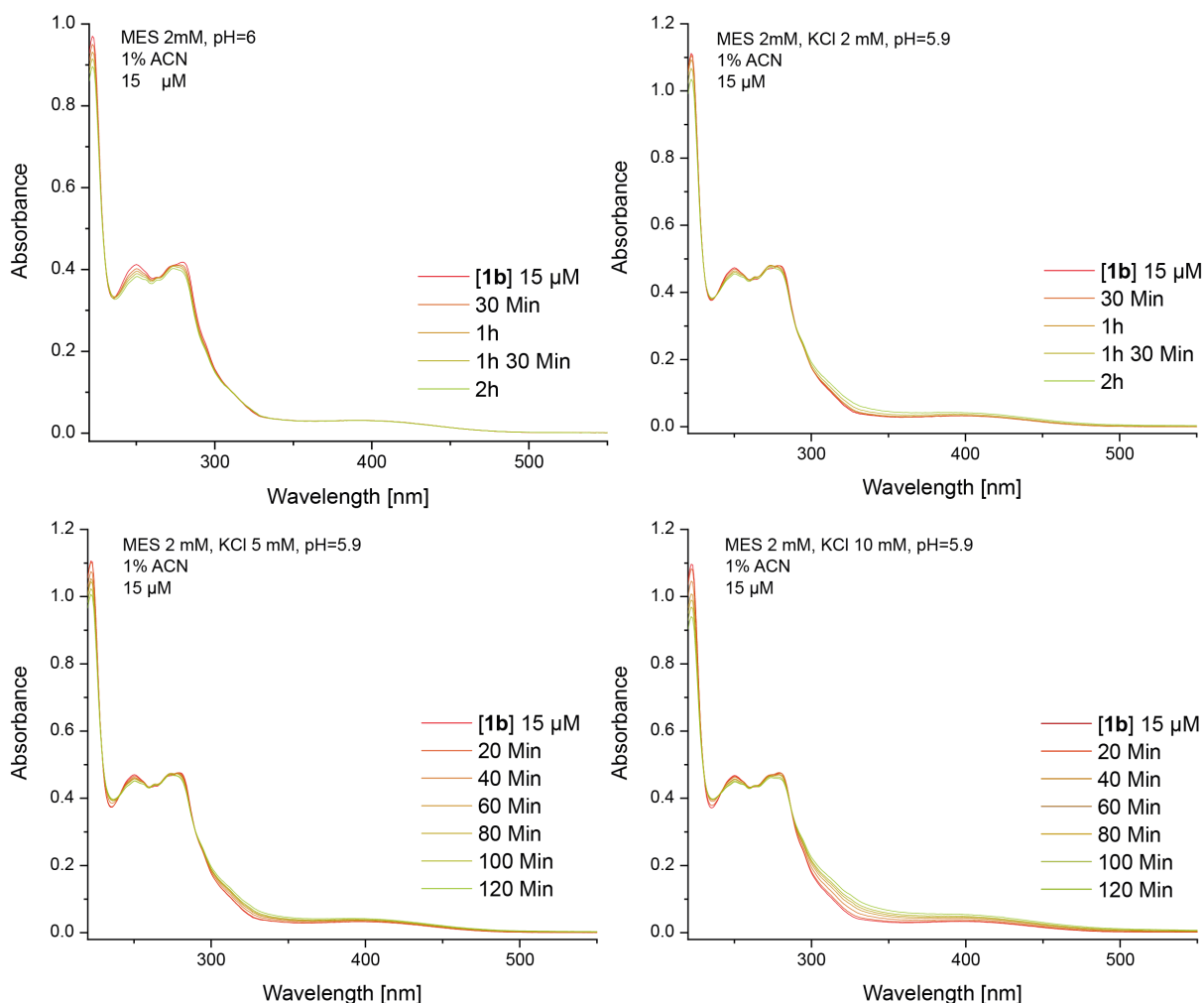


Fig. 15. Effect of potassium chloride on the short-term stability of compound **1b**. The pyridine complex **1b** is relatively stable over 2 h.

2.2.1.2. Effect of Irradiation

Since Ru–N bonds can potentially be photochemically cleaved under prolonged light exposure, solutions of pyridine complexes **1b** and **2b** were handled in the dark. To ascertain whether these complexes undergo ligand photosubstitution, aqueous solutions of **1b/2b** in MES buffer (pH 6) were irradiated with white light and studied by recording UV-Vis spectra. After 1 Min of irradiation, no changes were visible in the spectra; however, after 30 Min absorption of the irradiated samples decreased and a beginning release of the chelating ligand became visible, while in the dark no such changes could be observed (Fig. 16). These findings are in accordance with a gradual loss of the pyridine ligand, which destabilises the complexes.

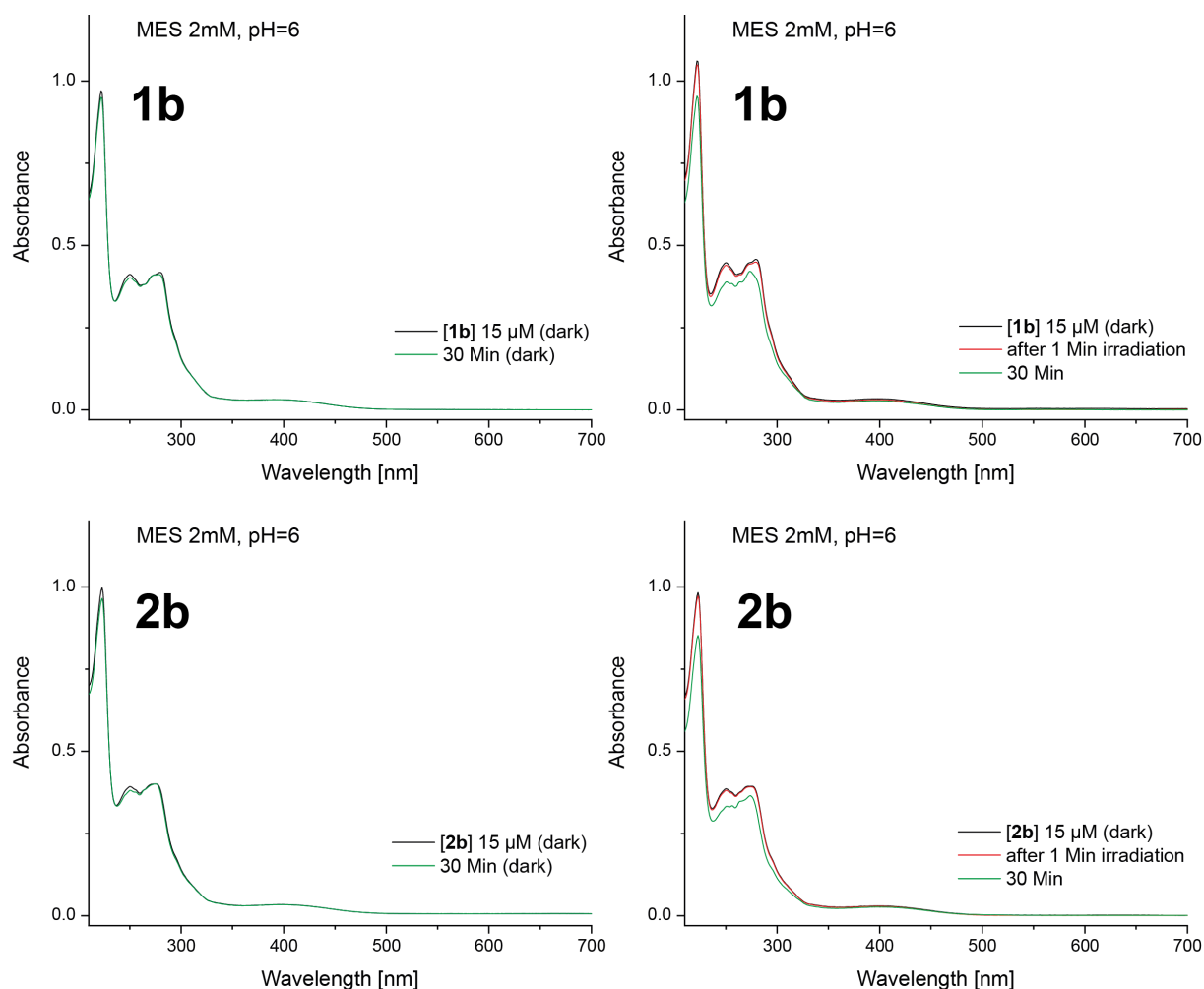


Fig. 16. Left: pyridine complexes **1b** and **2b** in the dark over 30 Min. Right: the same compounds after white light irradiation for 1 Min. After 30 Min, a relative increase of peaks associated with the free ligand can be observed.

FRET-labelled ODNs could not be irradiated, since the melting temperature of the oligonucleotides decreased drastically; however, mixtures of ODNs with samples of the metal compounds irradiated separately in buffer (potassium cacodylate 60 mM, pH 7.4) showed no significant changes in melting temperatures (see experimental section, Fig. E4). Because both FRET melting assays with G4s and cell vitality assays (see following sections) showed the loss of pyridine to have a detrimental effect on G4 interaction and cytotoxicity, the release of pyridine by photoexcitation was not further studied.

2.2.1.3. NMR Spectroscopy

To better understand the hydrolytic stability of our compounds, their behaviour in buffered aqueous solution was studied at different pH values. Compound **1b** was dissolved in three different buffer conditions and studied *via* ^1H -NMR.

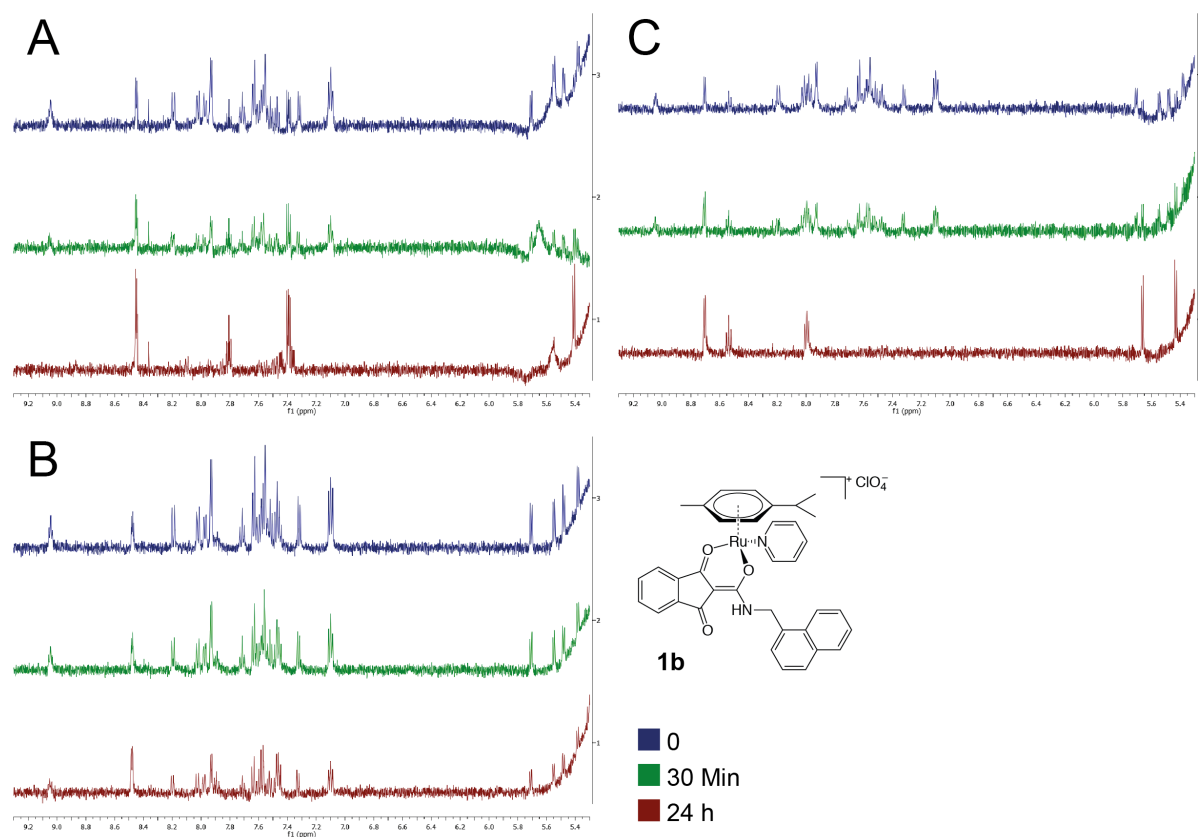


Fig. 17. Time-dependent ^1H -NMR spectra of compound **1b** (150 μM) in a mixture of 8 vol% d_6 -DMSO (compound stock solution), 10 vol% D_2O , and 82 vol% respective buffer. **A:** TRIS-HCl 10 mM, pH 7.5; **B:** MES 2 mM, pH 6; **C:** sodium citrate 10 mM, pH 3.4.

In both 10 mM TRIS-HCl buffer (pH 7.5) as well as 10 mM sodium citrate buffer (pH 3.4) signals arising from the naphthyl ligand system diminish over time as the ligand precipitates from solution. Merely peaks assigned to ruthenium-bound cymene and pyridine remain visible after 24 h. In MES buffered solution, all peaks present from the beginning were visible after 1 day.

Next, to ascertain whether the pyridine ligand is released in solution, free pyridine was added to a solution of **1b** in d_6 -DMSO. As shown in Fig. 18, peaks arising from free pyridine can be clearly distinguished from peaks arising from metal-bound pyridine. As none of the pyridine proton peaks in aqueous solutions shift over time, the pyridine leaving ligand is retained in aqueous solution within the timeframe of subsequent experiments.

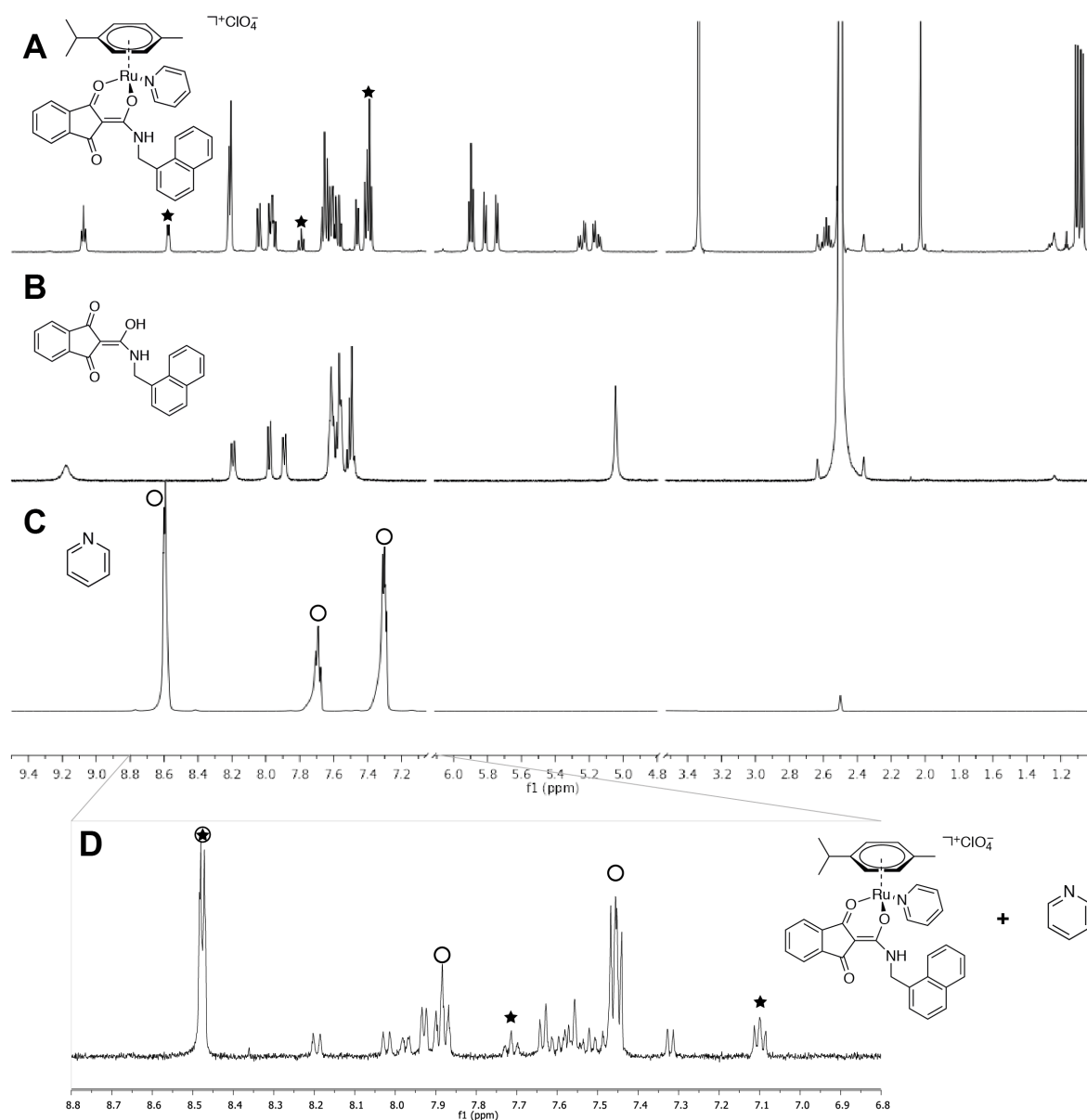


Fig. 18. A–C: spectra of **1b**, ligand **L1** for reference, and pyridine in d_6 -DMSO; peaks of metal-bound pyridine protons are highlighted with a star, peaks of free pyridine protons are highlighted with circles. **D**: enlarged section of a spectrum of a mixture of **1b** and free pyridine, with pyridine protons highlighted accordingly.

Concluding, metal complexes were stable at a slightly acidic pH in MES buffered solution, but tend to release the *O,O*-chelating ligand at physiological pH.

2.2.2. Interaction with B-DNA and G-quadruplexes

Stabilisation of duplex DNA and four G4 motifs (telomeric and oncogene-related) upon addition of our studied compounds was studied in FRET melting assays. Whether the ruthenium center of our compounds could bind to DNA was studied with the model compound 9-ethylguanine (9-EtG) *via* mass spectrometry. Next, conditions were established to record mass spectra of G4s in order to study the effect of our compounds on the folding of the *hTelo* and *c-KIT1* quadruplexes.

2.2.2.1. FRET melting assays

Five oligonucleotide sequences (ODNs) were selected for FRET assays to examine the effect of our compounds on G-quadruplexes: one ODN from the telomeric sequence (*hTelo*), three ODNs from oncogene promoters (*hTERT*, *c-KIT1*, *BCL-2*), and one ODN as a B-DNA model capable of folding into a hairpin-like structure (*dsDNA*). ODN sequences were labelled with the fluorescent dye couple FAM-TAMRA, affording signal when the ODN »melts« and loses its secondary structure (either G4 fold or B-DNA double helix).

As established via UV-Vis and $^1\text{H-NMR}$ spectroscopy (see above), metal compounds **1a/b** and **2a/b** are not stable at a pH above 7 and tend to release the *O,O*-dioxoindane ligand. However, FRET data is recorded over a temperature increase from 25°C to 95°C. Neither MES nor TRIS-HCl could be used in these experiments since their pK_a value changes significantly with temperature. Furthermore, the FAM-TAMRA dye couple cannot be used at an acidic pH, since the dye is being protonated and the signal is quenched. As a result, FRET melting assays were carried out in 60 mM potassium cacodylate buffer (pH 7.2). In these conditions complexes can be considered stable in the timeframe of FRET experiments (1h–2h), as shown via UV-Vis and $^1\text{H-NMR}$ (see experimental section).

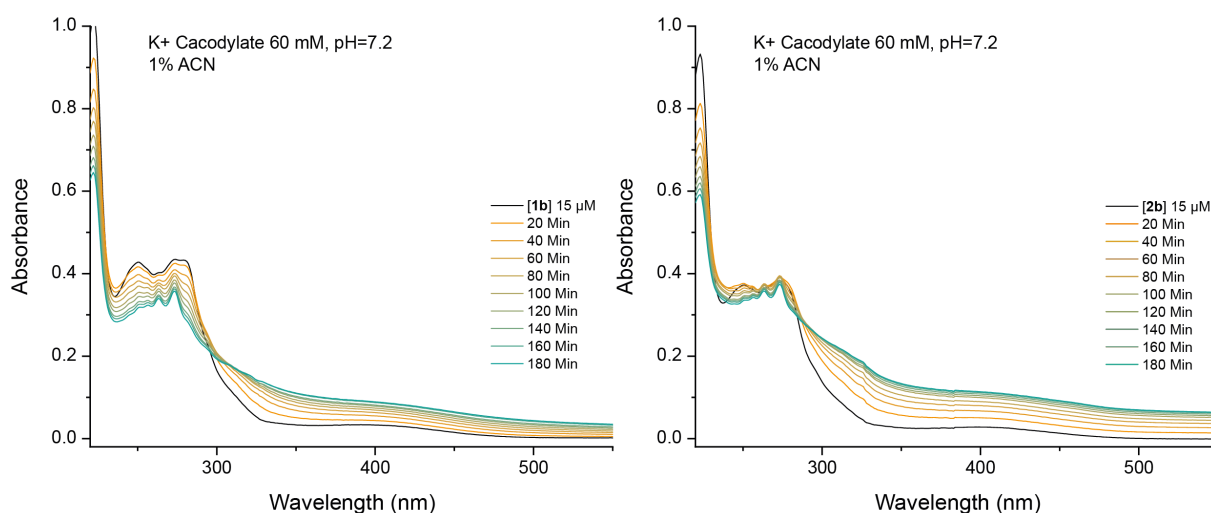


Fig. 19. Time-dependent UV-Vis spectra of pyridine complexes **1b** and **2b** in 60 mM potassium cacodylate buffer (pH 7.2). After 3 h, the release of the respective chelating ligand becomes visible in rising absorption peaks at 273 nm and 264 nm.

To ensure proper folding of the ODNs into G4s, ODNs were annealed by heating to 90°C and slowly cooling to room temperature. Annealed ODNs were then incubated in 96-well-plates with metal complexes and their corresponding ligands. Absorption profiles were measured in a PCR-cycler starting at 25°C and heated to 95°C. $\Delta T_{1/2}$ values indicate the relative stabilisation of a secondary structure incubated with compound as opposed to the free ODN. TMPyP4 was used as a non-selective, strongly stabilising control drug.

In all studied cases (Tab. 3), ligands **L1** and **L2** led to a decrease in melting temperature and thus destabilise the G4 structure. Chlorido complexes **1a** and **2a** across the board show only a negligible stabilising effect, the exception being **2a**, which significantly increases the melting temperature of *dsDNA*. Only pyridine complexes **1b** and **2b** exert a significant stabilising effect on the selected ODNs, especially in the case of the G4 sequence from the *c-KIT* gene promoter. **1a/b** exert the strongest stabilising effect on *c-KIT1* and, moreover, preferably stabilise *c-KIT1* over B-DNA model *dsDNA*.

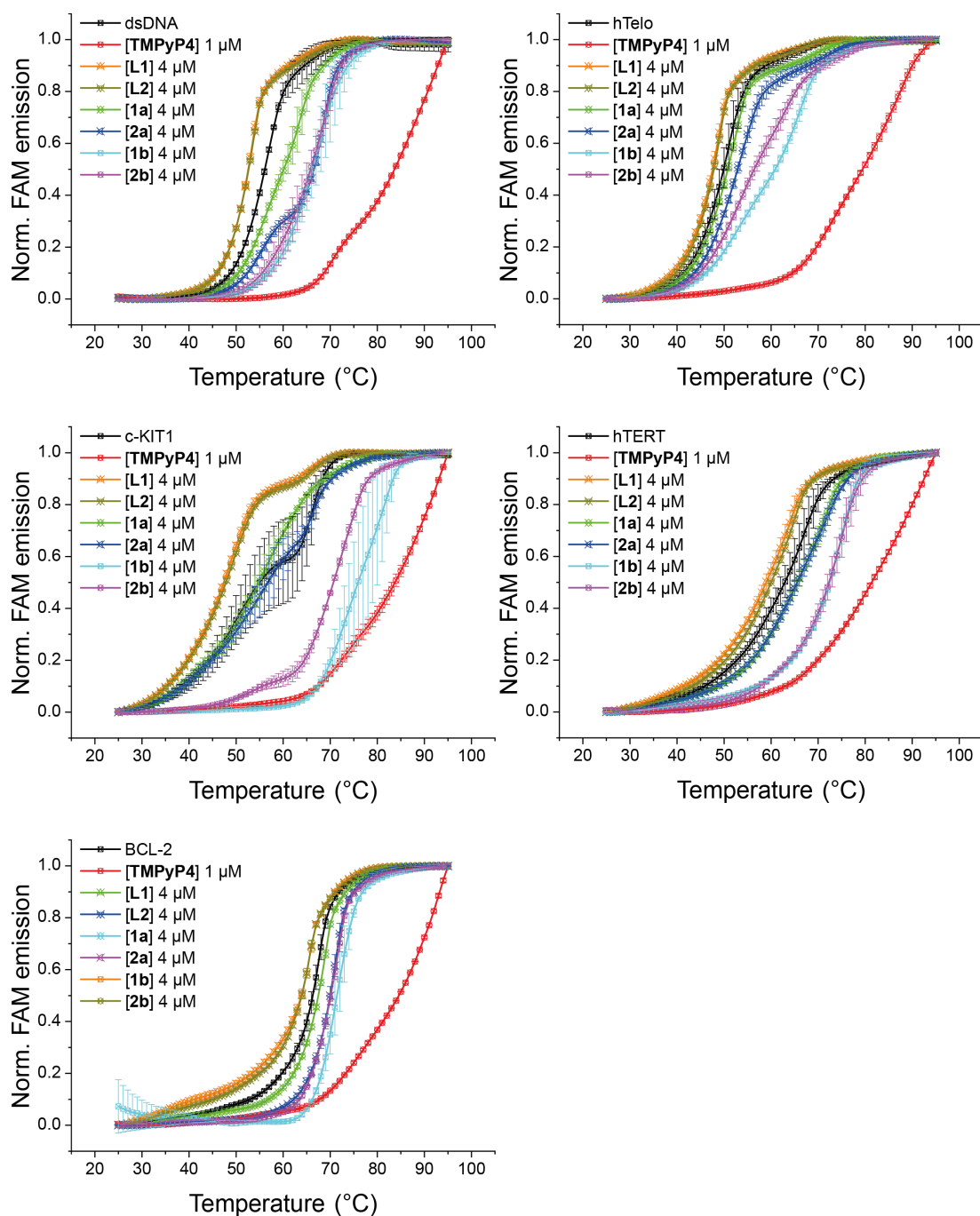


Fig. 20. FRET melting profiles of ODNs (0.2 μM) treated with 4 μM of studied compounds in 60 mM potassium cacodylate buffer (pH 7.2). $\Delta T_{1/2}$ values are reported in Table 3.

Since our metal complexes are known to release the ligand over time in the conditions described above, we ascertained whether the constituents of complexes **1b** and **2b** after ligand hydrolysis could exert as strong a stabilising effect on selected ODNs by an assay of control experiments. Complex **3** was synthesised according to the literature.^[97] ODNs were incubated with equimolar mixtures of ligands **L1** and **L2** with complex **3**. As shown in Table 3, $\Delta T_{1/2}$ values reported for mixtures of constituents were remarkably lower than for solutions of complexes **1b** and **2b**, proving that the intact complex is required for the maximal effect.

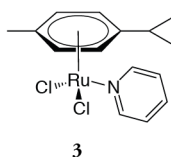


Fig. 21. Compounds **1a/b** and **2a/b** tend to release the *O,O*-chelating ligand in FRET-suited conditions over time (see section 2.2.1.3). Because pyridine is not released in the dark, the aquated species of compound **3** is the suggested solution species present after dissociation of the naphthyl-bearing ligand.

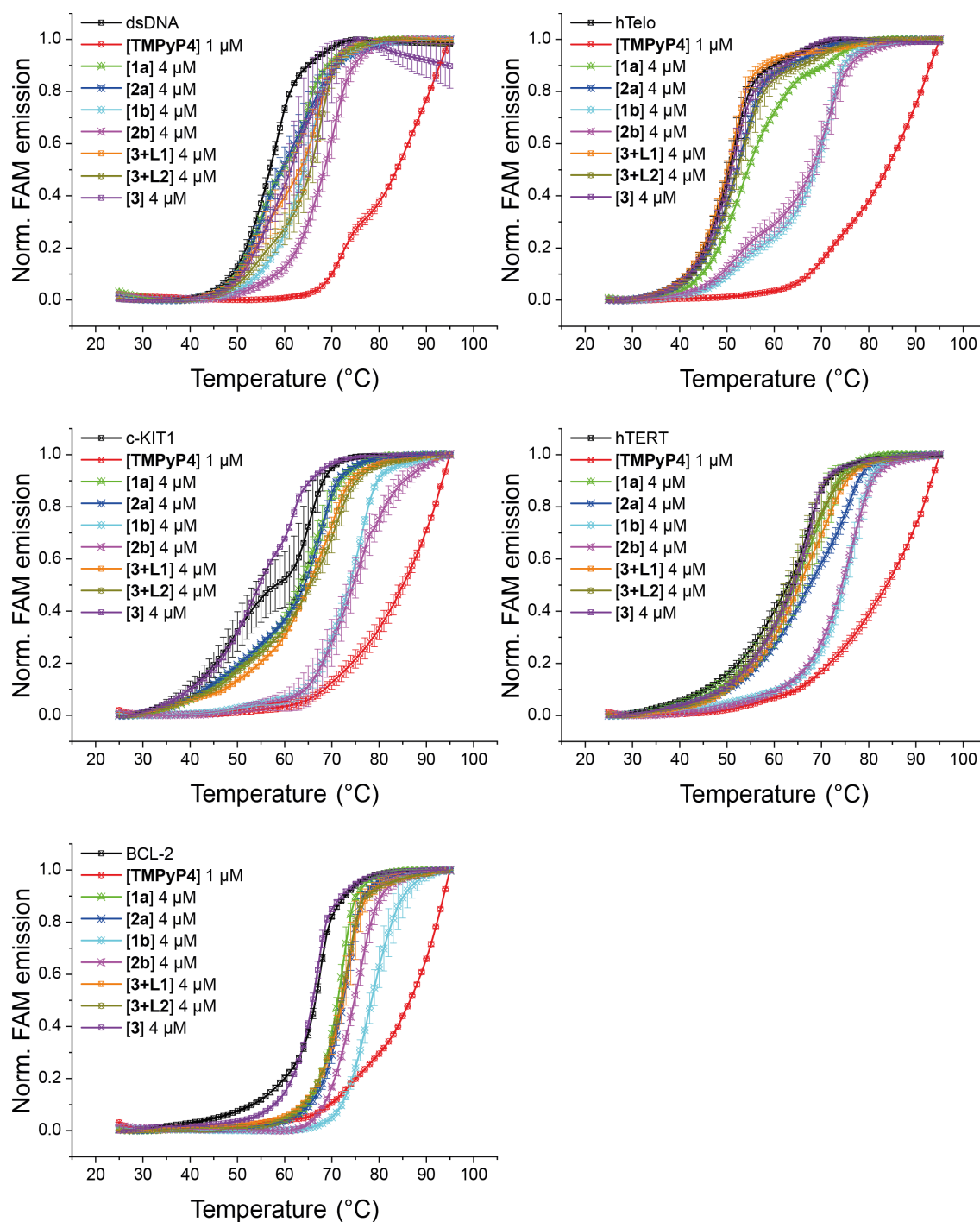


Fig. 22. Control experiment using mixtures of compound **3** with the metal-free ligands **L1** and **L2**. Compound **3** alone shows no stabilisation of ODNs. (potassium cacodylate 60 mM, pH 7.2). $\Delta T_{1/2}$ values are reported in Table 3.

Tab. 3. $\Delta T_{1/2}$ values obtained in FRET melting assays. Uncertainty is <0.5.

	<i>dsDNA</i>	<i>hTelo</i>	<i>c-KIT1</i>	<i>hTERT</i>	<i>BCL-2</i>
TMPyP4	27.2	31.4	27.8	19.7	19.2
L1	-3.5	-2.4	-7.6	-4.0	-2.4
L2	-3.4	-2.1	-7.1	-2.9	-2.3
1a	4.0	0.9	-0.1	2.7	1.2
1b	10.2	11.0	20.9	9.5	5.1
3+L1	6.3	-0.4	10.1	2.4	5.7
2a	10.4	3.0	1.8	2.6	3.7
2b	9.7	6.5	15.7	9.4	3.8
3+L2	8.6	1.8	10.5	0.7	5.4
3	4.4	0.4	-0.3	1.1	-0.6

The findings described above clearly indicate that the hydrolytic stability of the complexes is essential for their stabilising effect on G4s. Consequently, a shorter incubation time—and with it a larger fraction of intact complex—should result in a higher melting temperature. As can be seen in Figure 23, decreasing the incubation time by 1 h lead to a stabilising effect on *c-KIT1* even for the less stable chlorido complexes **1a** and **2a**.

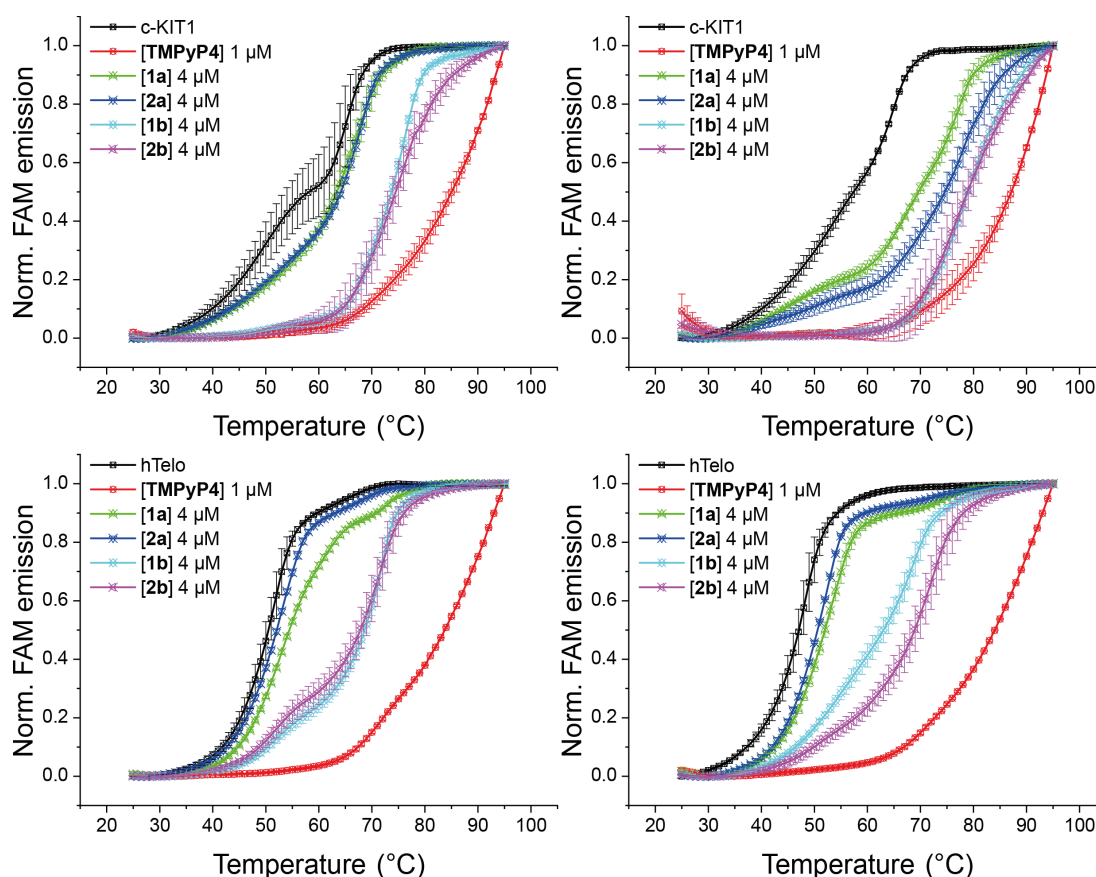


Fig. 23. Two exemplary FRET melting profiles for *c-KIT1* and *hTelo* oligonucleotides after 2 h incubation (left) and 1 h incubation (right). For $\Delta T_{1/2}$ see experimental section.

2.2.2.2. Mass Spectrometry

9-Ethylguanine. Since the preferred binding site of ruthenium to DNA is the guanine N7 position, compounds **1a/b** and **2a/b** were incubated (2 h in H₂O, MeOH 20%) with model compound 9-ethylguanine and subsequently analysed *via* ESI mass spectrometry. All complexes are able to coordinate to the 9-ethylguanine; in a subsequent series of experiments, this coordination was shown to occur relatively fast in under 30 Min.

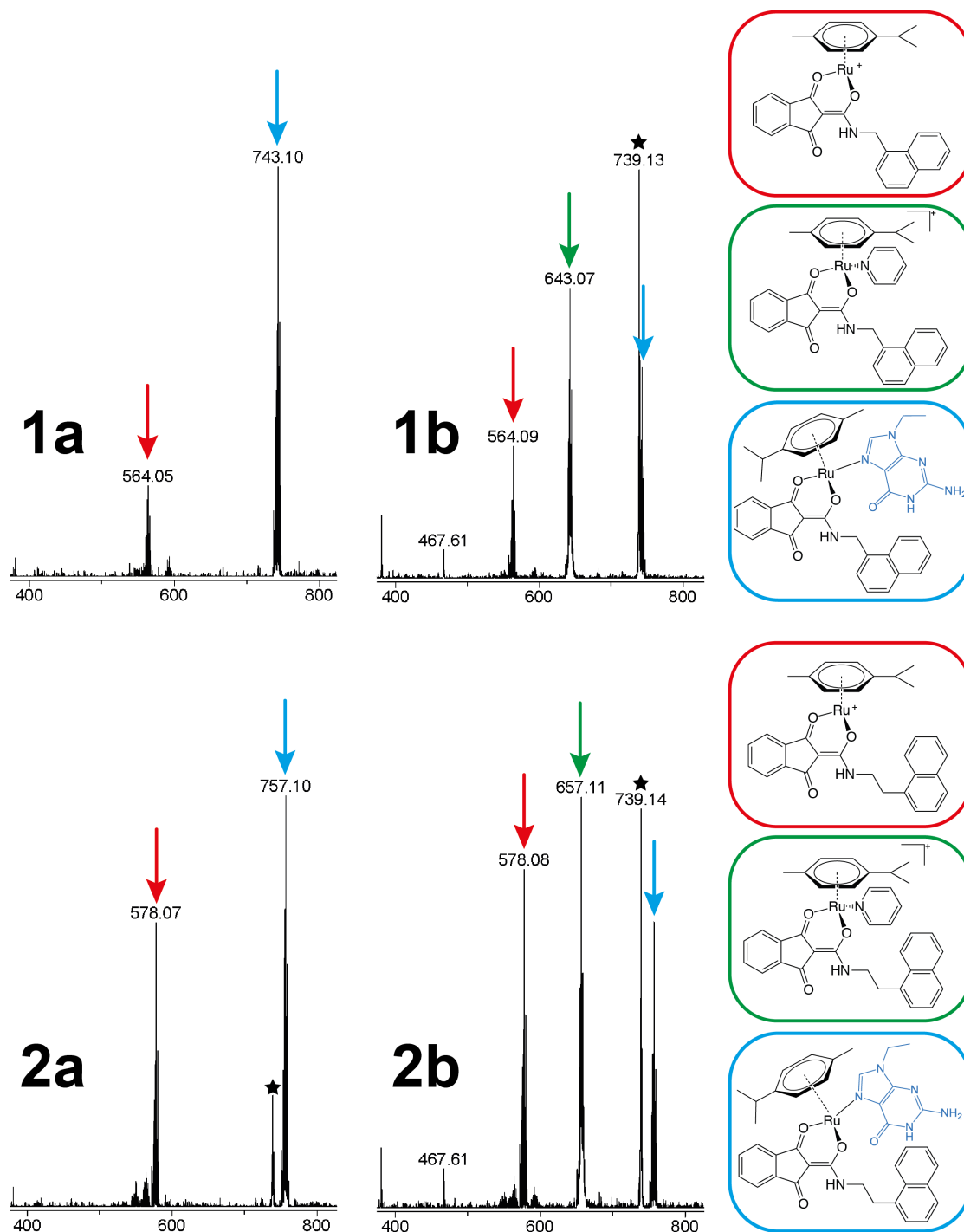


Fig. 24. Mass spectra of complexes **1a/b** and **2a/b** (0.2 mM) and 9-EtG (0.6 mM) in 20% MeOH/H₂O. All compounds form stable adducts with 9-EtG. For peak distributions (experimental and simulated) see experimental section. Peaks highlighted with a black star (739 m/z) correspond to aggregates of four molecules of 9-EtG (a G-tetrad) around trace amounts of sodium ions.^[98]

G-quadruplex interaction. Since nonvolatile potassium salts are not compatible with their use in buffers for ESI mass spectrometry, ammonium acetate was used instead to ensure the formation of G4.^[99] *hTelo* and *c-KIT1* were selected as G4 sequences, both containing stacks of three G-quartets and 2 monovalent ions in their folded structure. Experimental conditions were adjusted to allow ammonium ions coordinated between G-quartets to be retained, while ammonium ions associated to binding sites not specific for the G4 fold are mostly stripped during ionisation. Charge states of the oligonucleotide with the highest relative abundance (-5) were interpreted. The three most prominent peaks in each charge state correspond, respectively, to (1) the free G4, (2) G4 with one associated ammonium ion, and (3) G4 with two associated ammonium ions.

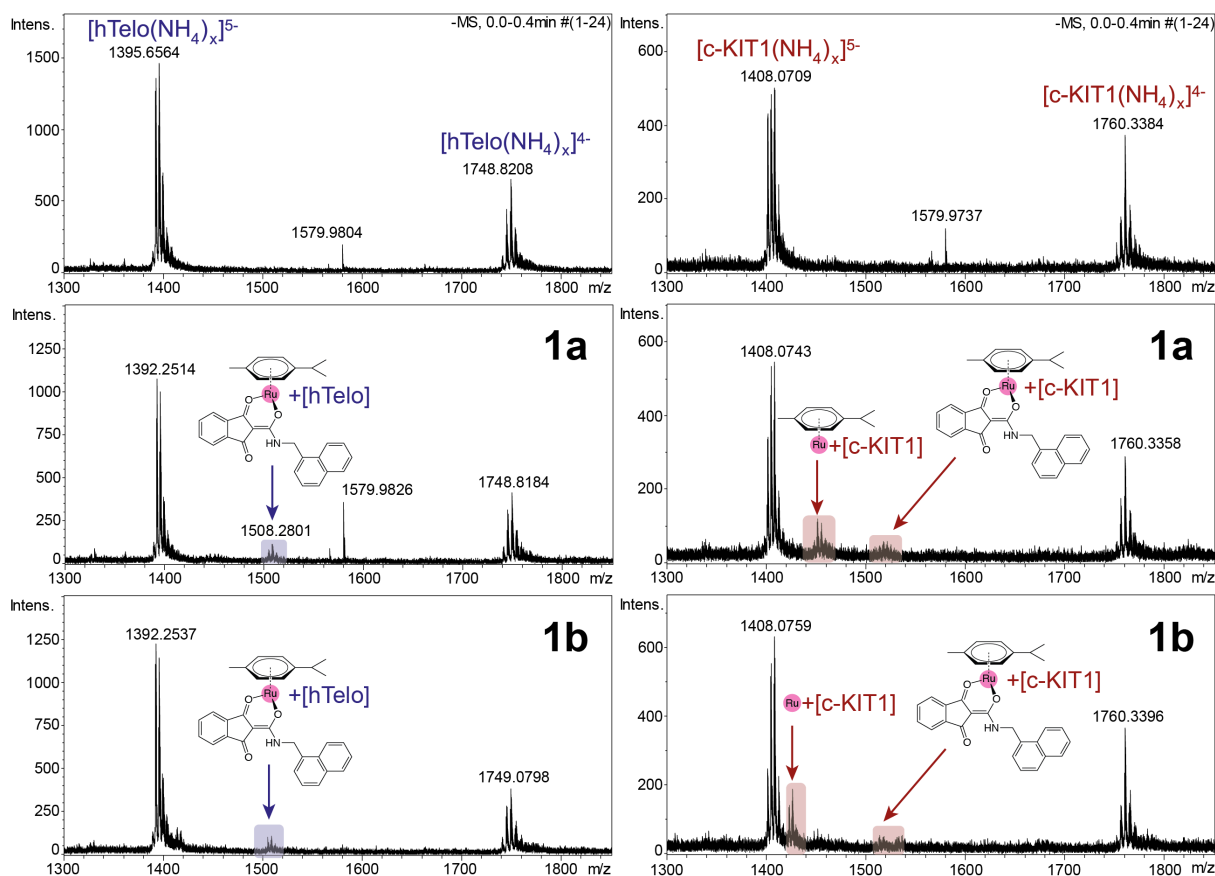


Fig. 25. Mass spectra of 10 μM *c-KIT1* and *hTelo*, respectively, in the absence (top) and presence of equimolar amounts of compounds **1a** and **1b**. Solutions were prepared in ammonium acetate (50 mM, pH 6.8, 1% ACN).

ESI-MS spectra of *hTelo*^[-5] showed peaks corresponding to 0–2 ammonium adducts, *hTelo*^[-5] with 1 associated ammonium ion being highest in abundance. As can be seen in Figure 26, addition of ruthenium complexes **1a/b** and **2a** lead to a shift in ammonium adduct distribution towards *hTelo*^[-5] with no associated ammonium. This indicates a minor destabilising effect of the complexes on the G4 structure, as the ammonium is more easily stripped away during ionisation.

c-KIT1^[-5] exhibits the opposite effect: upon addition of ruthenium complexes **1a/b** and **2a/b**, the relative abundance of *c-KIT1*^[-5] with 1–2 coordinated ammonium ions increases, indicating increased stability of the G4 structure as the ammonium is more readily retained.

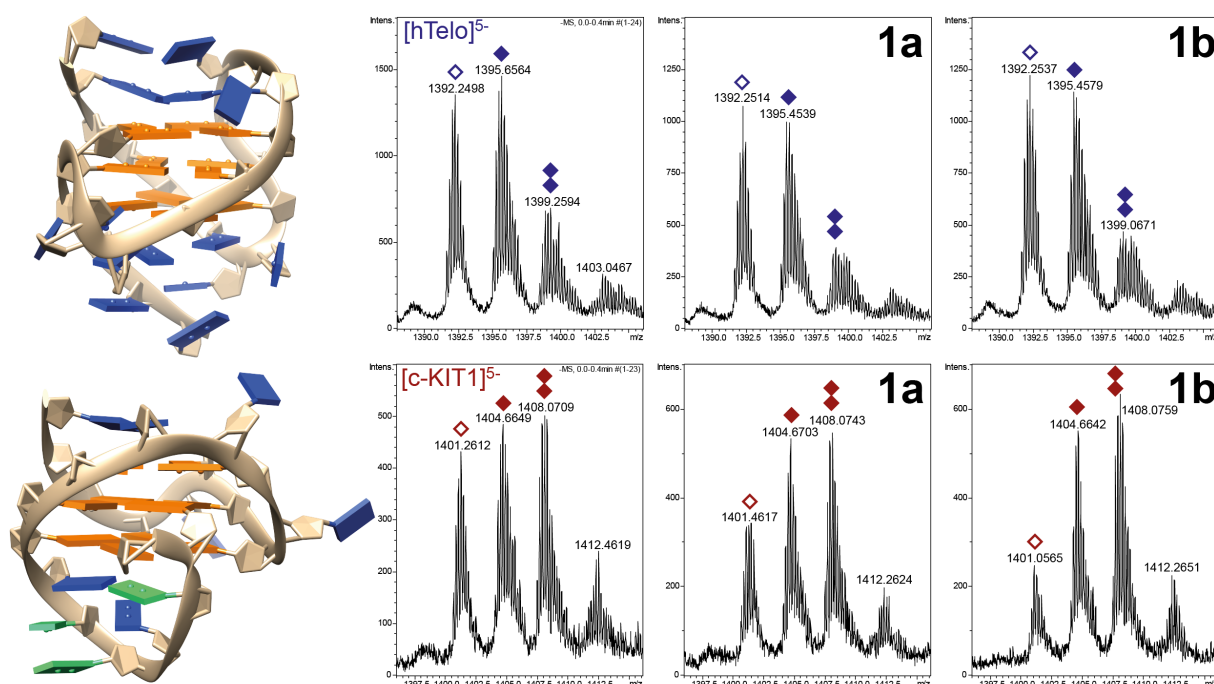


Fig. 26. Left: graphical representation of *c-KIT1* and *hTelo*, generated with Chimera (PDB 2HY9 and 2O3M). G-tetrads are highlighted in orange. Free guanine binding sides in *c-KIT1* are highlighted in green. Right: ammonium ion distribution in the (5-) charge state of *c-KIT1* and *hTelo*, respectively, of the spectra in Figure 25. The three peaks in each spectrum correspond to the free G4 without associated ammonium, one NH_4^+ ion adduct, and two NH_4^+ ion adduct (one/two diamonds, respectively).

The guanine N7 position is inaccessible to the ruthenium complex if the guanine is stacked in a G-quartet. The *hTelo* sequence contains no free guanines besides those participating in the G4 fold. Ruthenation of *hTelo* would therefore require a partial unfolding of the G4 structure, thereby releasing the monovalent ion between G-quartets. *c-KIT1*, on the other hand, features 3 non-stacked guanines, making them accessible as binding sites for ruthenium complexes.^[100]

Additionally, low-intensity peaks corresponding to 1:1 adducts of the complexes **1a/b** or their fragments could be found for both *hTelo* and *c-KIT1* (Fig. 25). This indication of the complex binding to the G4, in addition to our findings with 9-ethylguanine, lends credibility to the dual mode of binding strategy in that the ruthenium can coordinate to the guanine while simultaneously maintaining its effect on the stability of the G4 fold via π -stacking of the naphthyl group. The hypothesis is further substantiated by the fact that *c-KIT1* is the G4 sequence with the highest increase in melting temperature when exposed to complex **1b** in FRET melting assays, as *c-KIT1* has guanine binding sides outside the G4 fold, whereas *hTelo* does not.

2.2.3. Cytotoxicity

The cytotoxic activity of complexes **1a/b** and **2a/b** and their respective ligands were tested against the ovarian cancer cell line A2780 as well as osteosarcoma cell line U2OS. IC_{50} values were obtained in MTT-based vitality assays. Cells were treated with solutions of the compounds at concentrations of 0–75 μM and then incubated for 72 h at 37°C (5% CO_2).

As reported in Table 4, the most active compounds were the pyridine complexes **1b** and **2b** with IC_{50} values in the low micromolar range, well below that of ligands **L1** and **L2** and chlorido complex **1a**. Control compound **3** (the ruthenium-cymene-pyridine constituent *sans* the *O,O*-chelating ligand) showed almost no cytotoxicity as its effect was comparable to that of

DMSO, which was used as a solvent to prepare stock solutions. An equimolar combination of free ligands **L1** and **L2** with compound **3** (both being the constituents of complexes **1b** and **2b**, respectively) had an effect in cell viability comparable to that of the ligands alone, with IC_{50} values well above that of complexes **1b** and **2b**.

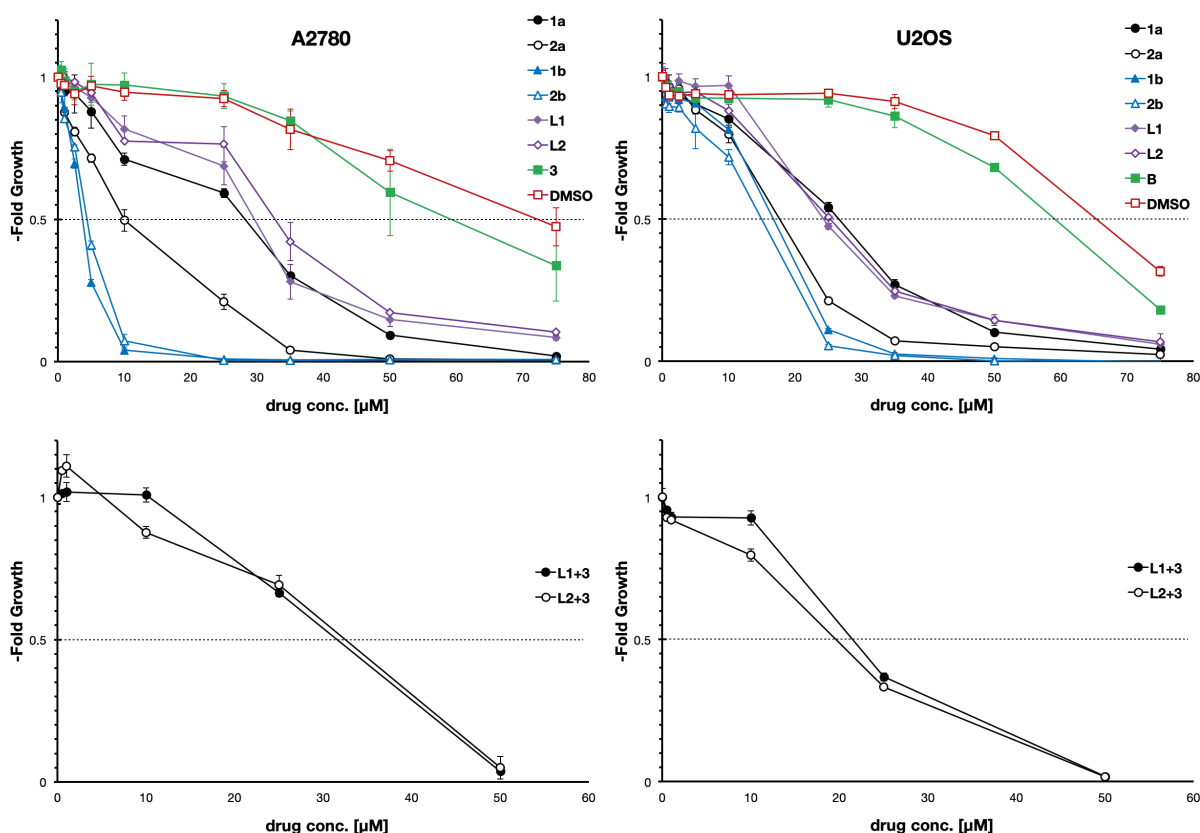


Fig. 27. Concentration dependent effect of compounds on cell viability as obtained in MTT-based cell vitality assays after 72 h incubation. IC_{50} values are reported in Table 4.

These findings are in remarkable accordance with cell-free experiments, wherein pyridine complexes showed the highest hydrolytic stability as well as the highest stabilisation of G4 structures. As cell viability is measured after 72 h, this class of compounds likely exerts its cytotoxic effect during the first hours of exposure, making the more stable pyridine compounds also the most active. As determined by UV-Vis spectroscopy (Fig. 28), **1a** releases the ligand fairly quickly in diluted cell culture medium (<1 h), whereas the release is slower for the more cytotoxic pyridine analogue **1b**. The IC_{50} value of **1a** is only slightly below that of the mixture of ligand **L1** and compound **3**, while **1b** is significantly lower than **1a**.

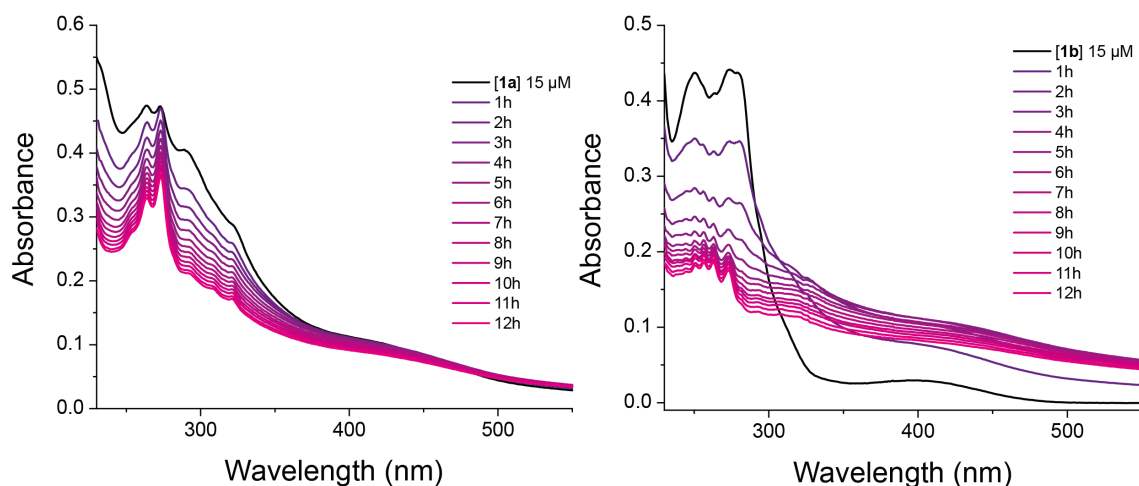


Fig. 28. Time-dependent UV-Vis spectra of **1a** and **1b** (15 μM) in 10-fold diluted cell culture medium.

Tab. 4. IC₅₀ values of naphthalene compounds obtained *via* MTT-based cell vitality assays after 72 h incubation against ovarian cancer cell line A2780 (mean ± SD from three separate experiments) and osteosarcoma cell line U2OS.

	IC ₅₀	
	A2780	U2OS
1a	28.2 ± 0.3	26.6
2a	10.4 ± 1.2	17.7
1b	3.7 ± 0.1	16.8
2b	4.4 ± 0.1	14.9
L1	29.6 ± 1.1	24.3
L2	33.1 ± 1.6	25.3
L1+3	31.6 ± 0.7	21.5
L2+3	32.4 ± 1.0	19.5
3	56.7 ± 8.1	59.1

2.3. Conclusion

Four novel organometallic ruthenium(II) complexes (two chlorido and two pyridine derivatives, respectively) with naphthyl-bearing 1,3-dioxindan-2-carboxamide ligands were investigated as G-quadruplex binders that can simultaneously stack on top of G-tetrads and coordinate to free guanine binding sites of the DNA.

Pyridine complexes **1b** and **2b** are hydrolytically more stable than their analogous chlorido counterparts **1a** and **2a**. This increase in stability drastically enhances the stabilising interaction with G-quadruplexes (as shown *via* FRET melting assays and mass spectrometry studies) as well as the cytotoxic activity towards ovarian cancer cell line A2780. As was shown *via* FRET and MTT-based assays, the complex as a whole is necessary for the maximum effect on G4s and cancer cells. Neither the ligands nor combinations of the ligand with the [Ru^{II}Cl₂(η⁶-*p*-cym)(pyr)] constituent were able to stabilise G4 structures or affect cell viability to the same extent as the full complexes **1b** and **2b**.

3. FLUORESCENT ORGANOMETALLIC RHODIUM(I) AND RUTHENIUM(II) METALLODRUGS WITH 4-ETHYLTHIO-1,8-NAPHTHALIMIDE LIGANDS

3.1. Background

While coordinative binding of metal complexes to DNA nucleobases is the assumed binding mode for many metal-based anticancer drugs, a large number of compounds has been reported to bind to DNA by intercalating between stacks of base pairs, thereby distorting DNA conformation.^{[44], [101]} Planar molecules and metal complexes with planar ligands exhibit rather high affinity for duplex DNA, but other ligand geometries continue to be explored to make the binding mode of these metal complexes more selective. In particular dual binding strategies are of enduring interest, by which a functional moiety with a different DNA binding mode is introduced in the ligand periphery of a metal center, which can itself bind covalently to DNA.^[101]

Noncovalently binding molecules, aside from their therapeutic interest as antiproliferative drugs, can become useful tools as fluorescent imaging agents. A tight association with DNA can alter the emission quantum yield of metal complexes in aqueous solution. This increase of emission intensity can be measured by fluorescence spectroscopy and made visible by confocal fluorescence microscopy, thus making DNA binding observable even in living cells. For example, ruthenium polypyridyl complex $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ is employed as a fluorescent intercalative switch-on probe for duplex DNA.^{[24], [102]}

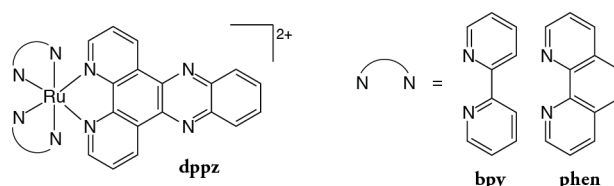


Fig. 29. Ruthenium complexes employed as fluorescent intercalative switch-on probes.

Ruthenium arene complexes in piano stool conformation have been modified as metallointercalators by functionalising the peripheral ligand scaffold with intercalating groups such as 1,8-naphthalimides. Naphthalimides can bind DNA by intercalation and act as topoisomerase II inhibitors;^[103] several compounds of this class have shown excellent antitumor activity and reached clinical trial stages, *e.g.* amonafide and mitonafide.^[104] Incorporating this intercalative binding moiety into metal complexes enhances the medicinal potential of these complexes considerably.

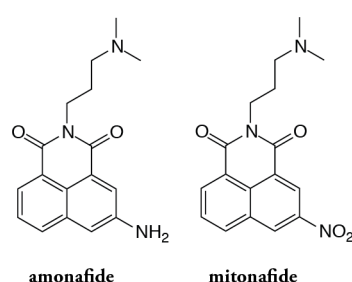


Fig. 30. Naphthalimide-based topoisomerase II inhibitors amonafide and mitonafide.

Kilpin *et al.* reported ruthenium arene complexes in which the 1,8-naphthalimide moiety is tethered in one case *via* the arene ligand to a RAPTA-type complex, and in another linked *via* an alkyl chain to an *N*-bound imidazole ligand.^[105] These pendant naphthalimide complexes exhibited a higher cytotoxic activity than prototype compound RAPTA-C against human ovarian cancer cell lines.

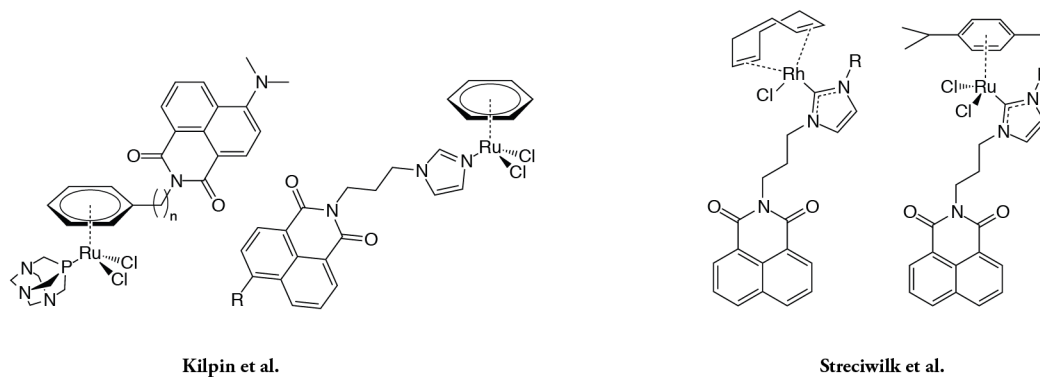


Fig. 31. Different design strategies for metal compounds containing a tethered naphthalimide moiety capable of stacking interaction with G4s.

Following a different approach, a series of complexes was conceived by Streciwilk *et al.* by tethering the naphthalimide moiety to an imidazole ligand to synthesise rhodium(I), ruthenium(II), and copper(I) complexes with antiproliferative properties. In contrast to the complexes described above, the naphthalimide-bearing imidazole ligand is bound by a metal-carbene bond on the C2 position of the imidazole ring.

Metal N-heterocyclic carbene (NHC) complexes are fairly well known for their applications in catalysis, but more recently also came into focus for their possible application as bioactive compounds.^[106] NHCs are neutral ligands with a divalent carbon bound to the metal center; they are readily accessible and provide ease of derivatisation alongside a tendency to form strong metal-carbon bonds. Metal NHC complexes of *i.a.* gold, silver, copper, nickel, platinum, palladium, and ruthenium have been investigated for their antimicrobial and antitumor properties.^[107]

In a previous work by Streciwilk *et al.*^[108], metal (Ru, Rh) arene (*p*-cym, cyclooctadiene COD) naphthalimide-NHC compounds (Fig. 31) were demonstrated to target DNA by combining intercalation of the naphthalimide moiety with coordinative binding by the metal center. The cytotoxic activity against human breast adenocarcinoma and colon carcinoma cells was significantly higher for rhodium NHC complexes (IC_{50} below 10 μ M) than for ruthenium, and only the 3-benzyl-imidazole ligand conferred favourable biological activity to the ruthenium NHC complex. The [(COD)(NHC)RhCl] and [(*p*-cym)(NHC)RuCl₂] complexes were stable in solution and proved to bind efficiently to DNA. Binding constants for the ethyl-imidazole ligand derivatives were obtained by UV-Vis and CD titrations with calf thymus DNA (ctDNA); the DNA binding constant K_b for the rhodium(I) complex was reported to surpass that of the metal-free naphthalimide ligands and the ruthenium(II) analogue by one order of magnitude.

The properties that make naphthalimides good intercalators (mainly the extended planar aromatic system) are the same characteristic features that make small molecules good G-quadruplex binders. This thesis deals with an extension of the work by Streciwilk *et al.* described above. Derivatives of the complexes shown in Figure 31—bearing an ethylthio

substituent in the C4 position of the naphthalimide—were analysed for their photophysical properties, DNA binding capabilities, and effect on selected G4 models. The structural change aimed to improve the fluorescence emission profile of the complexes in order to apply them in cellular imaging studies. While naphthalimides are generally presumed to act on DNA by intercalation, this thesis also seeks to elucidate the stabilising effect of these compounds on selected G-quadruplexes. From the series of compounds synthesised by Wojciech Streciwilk the most biologically active (against cancer cell lines MCF-7 human breast and HT-29 colon adenocarcinoma)^[108] were selected for further testing: [(COD)(NHC)RhCl] with either an ethyl- or benzyl-substituent (**4**, **5**) on the imidazole ring, and [(*p*-cym)(NHC)RuCl₂] with a benzyl side chain (**6**).

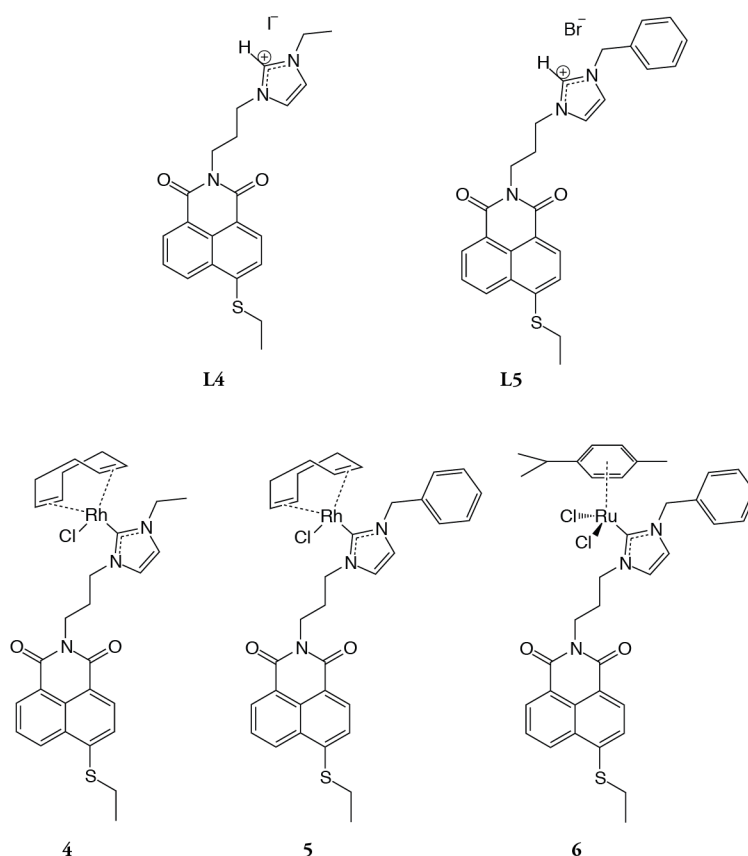


Fig. 32. 4-ethylthio-1,8-naphthalimide compounds studied herein.

3.2. Results and Discussion

The compounds depicted in Fig. 32 were examined for their properties in aqueous solution *via* UV-Vis and fluorescence spectroscopy. Next, the interaction of compounds **4**, **5**, and **6** (and for reference the metal-free ligands **L4** and **L5**) with DNA—either B-form DNA or G4-folded oligonucleotides (ODNs)—was measured *via* FRET melting assays, fluorescence and UV-Vis titrations, and circular dichroism; the affinity of the metal species for DNA base model 9-ethylguanine was gauged *via* mass spectrometry. The synthesis and further biological screening of these compounds is can be found in detail elsewhere.^[108]

3.2.1. B-DNA and G-quadruplex Interaction

3.2.1.1. UV-Vis Titrations

Time-dependent measurements over 24 h were conducted for compounds **4–6**. Spectra of ruthenium compound **6** virtually do not change over time, confirming that the compound is stable in solution. Compounds **4** and **5** showed a tendency to precipitate from solution, observable in the spectra as an overall decrease in absorbance. Since no wavelength shifts or intensity increases were detected for the rhodium compounds, it is reasonable to conclude that despite the precipitation, the NHC ligand is retained in solution.

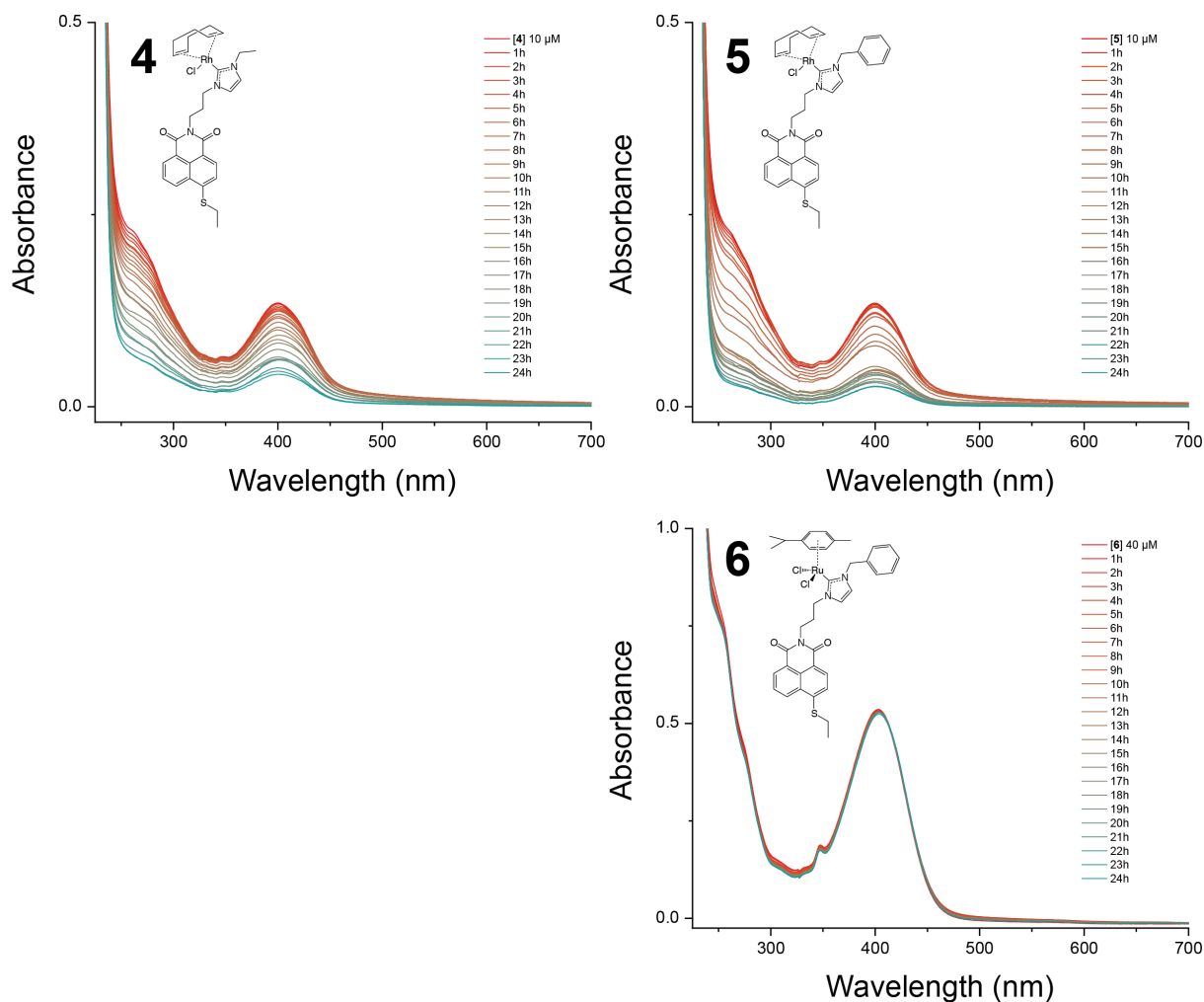


Fig. 33. Time-dependent UV-Vis spectra of compounds **4–6**. Rhodium complexes **4** and **5** (10 μ M) had a strong tendency to precipitate from solution. Ruthenium compound **6** (40 μ M) on the other hand was stable over 24 h and showed no signs of precipitation.

UV-Vis spectra of compounds **4**, **5**, **6**, **L4**, and **L5** were recorded at different concentrations (Fig. 34). All compounds showed a prominent peak at approx. 400 nm—a wavelength at which DNA is transparent, allowing for further titration experiments with DNA (*v.i.*). The relationship of concentration vs. extinction follows a linear trend; Lambert-Beer extinction coefficients are reported in Table 5.

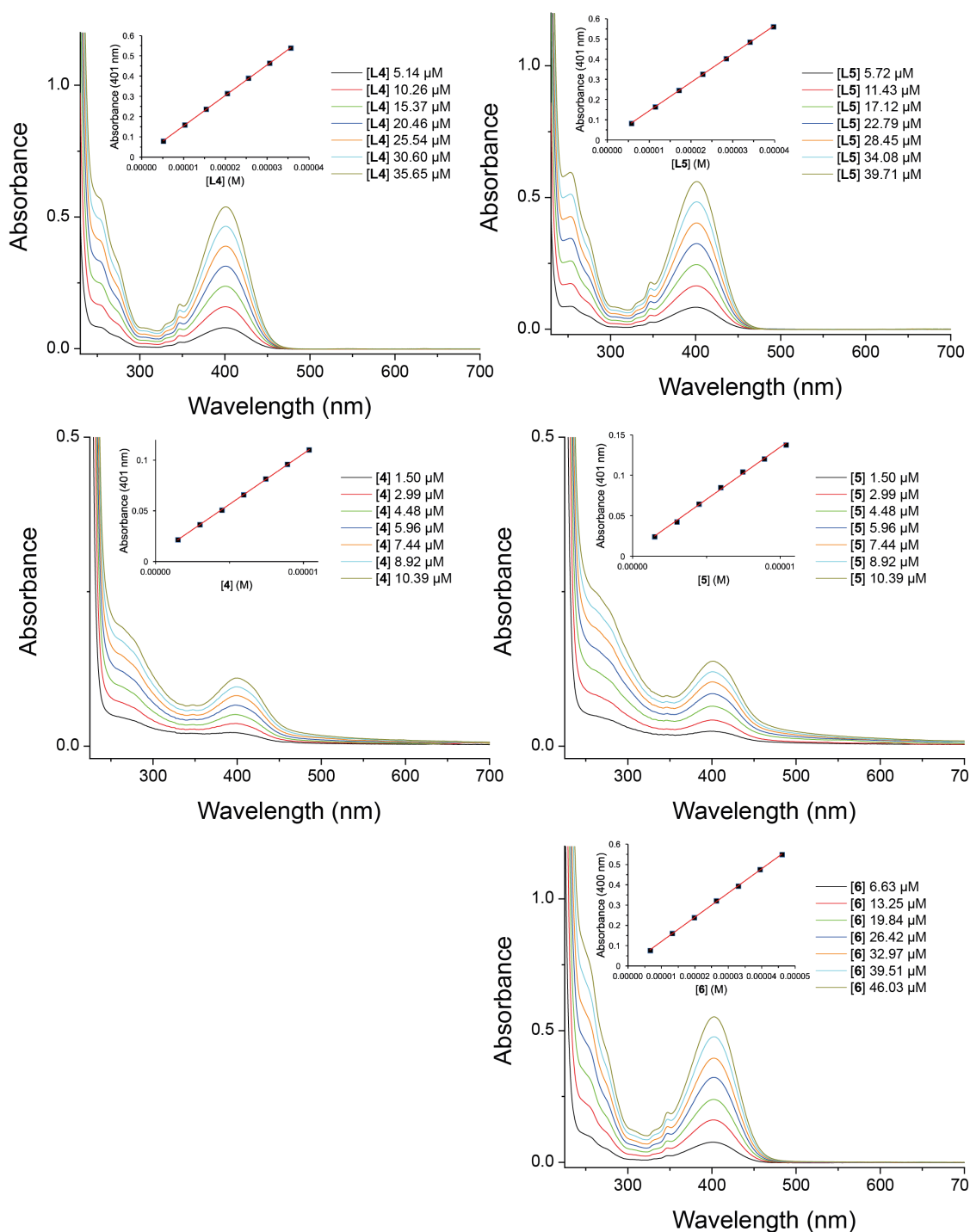


Fig. 34. UV-Vis spectra at different concentrations in buffer (TRIS-HCl 5 mM, KCl 50 mM, pH=7.8, 1% DMSO). Insets: linear trends of extinction *vs.* concentration.

Tab. 5. Lambert-Beer extinction coefficients at $\lambda=401$ nm.

	ϵ_{λ} ($\text{M}^{-1} \text{ cm}^{-1}$)
4	10017 ± 53
5	12908 ± 27
6	11998 ± 83
L4	15023 ± 60
L5	14049 ± 36

Using calf thymus DNA as a B-DNA model, spectra of solutions of compounds **L4**, **L5**, **4–6** were recorded upon incremental increase of DNA concentration (compound concentration was kept constant). Rhodium compounds **4** and **5** again proved difficult to measure and evaluate due to precipitation. Both ligands and metal complex solutions exhibit a strong hypochromic effect with increasing concentrations of DNA. For ligands **L4** and **L5** a bathochromic shift was observed, which was less pronounced for ruthenium compound **6**. These effects are in accord with previous observations on 1,8-naphthalimide derivatives and other intercalators, indicating a strong interaction with DNA, in particular with the metal-free ligands.

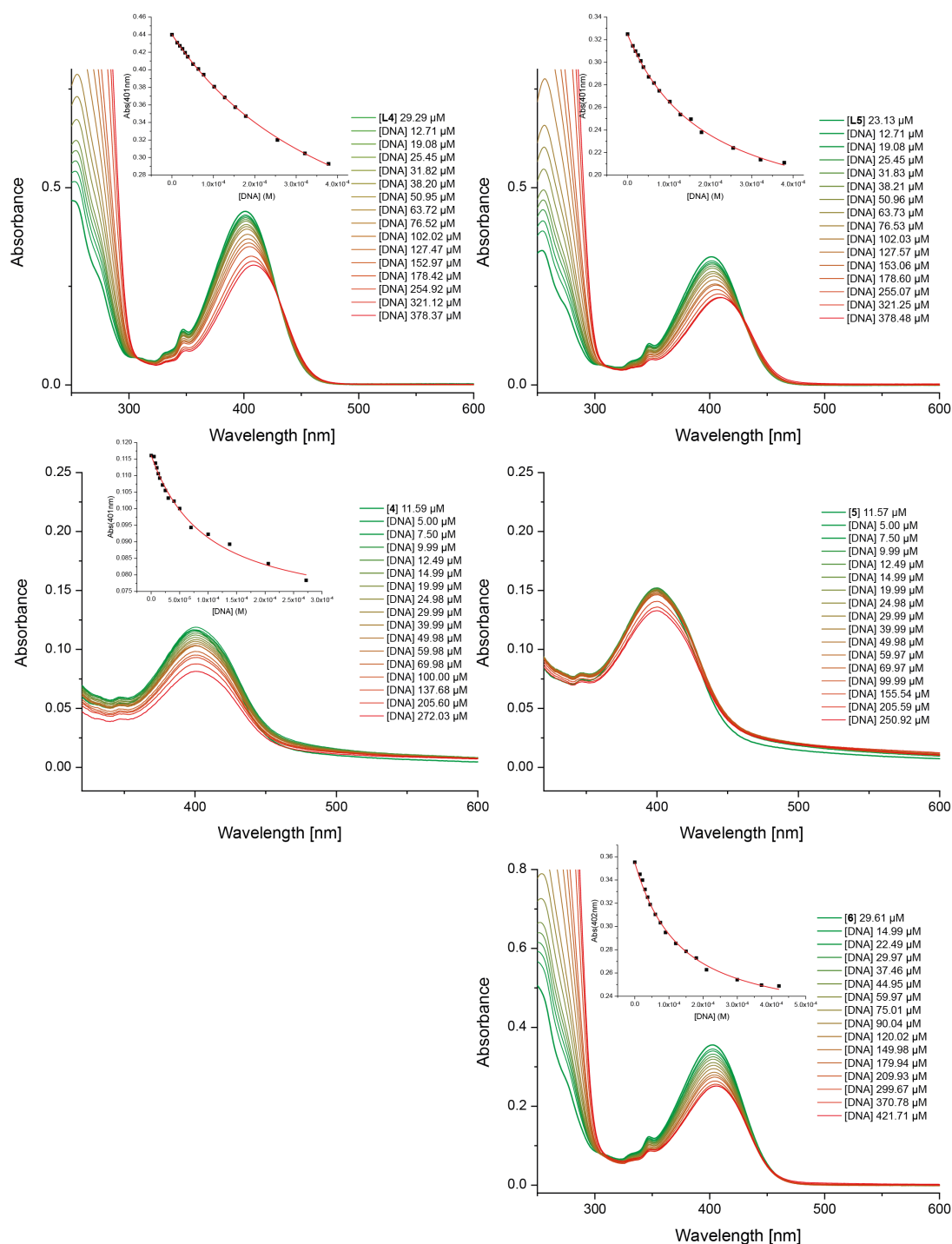


Fig. 35. UV-Vis titrations of metal-free ligands **L4–5** and metal compounds **4–6** with ctDNA. Insets: non-linear curve fitting plots of the extinction at $\lambda = 401$ nm *vs.* the concentration of DNA.

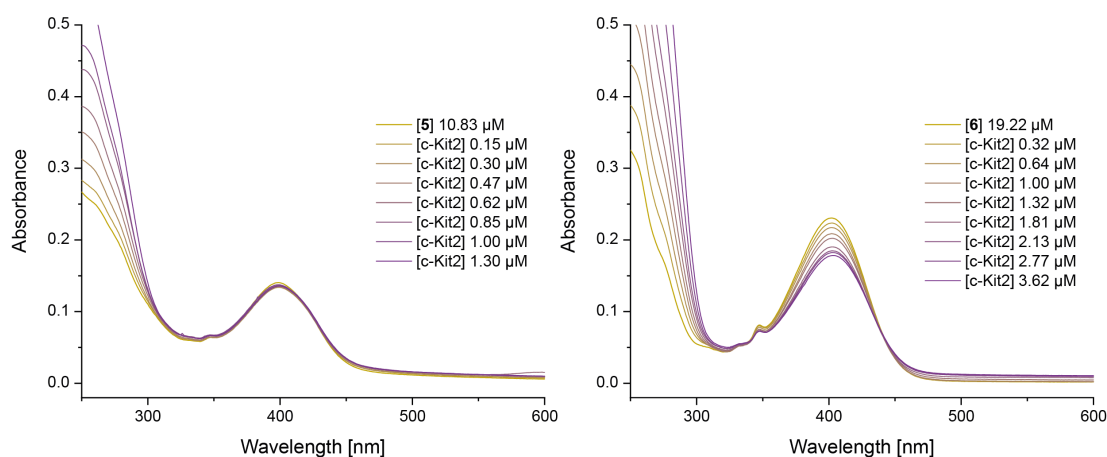


Fig. 36. UV-Vis titrations of compounds **5** and **6** (**4** had to be excluded due to precipitation) with *c-KIT2* quadruplex.

UV-Vis titrations were also conducted with *c-KIT2* quadruplex, which had proven to be selectively stabilized by ruthenium compound **6** as opposed to rhodium compounds **4** and **5**, which were largely unselective (see section 3.2.2.1 on FRET melting assays). While **4** could not be measured due to strong precipitation, increasing amount of G4 had a negligible effect on the absorption spectra of compound **5**. Compound **6** on the other hand exhibits hypochromic behaviour upon addition of *c-KIT2*, confirming a stronger interaction for compound **6**. However, no quantifiable binding constants could be obtained from UV-Vis titrations.

3.2.1.2. Fluorescence Spectroscopy

The fluorescence of 1,8-naphthalimides is generally attributable to photoinduced electron transfer (PET effect) from an electron-rich heteroatom in the sidechain to the electron-deficient tricyclic ring system of the naphthalimide. For the metal NHC complexes in Fig. 31, the fluorescence of the ligands is quenched upon binding to the metal complex. The addition of the electron-rich 4-ethylthio group on the naphthalimide was expected to enhance fluorescence by the PET effect.

Fluorescence spectra of compounds **L4**, **L5**, and complexes **4–6** were recorded at different wavelengths in buffered aqueous solution (Fig. 37). Ligands **L4** and **L5** showed a strong blue emission band at 503 nm when excited at 400 nm. Introduction of the metal center led to a decrease in emission intensity. This quenching effect was more pronounced for rhodium derivatives **4** and **5** than for ruthenium derivative **6**.

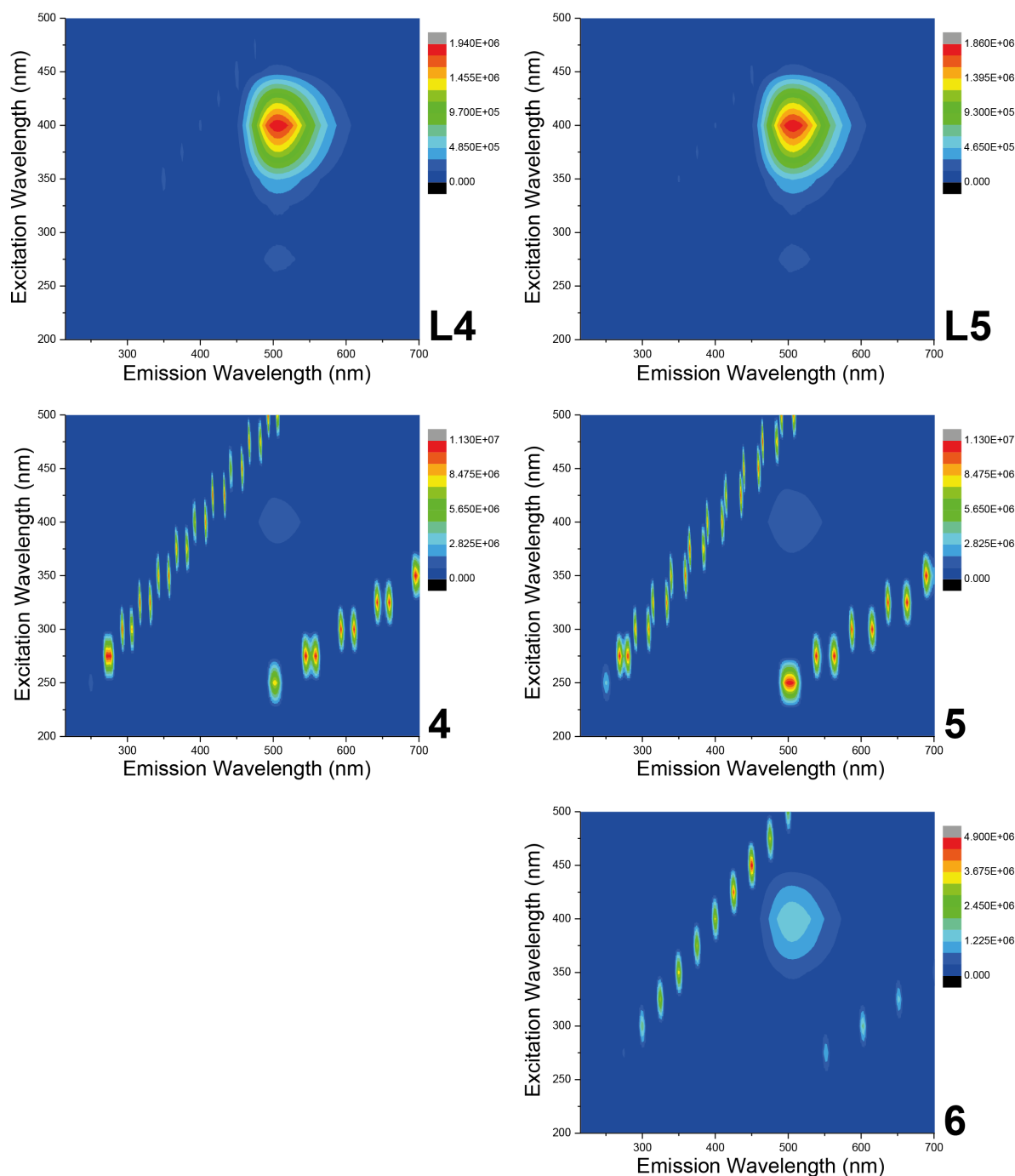


Fig. 37. 2D excitation-emission spectra of compounds **L4–5** and **4–6**. Diagonal ridges represent first and second order Rayleigh scattering.

Interaction between the compounds and DNA was studied in titration experiments tracking the change of fluorescence emission upon addition of DNA. Oligonucleotide *ds26* was used as a DNA model in fluorescence titrations (Fig. 38), alongside a G-quadruplex sequence (*c-KIT2*) from the *c-KIT* oncogene (Fig. 39). This G4 was selected after FRET measurements (discussed in detail below) indicated a selectivity of ruthenium complex **6** for *c-KIT2* over *dsDNA*.

Increasing amounts of ODN lowered the emission intensity of naphthalimide compounds in all cases. Through non-linear regression analysis (fitted through the binding model proposed by Thordarson^[109]) of the correlation between fluorescence intensity and ODN concentration, binding constants (K_b , M^{-1}) could be obtained and are presented in Table 6.

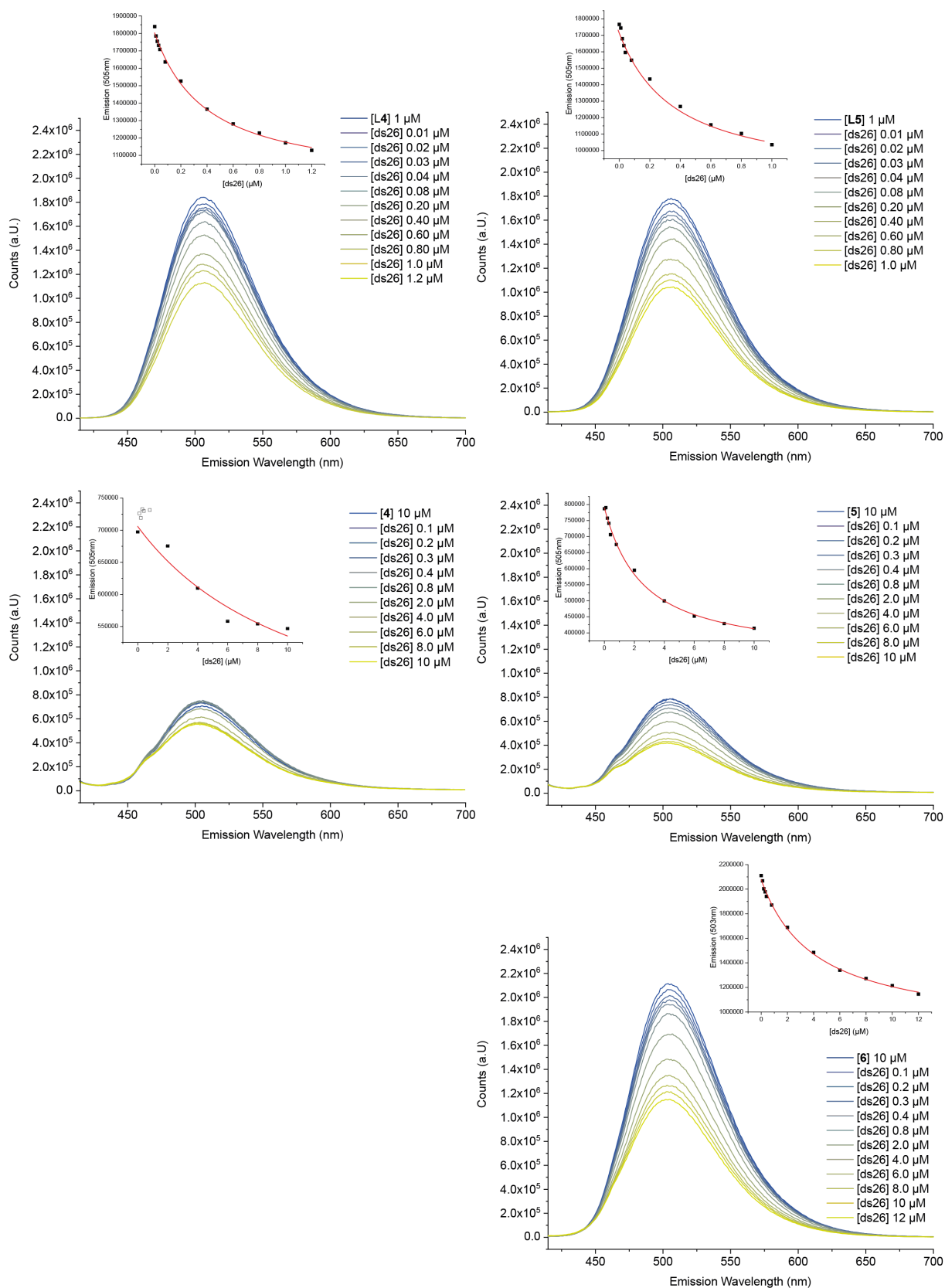


Fig. 38. Fluorescence spectroscopy titrations of B-DNA model *ds26* with compounds **L4-5** (1 μM) and **4-6** (10 μM). Insets: non-linear curve-fitting plots used to calculate binding constants presented in Table 6.

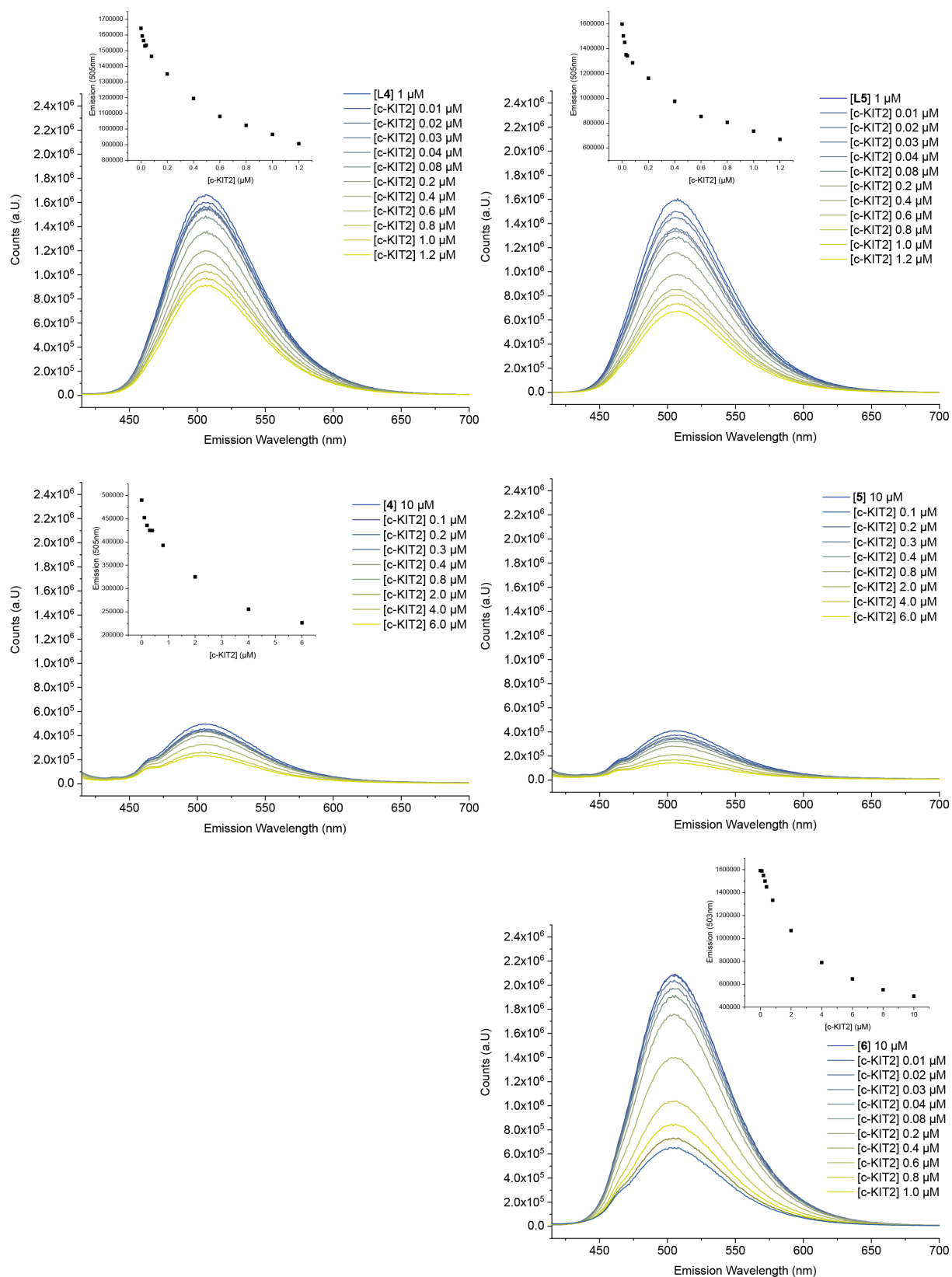


Fig. 39. Fluorescence spectroscopy titrations of G4 oligonucleotide *c-KIT2* with compounds **L4–5** (1 μM) and **4–6** (10 μM). Insets: non-linear curve-fitting plots used to calculate binding constants presented in Table 6.

Tab. 6. K_b (M^{-1}) binding constants obtained by fluorescence titrations.

	<i>ds26</i>	<i>c-KIT2</i>
4	$(3.4 \pm 0.4) \times 10^3$	$(2.3 \pm 0.7) \times 10^4$
5	$(1.7 \pm 0.3) \times 10^4$	$(5.7 \pm 0.9) \times 10^4$
6	$(1.0 \pm 0.1) \times 10^4$	$(1.6 \pm 0.1) \times 10^4$
L4	$(1.0 \pm 0.1) \times 10^5$	$(9.3 \pm 0.9) \times 10^4$
L5	$(1.1 \pm 0.3) \times 10^5$	$(1.6 \pm 0.5) \times 10^5$

K_b values were highest for metal-free ligands **L4** and **L5**, roughly one order of magnitude above metal compounds **4–6**. In this model assay, rhodium complexes **4** and **5** showed a slight preference for *c-KIT2* over *ds26*, while ruthenium compound **6** in this assay did not mirror the selectivity for *c-KIT2* displayed in FRET assays and UV-Vis titrations.

3.2.1.3. Mass Spectrometry

Besides non-covalent binding of DNA by the naphthalimide moiety, ruthenium and rhodium metal complexes are expected to metallate DNA nucleobases, the preferred binding site being the guanine N7 position. Compounds **4–6** were incubated with an excess of model nucleobase 9-ethylguanine and subsequently analysed *via* high resolution mass spectrometry (Fig. 40).

Compound-ethylguanine adducts could be confirmed for all metal compounds after loss of one chlorido ligand. Notably, aggregates of 4 units of 9-EtG and one Na^+ ion were found in all mass spectra (739 m/z), as 9-EtG forms G-tetrads, the base unit of a G-quadruplex structure, with monovalent ions in solution.^[98]

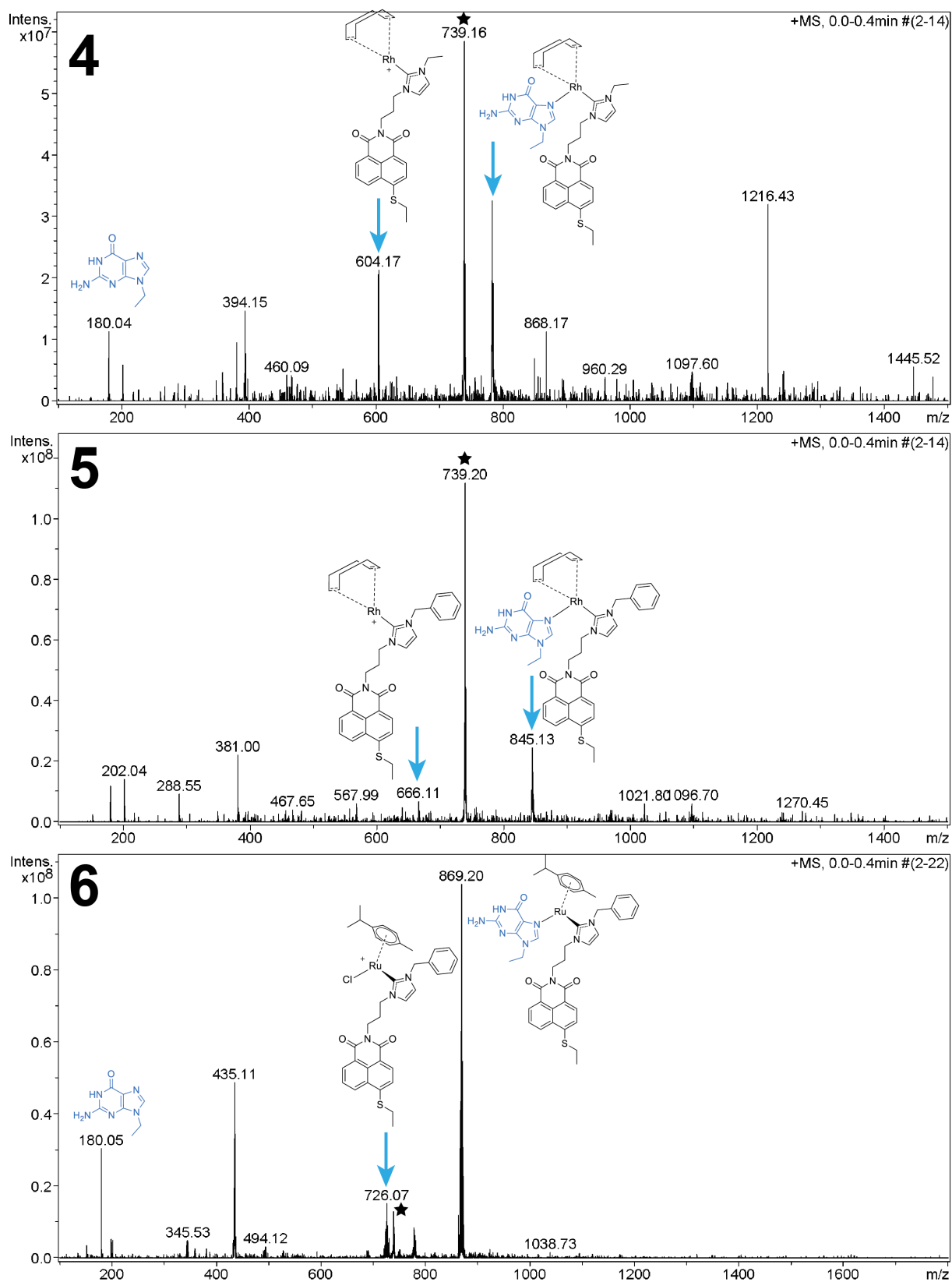


Fig. 40. Mass spectra of compounds **4–6** (0.2 mM) and 9-EtG (0.6 mM) in 20% MeOH/H₂O. All compounds form stable adducts with 9-EtG. Highlighted peaks (739 m/z ★) correspond to aggregates of four molecules of 9-EtG (a G-tetrad) around trace amounts of sodium ions.^[98]

3.2.2. G-quadruplex Stabilisation

3.2.2.1. FRET melting assays

The capacity of compounds **4–6** as G4 binders was further assessed in a FRET melting assays on model oligonucleotides (ODNs) for B-DNA (*dsDNA*), telomeric G4 (*hTelo*), and oncogene promoter G4s (*hTERT*, *BCL2*, *c-KIT1*, *c-KIT2*). Relative stabilisation of the ODN is indicated by an increase in melting temperature $T_{1/2}$; TMPyP4 was used as a non-selective, strongly stabilising control drug. Obtained values of $\Delta T_{1/2}$ are reported in Table 7.

Tab. 7. $\Delta T_{1/2}$ (°C) values for ODNs (0.2 μ M) upon interaction with 2–4 μ M G4 binders. x = not performed; * = not reported due to precipitation. Uncertainty ≤ 0.5

	conc.	<i>dsDNA</i>	<i>hTelo</i>	<i>hTERT</i>	<i>BCL-2</i>	<i>c-KIT1</i>	<i>c-KIT2</i>
TMPyP4	1 μ M	28.1	31.9	18.0	16.7	29.6	15.7
L4	2 μ M	0.0	2.3	5.2	0.7	9.0	x
	4 μ M	1.1	5.7	6.5	2.2	10.6	x
L5	2 μ M	4.9	10.4	6.7	3.3	10.7	x
	4 μ M	7.7	18.1	9.6	5.0	17.0	x
4	2 μ M	4.9	2.0	5.0	3.7	11.8	2.2
	4 μ M	10.8	9.4	10.9	13.9	15.3	6.1
5	2 μ M	9.9	6.1	7.8	9.7	11.1	4.2
	4 μ M	22.8	21.6	*	17.0	*	10.2
6	2 μ M	-2.2	-2.0	0.6	-0.1	-2.9	2.2
	4 μ M	0.0	-0.5	5.7	8.3	-4.0	15.0

3-Benzyl-imidazole ligand **L5** proved to have stronger interaction with both *dsDNA* and G4s than the 3-ethyl derivative **L4**. This trend is mirrored by rhodium complexes **4** and **5**, which both exhibit remarkably strong stabilisation of ODNs (in some cases even too high to be measured adequately), but are not selective of G4s over *dsDNA*. The less cytotoxic ruthenium compound **6** on the other hand shows little to no stabilising effect on most ODNs, especially not *dsDNA*; yet when applied at higher concentration (4 μ M), compound **6** exerts a strong and specific stabilising effect on *c-KIT2* in the range of control compound TMPyP4. This G4 sequence was subsequently selected for use in other binding studies, in particular fluorescence, UV-Vis and circular dichroism experiments.

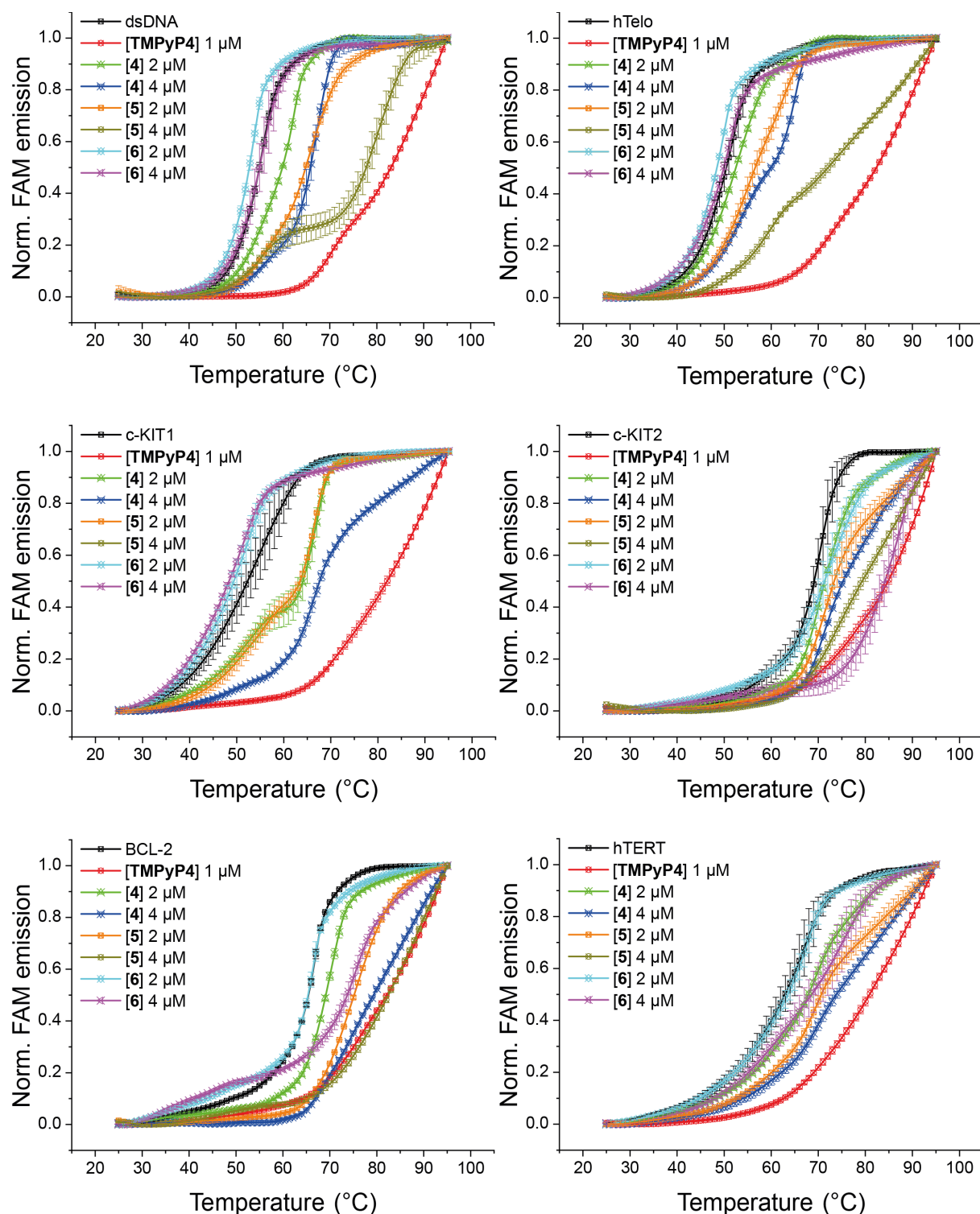


Fig. 41. FRET melting profiles of ODNs (0.2 μM) treated with 2–4 μM of studied compounds in 60 mM potassium cacodylate buffer (pH 7.8). $\Delta T_{1/2}$ values are reported in Table 7.

3.2.2.2. Circular Dichroism

To better understand the binding modes of our metal compounds to DNA and G4s, changes in the circular dichroism of ctDNA and *c-KIT2* were evaluated upon addition of increasing amounts of naphthalimide compounds. Unfortunately, rhodium complex **4** had to be excluded from these experiments, as the precipitation interfered too strongly with the measurement.

Circular dichroism spectra of ctDNA in buffered solution afforded the typically observed bands, a positive peak in ellipticity at approx. 275 nm and a negative one at approx. 245 nm. Addition of metal compounds **5** and **6** only moderately affected these bands, but presence of ruthenium compound **6** gave rise to an induced CD band (ICD) at approx. 425 nm. Presence of rhodium derivative **5** led to a fairly weak ICD at 400 nm. These results are in good agreement with an intercalative mode of binding for ctDNA.

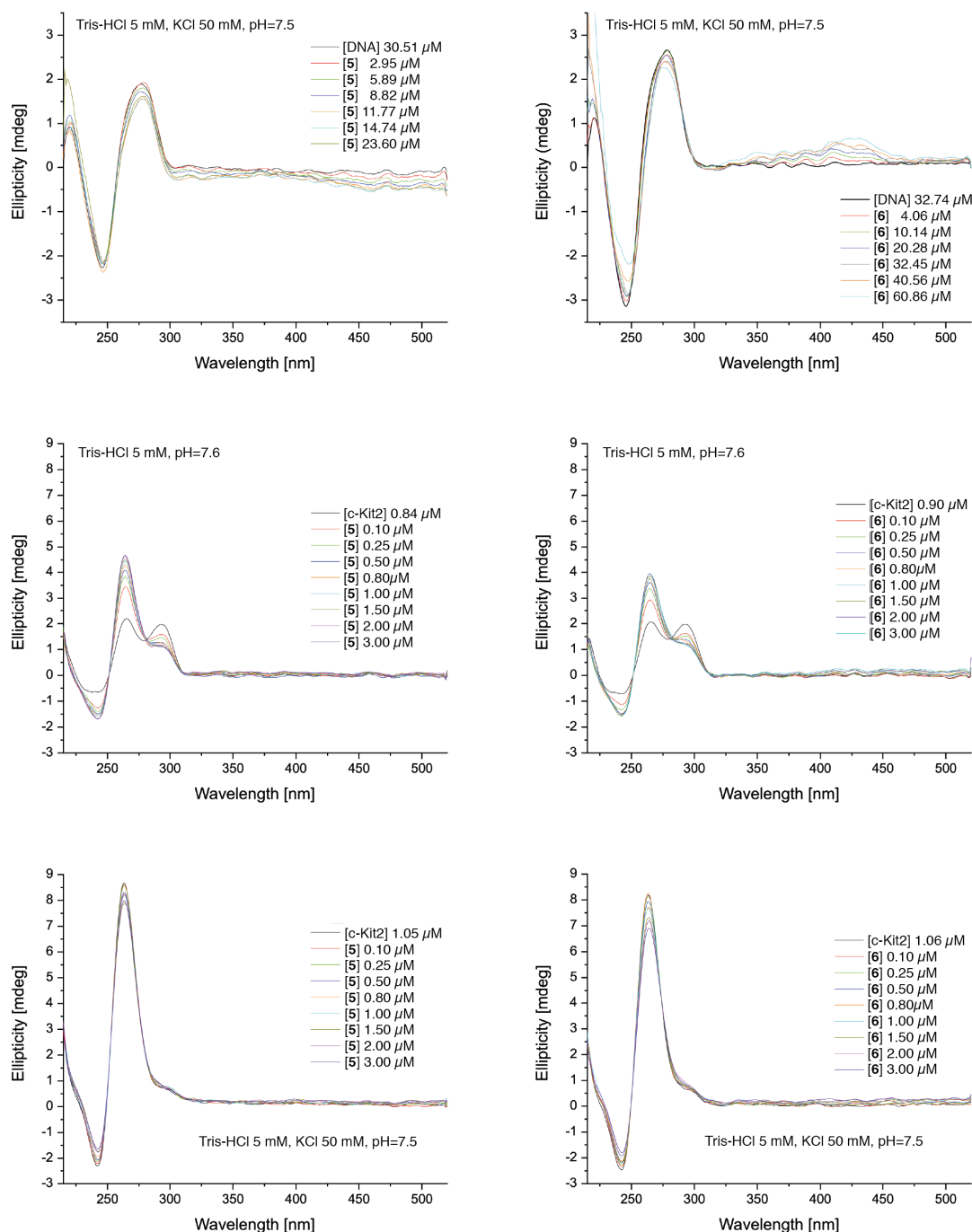


Fig. 42. CD titration-style spectra of ctDNA (top) and *c-KIT2* exposed to increasing concentrations of compounds **5** and **6**. Spectra in the absence of potassium ions in solution (middle: TRIS-HCl 5 mM, pH 7.6) show a transition of antiparallel strand topology towards parallel with an increase of binder concentration. G4 that was annealed in the presence of potassium (bottom: TRIS-HCl 5 mM, KCl 50 mM, pH 7.6) shows almost no changes upon addition of binder.

The characteristic CD bands of *c-KIT2* in potassium buffered solution declare its parallel G4 conformation. Addition of metal compounds **5** and **6** lead to little to no changes in the shape of the CD spectrum for *c-KIT2*, indicating that the G4 retains its parallel topology. This in turn suggests a top-stacking binding mode for compounds **5** and **6**, in which the naphthalimide moiety stacks on top of the outermost G-quartet without further perturbing the general G4 structure.

As has been established, compounds **4–6** do not induce any conformational changes in the fully folded structure of *c-KIT2* G-quadruplex. However, an interesting interaction could be observed between compound **5** and **6** and *c-KIT2* that was annealed in the absence of potassium and thus in conditions that are detrimental to G4 stability.

The CD spectrum of *c-KIT2* in potassium-free buffer (Fig. 42 middle) shows a markedly changed profile from that previously observed (Fig. 42 bottom). The positive band at 265 nm, typical of a parallel conformation of G4, denotes markedly less intense ellipticity than in potassium-buffered solution. Additionally, a second positive band at 295 nm is present, one that is characteristic for antiparallel G4 conformation. Incremental addition of compounds **5** and **6** (**4** had to be excluded for problems arising from precipitation) led to sharp increase of the band at 265 nm accompanied by a corresponding decrease of the band at 295 nm. This effect of compounds **5** and **6** on *c-KIT2* indicate that not only do they stabilise the G4 structure of *c-KIT2*, but can also induce the formation of the G4 structure in potassium-free solution.

3.3. Conclusion

Three novel naphthalimide-bearing metal arene NHC complexes were characterised for their solution phase spectral properties and their binding interaction with DNA.

The 4-ethylthio substituent on the naphthalimide moiety ameliorated the quenching effect previously observed upon introduction of the metal arene for both rhodium and ruthenium compounds. Previously developed analogous complexes lacking the 4-ethylthio group did not possess sufficient fluorescence emission to allow further fluorescence based studies. Compounds **4–6** on the other hand have since been applied in cellular imaging studies.^[108]

As elaborated elsewhere^[108], rhodium(I) compounds **4** and **5** proved to be more cytotoxic than their ruthenium counterparts. A dependency of the ligand was mostly observed in a higher activity of the more lipophilic 3-benzyl-imidazole based ligand system. Antiproliferative effects were also reported for several Gram-positive bacteria.

FRET melting assays showed rhodium complexes **4** and **5** stabilising DNA models strongly yet indiscriminately, with **5** exhibiting a moderately stronger effect. Prospects of further applications however have to take into consideration the unfavourable solution phase behaviour of these compounds, as they showed a strong tendency to precipitate from solution in different experimental set-ups. Time-dependent UV-Vis measurements nevertheless indicated that, despite the precipitation, the NHC ligand is preserved.

Ruthenium(II) compound **6** was notably less cytotoxic than its rhodium(I) derivative; yet this was the only compound found to have a selective stabilising effect of G4 model *c-KIT2* over *dsDNA* in FRET melting assays. The ruthenium arene fragment also had a weaker quenching effect than rhodium on the fluorescence emission of the naphthalimide ligand.

UV-Vis, Fluorescence, and CD titration experiments of compounds **4–6** with different DNA models suggest an intercalative binding mode for B-DNA and a top-stacking mechanism for G-quadruplexes. Binding constants obtained through fluorescence titrations revealed the strongest affinity for DNA by the metal-free ligands **L4** and **L5**, with rhodium compounds showing a binding preference for *c-KIT2* in this assay. The selectivity of ruthenium compound **6** observed in FRET assays could not be confirmed by quantitative comparison of binding constants.

Most interestingly, while neither compound perturbed the parallel G4 structure of *c-KIT2* in CD experiments, compounds **5** and **6** were able to induce the formation of parallel G4 in potassium-free solution.

In summary, this class of naphthalimide bearing metal NHC complexes combine the properties of its fluorescent DNA-intercalating ligand with the metallation aspect of the organometallic center, representing a novel framework for dual binding strategies between metallodrugs and DNA.

4. EXPERIMENTAL SECTION

4.1. Materials and Methods

Compounds **L1**, **L2**, **1a/b**, and **2a/b** were prepared by Stephan Mokesch at the Institute of Inorganic Chemistry, University of Vienna. The synthesis, characterisation via $^1\text{H-NMR}$, MS, elemental analysis data, X-ray structures, *etc.*, have been reported elsewhere.^{[96], [110]} All solutions of compounds **1b** and **2b** were handled in the dark to preserve the pyridine ligand.

Compounds **L4**, **L5**, **4**, **5**, and **6** were prepared by Wojciech Streciwilk at the Institute of Medicinal and Pharmaceutical Chemistry, Technische Universität Braunschweig. Synthesis, elemental analysis data, and characterisation via $^1\text{H-NMR}$ and MS can be found elsewhere.^[108]

All solvents and reagents were purchased from commercial suppliers. Milli-Q[®] water was used in the preparation of buffers. pH values were measured using a Mettler Toledo pH meter.

Oligonucleotides (ODN) were purchased from IDT Integrated DNA Technologies in HPLC purity grade. ODNs used in FRET studies were labelled with FRET label couple FAM-TAMRA. Nucleotide sequences are given in Table 8.

Tab. 8. 5'-3' sequences of oligonucleotides used in FRET assays and titrations.

ODN	Sequence
<i>dsDNA</i>	TATAGCTATA-(CH ₂ -CH ₂ -O) ₆ -TATAGCTATA
<i>ds26</i>	CAA TCG GAT CGA ATT CGA TCC GAT TG
<i>hTelo</i>	AGG GTT AGG GTT AGG GTT AGG G
<i>c-KIT1</i>	AGG GAG GGG GCT GGG AGG AGG G
<i>c-KIT2</i>	CGG GCG GGC GCG AGG GAG GGG
<i>hTERT</i>	GGG GGC TGG GCC GGG GAC CCG GGA GGG GTC GGG ACG GGG CGG GG
<i>BCL-2</i>	AGG GGC GGG CGC GGG AGG AAG GGG GCG GGA GCG GGG CTG

4.2. UV-Vis Spectroscopy

UV-VIS absorption spectra were recorded on a PerkinElmer LAMBDA 35 double beam spectrophotometer, equipped with a Peltier temperature controller. Measurements were carried out at 25°C using 1 cm path-length quartz cuvettes.

Stability in solution. Compounds **1a/b** and **2a/b** were dissolved in acetonitrile and diluted in designated buffer conditions to the desired concentration, with the final content of acetonitrile kept below 1%. Lambert-Beer extinction coefficients were determined in 2 mM MES buffer at pH 6 by adding compound stock solution in acetonitrile in small increments to the designated concentrations.

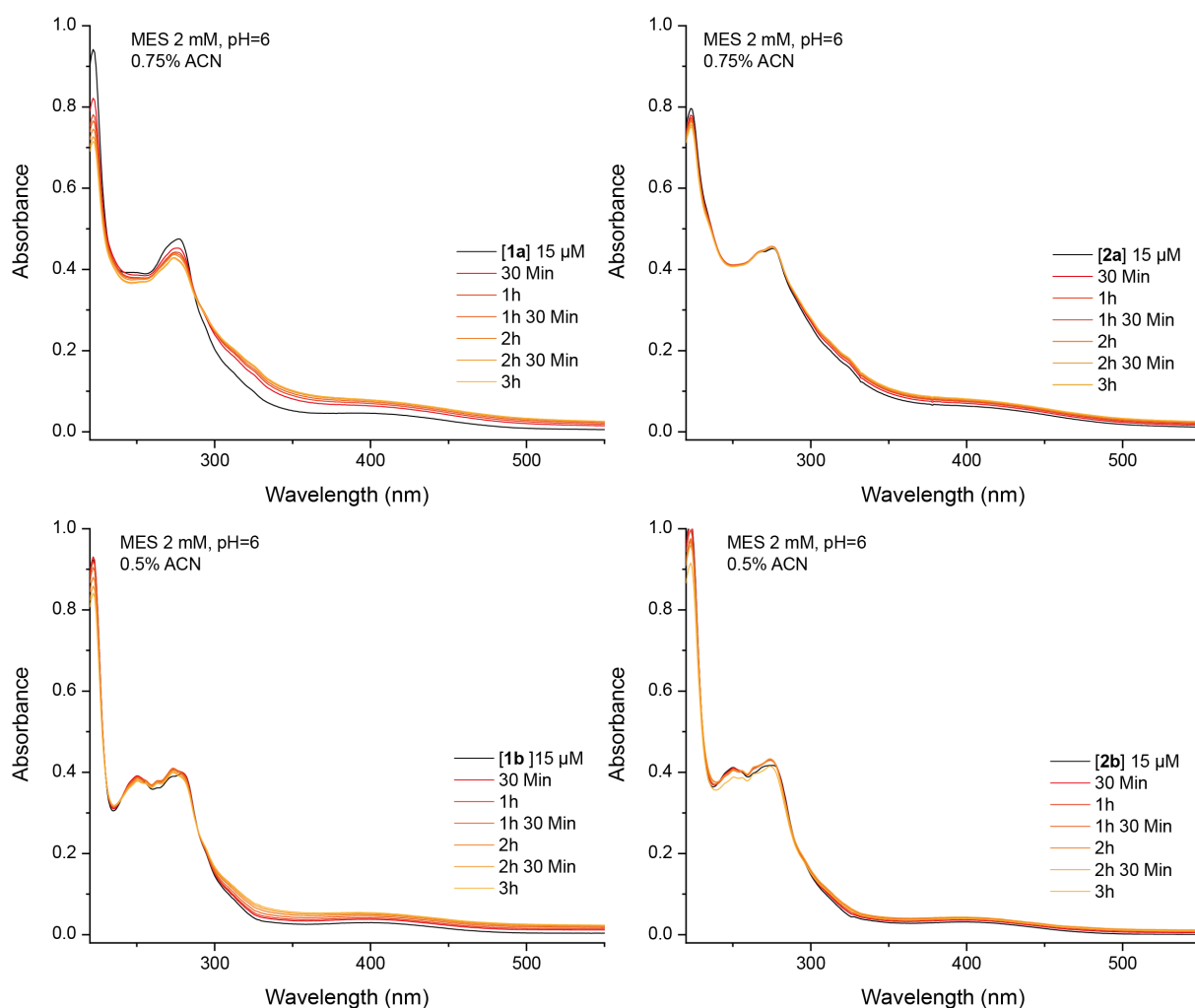


Fig. E1. Time-dependent spectra of compounds **1a/b** and **2a/b** in MES buffered solutions (2 mM, pH 6). Spectra remained virtually unchanged after 12 h.

Time-dependent (once every hour for 24 h) and concentration-dependent spectra of compounds **L4**, **L5**, **4–6** were measured in solutions of 5 mM TRIS-HCl, 50 mM KCl (pH 7.8). Compound stock solutions were prepared in DMSO, the final concentration of DMSO was less than 1%. Lambert-Beer extinction coefficients were calculated by linear regression.

UV-VIS DNA titrations. Lyophilised ctDNA (Sigma Aldrich) was resuspended in 1 mM TRIS-HCl buffer (pH 7.5). DNA base concentration was calculated from the absorption at 260 nm (molar absorption coefficient $\epsilon_{260\text{nm}} = 6600 \text{ M}^{-1}\text{cm}^{-1}$). Oligonucleotide strand concentration was calculated from the extinction coefficient provided by the manufacturer. ODNs were diluted in potassium-containing buffer to desired concentration and annealed by heating to 90°C for 5 Min before being allowed to slowly cool to room temperature overnight. Compound stock solutions (1 mM) were prepared in DMSO, with the final percentage of DMSO in measured solutions less than 1%. Titrations were performed by adding aliquots of DNA (ctDNA, *c-KIT2*) to a solution of the compounds in buffer (TRIS-HCl 5 mM, KCl 50 mM, pH 7.8) at constant compound concentration (kept constant by adding an equal-volume aliquot of doubly concentrated compound solution to the mixture). K_b values were obtained by fitting absorption–concentration data plots through the binding model proposed by Thordarson.^[109] Non-linear curve fitting was carried out in Origin 9.5 utilising a script as reported by Hargrove *et al.*^[111] Rhodium compounds

4 and **5** precipitated from solution at higher DNA concentrations and could not be analysed properly.

4.3. NMR Spectroscopy

NMR spectra were recorded at 25°C on a Bruker FT-NMR spectrometer Advance III™ 500 MHz. Chemical shifts are presented in parts per million (ppm) referenced to residual solvent peaks.

Stability in solution. Compound stock solution of **1b** (1875 µM) was prepared in d₆-DMSO. ¹H-NMR spectra were recorded of a mixture of 8 vol% compound stock, 10 vol% D₂O, and 82 vol% working buffer, with the final concentration of **1b** being 150 µM. Aqueous buffers used in this experiment were chosen to cover a pH range from acidic to mildly alkaline. These were: 10 mM sodium citrate (pH 3.4), 10 mM TRIS-HCl (pH 7.5), 2 mM MES (pH 6). Spectra were recorded directly after mixing, after 30 min, and after 24 h. FRET buffer (potassium cacodylate) was measured in a similar fashion over 2 h (Fig. E2).

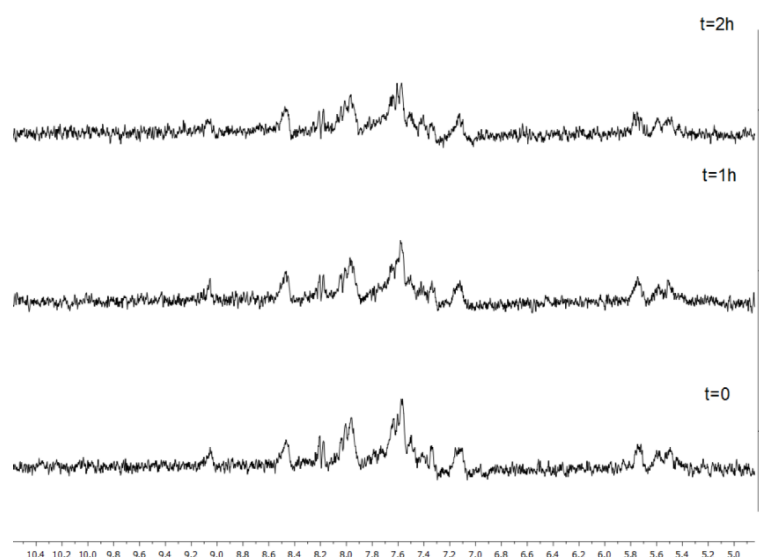


Fig. E2. Spectra of **1b** in 60 mM potassium cacodylate buffer (pH 7.4, 8% d₆-DMSO, 10% D₂O) directly after mixing, after 1 h, and after 2 h, respectively. The spectra do not change significantly over the 2h-timeframe of FRET experiments.

4.4. FRET Melting Assays

FRET studies were performed on an Applied Biosystems® 7500 Real-Time PCR Cycler equipped with a FAM filter (λ_{ex} =492 nm, λ_{em} =516 nm). Lyophilised oligonucleotides were dissolved in IDTE buffer (10 mM TRIS, pH 7.5, 0.1 mM EDTA, Integrated DNA Technologies) to a concentration of 100 µM stock solutions. ODN stock solutions were then diluted in 60 mM potassium cacodylate buffer (pH 7.4) to a concentration of 400 nM prior to annealing. To ensure G4 folding, diluted oligo solutions were annealed by heating to 90 °C for 5 min and allowed to slowly cool to room temperature overnight. All FRET experiments were performed in 96 well plates (total volume per plate: 30 µL) with a final ODN concentration of 200 nM. Compounds were dissolved in organic solvent (1 mM in DMSO or ACN as specified) and diluted in 60 mM potassium cacodylate to desired the concentration. Final concentration of organic solvent was below 1%.

The PCR cycler ramp temperature program operated on 1°C increase every 30 seconds starting at 25°C and heating to 95 °C. Measurements were recorded at increments of 1°C. FAM emission data was normalised (0–1) for better comparison between data sets. $\Delta T_{1/2}$ values are given as the difference of $T_{1/2}$ of the ODN-compound-mixture and $T_{1/2}$ of the ODN in absence of compound, where $T_{1/2}$ is the temperature at which normalised FAM emission reached 0.5. All measurements were made in duplicates. Data analysis was carried out using Origin 9.5 (OriginLab Corp.).

Incubation time. FRET assays were generally measured 2 h after mixing compound solutions with ODN solutions. Compounds **1a/b** and **2a/b** were incubated for only 1 h for the experiment shown in Figure E3 (melting temperatures and incubation times are listed in Table 9).

Table 9. $\Delta T_{1/2}$ (°C) values for ODNs (0.2 μ M) upon interaction with 4 μ M G4 binders. Uncertainty is ≤ 0.5

	<i>dsDNA</i>	<i>hTelo</i>	<i>c-KIT1</i>	<i>hTERT</i>	<i>BCL-2</i>
TMPyP4	27.2	31.4	27.8	19.7	19.2
1a (2 h)	4.0	0.9	-0.1	2.7	1.2
1a (1 h)	4.1	4.9	12.2	1.2	4.0
1b (2 h)	10.2	11.0	20.9	9.5	5.1
1b (1 h)	3.4	15.9	21.6	13.1	8.3
2a (2 h)	10.4	3.0	1.8	2.6	3.7
2a (1 h)	2.2	3.5	17.2	5.1	3.7
2b (2 h)	9.7	6.5	15.7	9.4	3.8
2b (1 h)	6.1	21.8	21.5	11.5	10.4
L1+3 (2 h)	6.4	-0.4	7.0	2.4	5.7
L1+3 (1 h)	1.9	3.2	16.4	2.6	4.9
L2+3 (2 h)	8.6	1.8	7.3	0.7	5.5
L2+3 (1 h)	3.4	1.1	14.2	1.0	3.4
3 (2 h)	4.4	0.4	-3.5	1.1	-0.6
3 (1 h)	1.2	2.0	5.0	2.2	-1.1

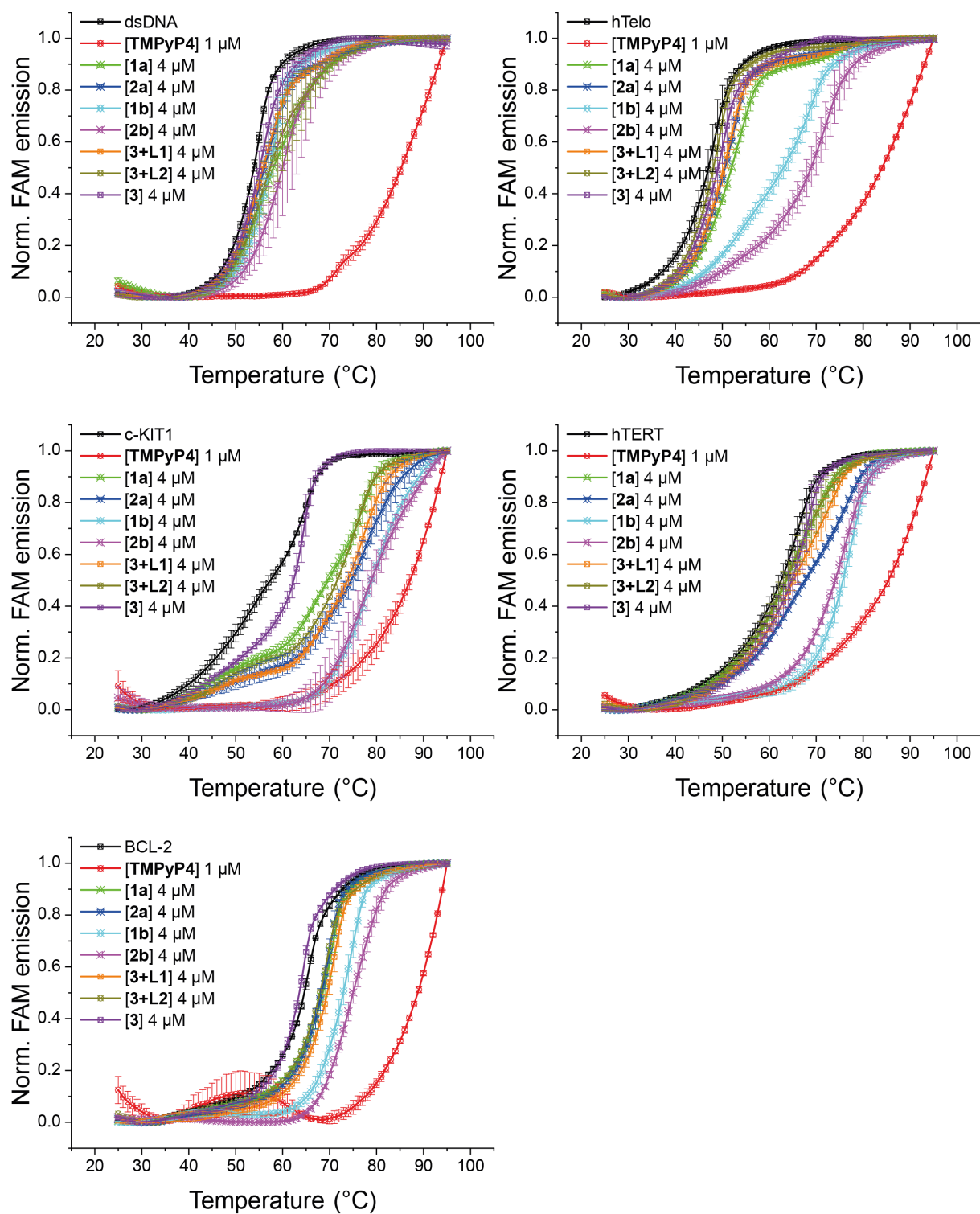


Fig. E3. FRET melting profiles of ODNs (0.2 μM) incubated for 1 h with compounds at the indicated concentrations (potassium cacodylate 60 mM, pH 7.4).

FRET Experiments involving Irradiation. ODN solutions were prepared as described above. Compound stock solutions of **1b/2b** in ACN were diluted with 60 mM potassium cacodylate (pH 7.4) and irradiated with blue light for 1 minute prior to mixing with the ODNs.

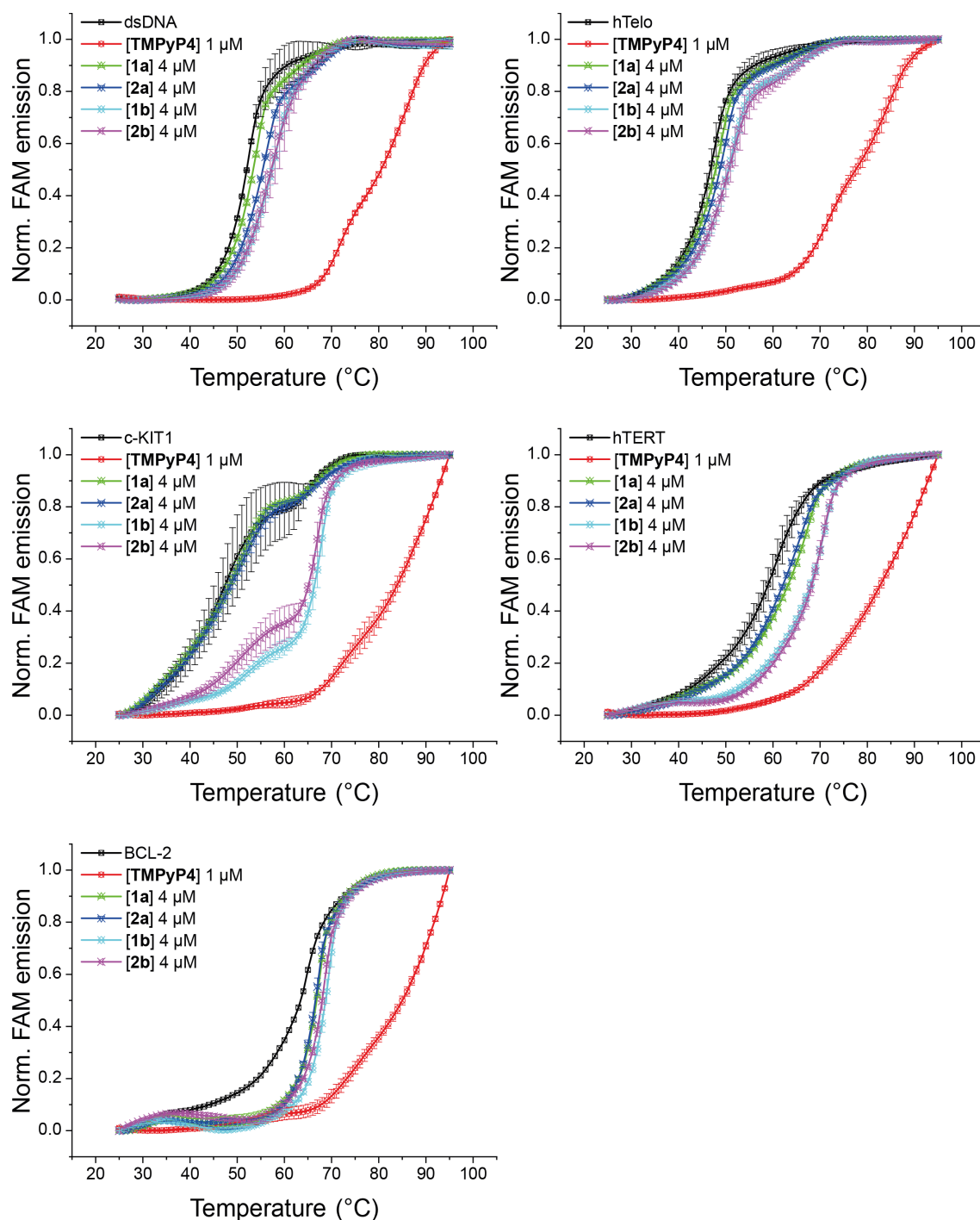


Fig. E4. FRET melting profiles of ODNs (0.2 μ M) treated with irradiated samples of compounds **1a/b** and **2a/b** in 60 mM potassium cacodylate buffer (pH 7.4).

4.5. Circular Dichroism

CD spectra were recorded on a ChirascanTM CD spectrometer (AppliedPhotophysics) using 1 cm path-length quartz cuvettes at 25°C. Titrations were carried out by incremental addition of compound stock solution aliquots to a mixture of DNA or G4 at constant concentration. All reported experiments used TRIS-HCl buffered solutions as indicated.

4.6. Fluorescence Spectroscopy

Fluorescence spectra were recorded on a Horiba FluoroMax®-4 spectrofluorometer. Data was processed with the FluorEssence v3.5 software.

Emission Screening. Emission spectra were recorded in 5 mM TRIS-HCl, 50 mM KCl (pH 7.8) at a stepwise increase by 25 nm over a range of excitation wavelengths of 200–500 nm. Emission was recorded over a range of 225–700 nm. Excitation and emission slit widths are specified per data set. Diagonal ridges in full excitation-emission 2D spectra represent first and second order Rayleigh scattering.

Tab. 10. Slit widths and concentrations of naphthalimide compounds used in fluorescence studies to determine the excitation/emission range.

	conc.	slit width
L4	5 μ M	1 nm
L5	5 μ M	1 nm
4	10 μ M	5 nm
5	10 μ M	7.5 nm
6	20 μ M	2.5 nm

Fluorescence titrations. *c-KIT2* was diluted in potassium-containing buffer to desired concentration and annealed by heating to 90°C for 5 Min before being allowed to slowly cool to room temperature overnight. Compound stock solutions (1 mM) were prepared in DMSO, with the final percentage of DMSO in measured solutions less than 1%. Titrations were performed by adding aliquots of DNA (*ds26*, *c-KIT2*) to a solution of the compounds in buffer (TRIS-HCl 5 mM, KCl 50 mM, pH 7.8) at constant compound concentration (kept constant by adding an equal-volume aliquot of doubly concentrated compound solution to the mixture). K_b values for compounds were obtained by fitting emission–concentration data plots through the binding model proposed by Thordarson.^[109] Non-linear curve fitting was carried out in Origin 9.5 utilising a script as reported by Hargrove *et al.*^[111]

4.7. Mass Spectrometry

All ESI-TOF-MS experiments were performed on a maXis classic (Bruker Daltonik GmbH, Bremen, Germany) hybrid ESI-Qq/oa-TOF mass spectrometer. Data sets were analysed with the instrument software Bruker Daltonics DataAnalysis.

9-Ethylguanine binding studies. Compound stock solutions were prepared in methanol. Stock solutions were diluted in Milli-Q water and mixed with a solution of 9-EtG in Milli-Q water. The resulting mixture was stirred at room temperature for 2 h (30 Min for **1a/b** and **2a/b**) before measurement. Samples were diluted prior to injection with ACN/MeOH 1% H₂O and spectra were acquired *via* direct infusion. MS spectra were recorded in positive ion mode using the following parameters: flow rate 3 μ L/min; capillary voltage -4500 V; dry gas flow 4.0 L/min (nitrogen); dry temperature 180 °C; Resolution: 20000 FWHM; Mass accuracy < 5ppm.

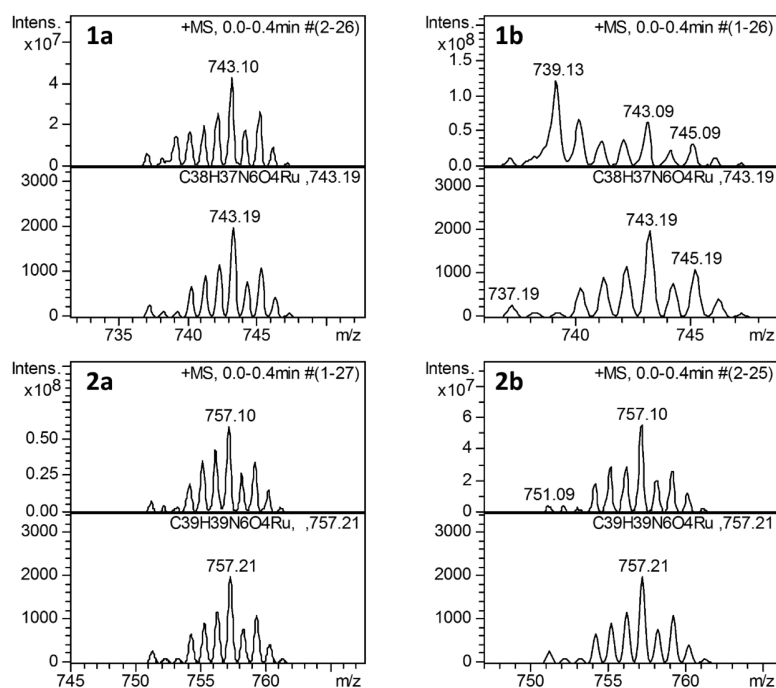


Fig. E5. Experimental and simulated m/z peaks corresponding to adducts of 9-EtG with ruthenium complexes **1a/b** and **2a/b** upon the loss of the leaving group ligand (chlorido or pyridine).

G4 binding studies. All ESI-TOF-MS experiments involving G4s were carried out in negative ion mode with settings as reported in the literature^[112]: capillary voltage +3.500 V; nebuliser 1 bar, dry gas flow (nitrogen) 4.0 L/min at 120 °C; analyser background pressure 3×10^{-7} mbar; rate of sample infusion 3 μ L/min, end plate offset voltage 500 V, resolution 20000 FWHM, mass accuracy <5 ppm. 100 μ M stock solutions of oligonucleotides *hTelo* and *c-KIT1* were prepared in 100 mM ammonium acetate buffer (pH 6.8) and heated to 90 °C for 5 min before being allowed to slowly cool to room temperature. Compounds **1a/b** and **2a/b** were prepared as 1 mM stock solutions in acetonitrile and diluted with MilliQ water to a concentration of 20 μ M. An equimolar mixture of drug and G4 (10 μ M, respectively) was injected, containing 50 mM ammonium acetate. Peak patterns were interpreted as previously reported.^[113]

4.8. Biological Activity

Ovarian cancer cell line A2780 (Sigma Aldrich) was cultured in RPMI 1640 medium supplemented with 2 mM glutamine and 10% foetal bovine serum (FBS South America, Biowest, Nuaille, France). Cell cultures were incubated at 37°C and 5% CO₂ and screened regularly for *Mycoplasma* contamination (*mycoplasma* stain kit, Sigma, USA). Cells were seeded ($2\text{--}3 \times 10^4$ cells per mL) in 96 well plates and left to adhere over night. Cells were treated with solutions of compounds in DMSO to a final drug concentration as specified (0–75 μ M) for 72 h. Cell viability was determined in a MTT-based vitality assay (EZ4U, Biomedica, Vienna, Austria) according to instructions by the manufacturer. Dose–Response curves were generated in GraphPad Prism 5 software. Data points represent the mean $\pm$ standard deviation of three replicates. IC₅₀ values were calculated from dose–response curves as the compound concentration leading to a 50% decrease in cell count compared to untreated control.

5. SUMMARY AND OUTLOOK

The research effort in the field of metal-based anticancer drugs continues to explore a wider and wider range of new targeting strategies and improvement of drug performance. Progress is being made in the understanding of tumor biology as well as the mechanisms of actions for different classes of drugs, and with that understanding, strategies are being developed to combine different approaches to drug targeting within a single applicable drug. Since DNA ranks among the most promising drug targets for antitumor activity, developing drugs with dual modes of binding to that all-important biomolecule will hopefully lead to more effective, more specific, and better understood cancer treatment in the future.

Within this work, two classes of metal arene complexes have been investigated for their covalent and non-covalent binding to DNA in general and their effects on G-quadruplex structures in particular. G-quadruplexes are secondary motifs that occur non-randomly in the genomic sequence, and several specific sites occur in cancer-related sequences, be that oncogene promoters or the human telomeric sequence.

The first class of compounds to be explored featured a DNA-coordinating metal center (Ru), a facial arene ligand (*p*-cym), a leaving group ligand (Cl, pyr), and an *O,O*-chelating bioactive ligand (1,3-dioxindan-2-carboxamide) in turn connected *via* an alkyl sidechain (methyl-, ethyl-) to a G4-binding moiety (naphthalene). Varying the length of the alkyl-linker did little to affect drug activity, but exchanging the chlorido ligand for a pyridine greatly enhanced the hydrolytic stability of the complex as a whole, and with it, its capabilities to act on selected G4 models. Chlorido complexes **1a** and **2a** quickly released the *O,O*-chelating ligand in aqueous solution at a pH above 7 and were both weaker overall G4 binders and less cytotoxic against ovarian cancer cell line A2780 than their pyridine derivatives. Pyridine complexes **1b** and **2b** retained the dioxindan ligand for a longer period of time at physiological pH and were much more effective than their metal-free ligands in terms of cytotoxicity as well as binding and stabilising G4 motifs (determined *via* FRET). Most importantly, these compounds showed a selective stabilising effect on *c-KIT1*, a G4 motif from the *c-KIT* oncogene that features guanine binding sites that are not involved in G-tracts. MS binding studies confirmed this selectivity and substantiated evidence that the compounds can bind G4s both by ruthenation of guanine and π -stacking interactions. Finally, control experiments confirmed that the complex applied as a whole exhibits higher activity than the sum of its parts, *i.e.* ruthenium-arene-pyridine fragment **3** and metal-free ligand applied in combination.

Overall, ruthenium arene complexes with a linked naphthalene moiety proved to be fairly good G4 binders. Increasing the hydrolytic stability of these compounds leads to a markedly improved activity, and should therefore be the first direction for developing this class of compounds further. A possible direction is the replacement of the donor atoms of the *O,O*-chelating motif with other donor atoms such as nitrogen or sulfur.

The second project comprised metal arene complexes (Rh-cyclooctadiene, Ru-*p*-cymene) with *N*-heterocyclic carbene ligands (imidazole-2-ylidene) carrying side-chains (3-ethyl-, 3-benzyl-) and a linked (1-propyl) moiety of G4 binder (*N*-(4-ethylthio-1,8-naphthalimide)). Rhodium complexes **4** and **5** proved to be the most potent G4 stabilising agents and bonded more strongly to DNA models (ctDNA, *ds26*) than the ruthenium derivative; the benzyl sidechain of complex **5** further enhanced this activity. However, rhodium compounds tended to precipitate from

solution, thereby narrowing their prospects for application. Ruthenium compound **6** did not suffer from this problem, and in addition had a higher fluorescence emission yield than the rhodium compounds. Compound **6**, although less cytotoxic, also showed a selective stabilisation of *c-KIT2* quadruplex over dsDNA in FRET melting assays, whereas compounds **4** and **5** were mostly unselective. All three metal compounds proved capable of coordinating to guanine model 9-EtG, while fluorescence and CD studies both presented evidence of an intercalative binding mode to DNA. Furthermore, while neither compound **5** nor **6** perturbed the G4 structure of *c-KIT2*, the metal compounds were able to induce the formation of parallel G4 even in the absence of potassium. The 4-ethylthio substituent preserved the fluorescence emission of the naphthalimide ligand even after metallation, making the metal compounds suitable for fluorescence microscopy.

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