



universität
wien

DISSERTATION / DOCTORAL THESIS

Titel der Dissertation /Title of the Doctoral Thesis

„ *In vitro* investigations of ruthenium, osmium and tungsten compounds as novel anticancer agents “

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of
Doktorin der Naturwissenschaften (Dr.rer.nat.)

Wien, 2023 / Vienna 2023

Studienkennzahl lt. Studienblatt /
degree programme code as it appears on the student
record sheet:

UA 796 605 419

Dissertationsgebiet lt. Studienblatt /
field of study as it appears on the student record sheet:

Chemie

Betreut von / Supervisor:

o. Univ.-Prof. Dr. Dr. Bernhard Keppler

Acknowledgements

I am deeply grateful to O. Univ.-Prof. Dr. Dr. Bernhard K. Keppler for giving me the opportunity to pursue my doctoral work at the Institute of Inorganic Chemistry (University of Vienna). I would like to express my deepest gratitude to Dr. Michael A. Jakupec who has been greatly supportive and always sharing his advice, suggestions, and feedback during both my MSc and doctoral studies.

I could not have completed my doctoral studies without my valuable colleagues from the Institute of Inorganic Chemistry (University of Vienna, Austria), who synthesised, characterised, and provided their compounds for my laboratory work; in particular I would like to thank Dr. Wolfgang Kandioller, Dr. Michael Malerek, Dr. Heiko Geisler, Dr. Stephan Mokesch, Dr. Iker Berasaluce, Alexandra Banc MSc. and Valentin Fuchs MSc. I am deeply indebted to Prof. Dr. Gunda Köllensperger, Dr. Andreas Schweikert and Martin Schaier MSc. from the Department of Analytical Chemistry (University of Vienna, Austria) for their cooperation.

I would like to thank to Mag. Veronika Knoll and Mag. Elfriede Limberger for the administrative support during my MSc and doctoral studies.

Words cannot express my gratitude to Dr. Debora Wernitznig who generously shared her expertise, led me to new research paths during my whole doctoral work and in the process became a dear friend. I would like to extend my sincere thanks to Dr. Konstantinos Kiakos for the academic support, friendship and practicing the English language with me. Thank you for always believing in me and for your friendship!

I am particularly grateful for the assistance given by Dr. Anton Legin with any problem I encountered during my laboratory work. I also received support from lab colleagues, Michaela, Ekaterina, Verena, and Dominik. Many thanks for the good atmosphere and all the nice chats as well as lunch breaks.

And last, but not at least I would like to say thanks to my best female friend Rita, who always helps me out with her expertise. Special thanks to Peti (or more formally Dr. Péter Velicsányi ☺), who provided me with insightful comments and suggestions, as my best male friend. I would like to highlight the fundamental role of friends in my life, as they are the ones who keep supporting me throughout my life and have helped me stay healthy both physically and mentally!

Finally, I would like to express my heartfelt gratitude to my family my parents Szilvia and Benjamin, my sister Evelin and to my boyfriend Shin. Thank you for your warmth, unconditional and unwavering love and the all-important financial support. I love you all so much!

Köszönöm a támogatást "Kis család"!

Abstract

Cancer is one of the leading causes of mortality worldwide. Platinum-based drugs are one of the most widely used and most effective classes of anticancer agents in chemotherapy. Despite their clinical success and high efficacy in certain malignancies, platinum drugs have various limitations. Patients suffer from a series of adverse effects, while treatment failure often results from acquired drug resistance. In the past decades researchers have sought to rationally design potential anticancer agents to evade and/or overcome chemoresistance. As such, ruthenium, osmium, and tungsten compounds have been recognized as lead drug candidates. Currently, two prominent coordination compounds of ruthenium are under clinical investigation (the Ru(III) complex BOLD-100 and Ru(II) complex TLD-1433), while organometallic ruthenium(II) complexes are also being developed. No clinical studies have evaluated the impact of osmium and tungsten complexes so far, but impressive anticancer activity *in vitro* and *in vivo* has been recently reported.

The aim of this doctoral thesis is to clarify several aspects of the biological activity of novel metallodrugs and to further elucidate their mode of action in cancer cells. In particular, this PhD thesis examines the following three groups of anticancer drug candidates: (i) 2-phenylbenzothiazole derivatives and their ruthenium(II) as well as osmium(II) metallacycles; (ii) naphthoquinone-based ruthenium(II) arene complexes; and (iii) tungstenocenes with a diverse set of (O,O-), (O,S-) and (O,N-) chelates.

In the first project, a series of 2-phenylbenzothiazoles and their corresponding ruthenium(II) as well as osmium(II) half-sandwich complexes have been investigated on their biological properties. All of these compounds exhibited promising IC₅₀ values in the low μ M range in a number of human cancer cell lines. The osmium complexes showed more potent antiproliferative activity than the ruthenium analogues; moreover, all metal-containing complexes exerted higher anticancer activity in comparison to the free ligands. Intriguingly, an apoptosis/necrosis assay yielded results not mirroring those of the cytotoxicity tests, with the ruthenium compounds being more potent in inducing apoptotic and necrotic events than the osmium analogues. A finding of particular relevance for their cellular pharmacology was the colocalization of a selected fluorescent 2-phenylbenzothiazole ligand and its corresponding ruthenium arene complex in acidic organelles, such as lysosomes, in human colon cancer cells.

In the second project, ruthenium(II) organometallics with coordinating naphthoquinones were investigated on their biological properties. Naphthoquinones are bioactive molecules of plant origin, which exhibit auspicious anticancer and antibacterial activity. The naphthoquinone-based ruthenium(II) arene complexes yielded the expected *in vitro* anticancer activities in short- and long-term colorimetric assays. The most striking result to emerge from the

findings is that higher cytotoxicity has been found in SW480 colon cancer cells than in the broadly chemosensitive CH1/PA-1 ovarian teratocarcinoma cells. The two most cytotoxic complexes with (*p*-cymene and biphenyl as their arenes), had the highest aqueous stability, and showed a positive correlation between their cellular accumulation and lipophilicity. Furthermore, the naphthoquinone derivatives have been investigated on their mechanisms of action, revealing a pronounced influence on the cell cycle (especially in SW480 cells), a significant induction of apoptosis and partial inhibition of NAD(P)H quinone dehydrogenase 1 (NQO1) activity. Other effects known from the literature on naphthoquinones, such as reactive oxygen species (ROS) generation and DNA interactions, could not be observed.

In the third project, tungstenocenes with various biologically active (*O,O*-), (*S,O*-) and (*N,O*-) chelates were characterised. Recent developments in *in vitro* cancer research suggest a need for using three-dimensional cell culture techniques; therefore, experiments were performed with monolayer as well as multicellular tumour spheroid cultures. Promising antiproliferative activity was detected for compounds bearing (thio)flavone ligands in both cellular models. These two complexes were capable of slowing down or completely stopping spheroid growth, detected by capturing the diameter of spheroids and by immunofluorescence staining with the KI67 proliferation marker. Tungsten uptake and subcellular distribution were investigated in cryo-sectioned spheroids *via* LA-ICP-TOF-MS, resulting in different signal intensity maps of the metal. Moreover, ROS generation and apoptosis/necrosis induction could be detected in a concentration-dependent manner for some of the tested compounds. The (*S,O*-) chelate bearing complexes tend to be more active than their (*O,O*-) and (*N,O*-) counterparts, and the most promising candidates of this series are compounds bearing (thio)flavone ligands.

In summary, structure-activity relationships could be elucidated, the most potent representatives identified and previously unknown aspects of their biological activity in cancer cell models revealed for all three classes of novel antitumour metal complexes, illustrating the innovative potential still inherent in this field of research.

Zusammenfassung

Krebs ist eine der häufigsten Todesursachen weltweit. Platinhaltige Therapeutika sind eine der am häufigsten angewendeten und wirksamsten Klassen von tumorhemmenden Verbindungen in der Chemotherapie. Trotz des klinischen Erfolges und der hohen Effektivität bei bestimmten malignen Tumoren ist die Wirksamkeit von Platinmedikamenten eingeschränkt. Die Patienten leiden unter einer Reihe von unerwünschten Nebenwirkungen und die Behandlung scheitert häufig an einer erworbenen Arzneimittelresistenz. In den letzten Jahrzehnten haben sich WissenschaftlerInnen bemüht, potenzielle Krebsmedikamente zu entwickeln, um Chemoresistenz zu vermeiden oder zu überwinden. Hierfür wurden Ruthenium-, Osmium- und Wolframverbindungen als vielversprechende Wirkstoffkandidaten identifiziert. Derzeit werden zwei prominente Ruthenium-Koordinationsverbindungen in der Klinik untersucht (der Ru(III)-Komplex BOLD-100 und der Ru(II)-Komplex TLD-1433), während gleichzeitig auch organometallische Ruthenium(II)-Komplexe entwickelt werden. Osmium- und Wolframkomplexe wurden bisher in klinischen Studien nicht untersucht, aber eine beeindruckende tumorhemmende Wirkung *in vitro* und *in vivo* wurde in jüngster Zeit berichtet.

Das Ziel dieser Dissertation ist es, verschiedene Aspekte der biologischen Aktivität der neuartigen Metallkomplexe zu klären und ihre Wirkmechanismen in Krebszellen besser zu verstehen. Im Speziellen wurden in dieser Dissertation die folgenden drei Gruppen potentieller antitumoraler Wirkstoffe untersucht: (i) 2-Phenylbenzothiazol-Derivate und ihre Ruthenium(II)- sowie Osmium(II)-Metallcyclen; (ii) Ruthenium(II)-Aren-Komplexe auf Naphthochinon-Basis; und (iii) Wolframocene mit einer Reihe verschiedener (O,O-), (O,S-) und (O,N-)-Chelate.

Im ersten Projekt wurde eine Serie von 2-Phenylbenzothiazolen und entsprechenden Ruthenium(II)- sowie Osmium(II)-Halbsandwich-Komplexen auf ihre biologischen Eigenschaften untersucht. Alle diese Verbindungen wiesen in einigen humanen Krebszelllinien vielversprechende IC₅₀-Werte im niedrigen mikromolaren Bereich auf. Die Osmium-Komplexe zeigten eine stärkere antiproliferative Aktivität als die Ruthenium-Analoga; darüber hinaus wiesen alle metallhaltigen Komplexe eine bessere tumorhemmende Aktivität im Vergleich zu den freien Liganden auf. Bemerkenswerterweise entsprachen die Ergebnisse von Apoptose/Nekrose-Tests nicht jenen der Zytotoxizitätstests, da die Ruthenium-Verbindungen in höherem Maß apoptotische und nekrotische Vorgänge auslösten als die Osmium-Analoga. Von Bedeutung für die zelluläre Pharmakologie dieser Verbindungen ist die Lokalisierung eines ausgewählten fluoreszierenden 2-Phenylbenzothiazol-Liganden und des entsprechenden Ruthenium-Aren-Komplexes mit sauren Organellen, wie etwa Lysosomen, menschlicher Darmkrebszellen.

Im zweiten Projekt wurden Ruthenium(II)-Organometallverbindungen mit koordinierenden Naphthochinonen auf ihre biologischen Eigenschaften untersucht. Naphthochinone sind bioaktive Moleküle pflanzlichen Ursprungs, die eine vielversprechende krebshemmende und antibakterielle Aktivität aufweisen. Die Ruthenium(II)-Aren-Komplexe auf Naphthochinon-Basis zeigten in kolorimetrischen Kurz- und Langzeitexperimenten an Krebszellen die erwarteten *in-vitro*-Aktivitäten. Das bemerkenswerteste Ergebnis ist, dass in SW480-Kolonkarzinomzellen eine höhere Zytotoxizität festgestellt wurde als in den weitgehend chemosensitiven CH1/PA-1-Teratokarzinomzellen. Die beiden zytotoxischsten Komplexe (mit *p*-Cymol und Biphenyl als den Arenen), welche die höchste Stabilität in wässriger Lösung aufweisen, zeigten eine positive Korrelation zwischen ihrer zellulären Akkumulation und ihrer Lipophilie. Darüber hinaus wurden die Naphthochinon-Derivate auf ihre möglichen Wirkmechanismen untersucht, wobei sich ein ausgeprägter Einfluss auf den Zellzyklus (insbesondere in SW480-Zellen), eine signifikante Apoptose-Induktion und eine teilweise Hemmung der Aktivität der NAD(P)H-Dehydrogenase 1 (NQO1) zeigten. Andere aus der Literatur zu Naphthochinonen bekannte Effekte, wie die Bildung reaktiver Sauerstoffspezies (ROS) und DNA-Interaktionen, konnten nicht beobachtet werden.

Im dritten Projekt wurden Wolframocene mit unterschiedlichen biologisch aktiven (O,O-), (S,O-) und (N,O-)-Chelaten charakterisiert. Aktuelle Entwicklungen der *In-vitro*-Forschung legen eine Notwendigkeit der Verwendung dreidimensionaler Zellkulturtechniken nahe; daher wurden Experimente sowohl an Monolayer- als auch an multizellulären Tumorsphäroid-Kulturen durchgeführt. In beiden Zellkultur-Modellen wurde eine vielversprechende antiproliferative Aktivität für Verbindungen mit (Thio-)Flavon-Liganden festgestellt. Diese beiden Komplexe waren fähig, das Wachstum der Sphäroide zu verlangsamen oder vollständig zu stoppen, wie mittels Bestimmung der Durchmesser der Sphäroide und Immunfluoreszenzfärbung des Proliferationsmarkers KI67 nachgewiesen wurde. Die Aufnahme und subzelluläre Verteilung von Wolfram wurde in Gefrierschnitten von Sphäroiden mittels LA-ICP-TOF-MS untersucht, was unterschiedliche Signalintensitätsverteilungen des Metalls ergab. Des Weiteren konnten für einige der getesteten Verbindungen die Bildung von ROS und konzentrationsabhängige Apoptose-/Nekrose-Induktion nachgewiesen werden. Die (S,O-)-Chelat-Komplexe sind tendenziell aktiver als ihre (O,O-)- und (N,O-)-Analoga, und die vielversprechendsten Kandidaten dieser Serie sind Verbindungen mit (Thio-)Flavon-Liganden.

In Summe konnten für alle drei Klassen neuartiger antitumoraler Metallkomplexe Struktur-Aktivitäts-Beziehungen aufgeklärt, die potentesten Vertreter identifiziert und bisher unbekannte Aspekte ihrer biologischen Aktivität in Krebszellmodellen aufgezeigt werden. Dies illustriert das Innovationspotential, welches diesem Forschungsfeld nach wie vor innewohnt.

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1. Introduction

1.1. Cancer

Cancer is a major public health problem with a very high mortality rate [1]. The estimated new incidences in 2020 were around 19 million worldwide, including all ages in both genders. The estimated mortality in 2020 was around 10 million worldwide [2].

Cancer is often regarded as a disease of aging, as the estimated number of cancers can vary greatly according to age in either gender (Figure 1) [3]. Among individuals in the age group of 0–24 years, 185.578 (female) and 208.026 (male) new cancer incidences were estimated in 2020. The occurrence of cancer cases substantially increases among individuals of the cohort 25–49 years of age: 1.867.350 (female) and 1.051.249 (male). The highest estimated number of cancers arise in individuals over the age of 50, reflecting the influence of aging on the development of malignant neoplasms, with 7.174.556 (female) and 8.806.030 (male) cases reported [2]. There is strong biological evidence, statistically verified, that aging dramatically influences tumour progression due to the time-dependent accumulation of cellular damage, alterations in stromal homeostasis and changes of the immune system [3]. The other prominent aspect influencing cancer statistics is sex-dependent differences [4, 5]. For young individuals (ages 0–24) the two most common cancer types are leukaemia (20.8%) and thyroid cancer (20.8%) in females, leukaemia (20.8%) and brain and central nervous system cancer (10.3%) in males (Figure 1). Among individuals of 25–49 years of age the sex-dependent emergence of certain cancer types is very conspicuous, e.g. the increased incidence of breast (35.3%) and cervical/uterine (13.4%) cancers in females. On the other hand, the estimated incidences of the most prevalent cases in males, with liver (9.5%), colorectal (9.0%) and lung cancers (7.6%) are not related to gender specificities. The most significant sex-dependent differences occur over the age of 50, when the reproductive organs are most susceptible to neoplastic disease, especially among males. Prostate cancers (15.8%) represented the most abundant new cases in 2020. These statistical data underpin the relevance of sex differences for developing novel anticancer agents and targeted therapies.

According to the World Health Organization (WHO) classification system, cancers are divided into six groups based on histological type. The international classification/nomenclature of cancers is based on the tissue of origin and laid down in the International Classification of Diseases for Oncology, Third Edition (ICD-O-3): carcinoma (malignant neoplasm with epithelial origin), sarcoma (originates in supportive and/or connective tissue), myeloma (originates in plasma cells of the bone marrow), lymphoma (originates in the

glands and nodes of the lymphatic system), leukaemia (“blood cancer”) and mixed types of different origins [6].

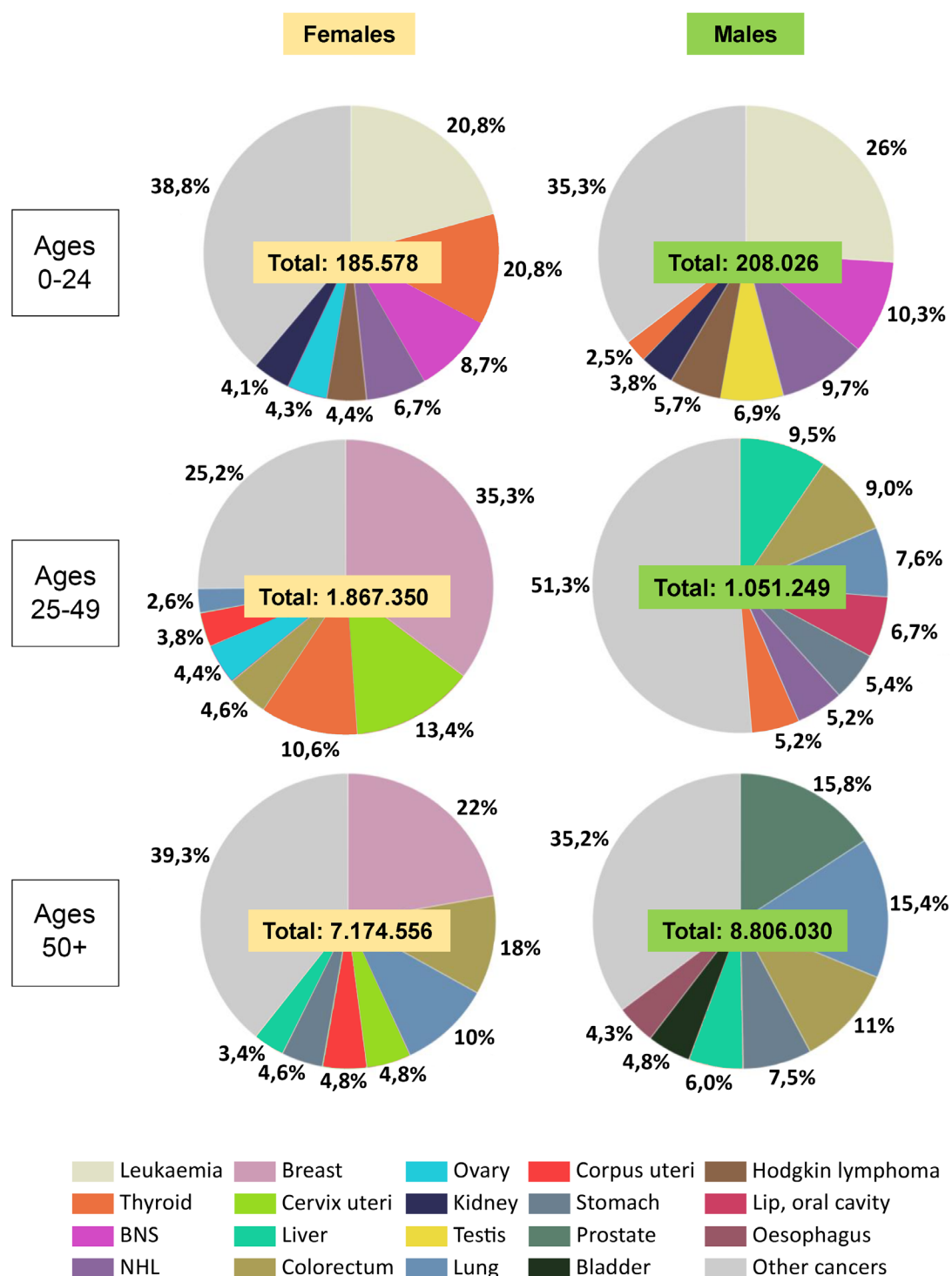


Figure 1: Estimated number of new cancer cases in 2020, worldwide in females (left) and males (right) of the age of 0–24 (top), 25–49 (middle) and over 50 years (bottom); BNS: Brain and central nervous system; NHL: Non-Hodgkin lymphoma. Globocan 2020 was used as data source, diagrams are adapted from reference [2].

1.2. Hallmarks of cancer

When a single cell undergoes malignant transformation, it starts proliferating uncontrollably and can develop into a malignant neoplasm, which can then spread to other tissues and organs. The damaged cells manipulate existing molecular and cellular pathways for their sustained proliferation. The perturbation of signalling pathways and networks are key factors for tumour development, adding to the complexity of cancer (genetics and biology). In 2000 Hanahan and Weinberg summarized the key mechanisms, identified as fundamental for cancer progression. These “hallmarks of cancer” offer a better understanding of the multistep processes of tumour pathogenesis [7]. In this original framework Hanahan and Weinberg defined six hallmarks of cancer, which in 2011 they expanded with the addition of two more hallmarks and two enabling characteristics [7, 8]. In 2022 Hanahan published an update on these hallmarks, extending the framework with four new facets. Collectively, nine hallmarks of cancer and five enabling characteristics have so far been recognized and are shown in Figure 2 [9].

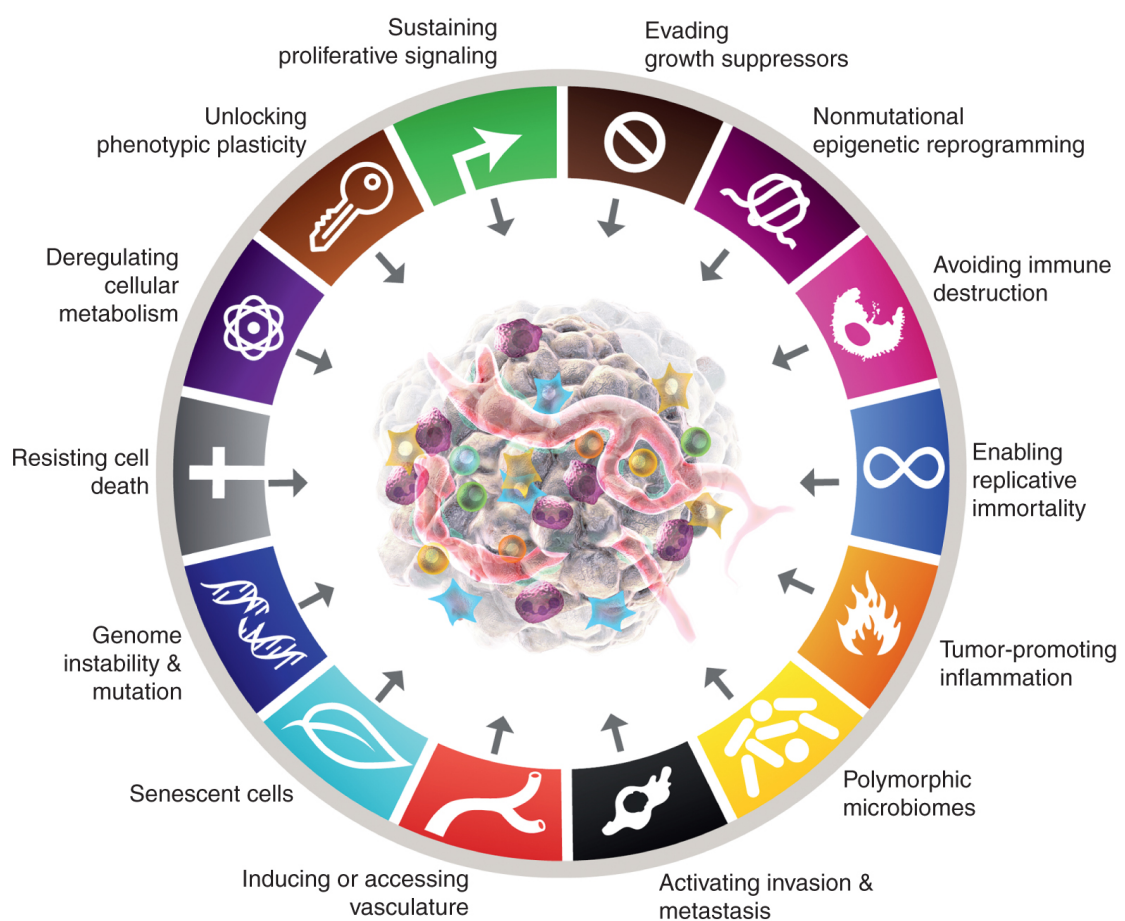


Figure 2: Hallmarks of cancer, including all the latest additions (adopted from reference [9] with permission from the American Association for Cancer Research).

The original six core hallmarks

Sustaining proliferative signalling (Self-sufficiency in growth signals)

Cell division and growth in normal tissues are processes tightly controlled by mitogenic growth signals (GS). The GS from neighbouring cells initiate intracellular signalling cascades (such as the Akt, MAPK/ERK and mTOR pathways), which are essential for maintaining tissue homeostasis and for controlling cell proliferation. Cancer cells can stimulate their own cell cycle, they are not dependent on outside signals such as epidermal growth factor (EGF) for their division, which leads to uncontrolled growth and self-sufficiency [7, 8].

Evading growth suppressors (Insensitivity to anti-growth signals)

In order to maintain an equilibrium between cell number and tissue homeostasis normal cells produce growth suppressors, which act as stop signals. Cancer cells can evade these external growth-inhibitory signals. Therefore, in a tumour tissue, cells bearing DNA damage are not prevented from dividing. There are two key proteins, retinoblastoma-associated (RB) and TP53 (p53), which are suppressors of proliferation. RB regulates the growth-and-division cycle, whereas p53 can trigger apoptotic programmes. The functions of these proteins are disrupted in cancer cells which become resistant to apoptotic signalling and cannot enter the G0 resting phase [7, 8].

Resisting cell death (Evading apoptosis)

Normally, damaged cells undergo programmed cell death (called apoptosis) to maintain tissue homeostasis. The apoptotic machinery works by the activation of death receptors through upstream regulators and downstream effectors. Apoptosis can occur by signals of extracellular origin (extrinsic pathway) or by intracellular signals (intrinsic pathway). The intrinsic apoptotic programme is more widely implicated as a barrier to cancer pathogenesis. Cancer cells can limit or evade apoptosis through the dysregulation of several proteins, such as the TP53 tumour suppressor (loss of function); Bcl-2, Bcl-XL, Bcl-W antiapoptotic regulators (increased expression); Bax, Bak, Bid, Bim, Puma proapoptotic factors (downregulation) or IGF1/2 survival signals (increased expression) [7, 8].

Enabling replicative immortality (limitless replicative potential)

Usually, normal cells do not have unlimited replicative potential, and their capacity for dividing tends to enable around 60–70 divisions, also called the Hayflick limitation [7]. This is caused by the shortening of the telomeres (repetitive sequences at the end of chromosomes) during the DNA replication of each cell cycle, inducing senescence and apoptosis in healthy cells and thereby providing proliferative barriers. Cancer cells are able

to produce their own telomerases (enzymes which elongate telomeres) leading to abnormally high levels of the enzymes and resulting in sustained cellular division [7, 8].

Inducing or accessing vasculature (Sustained angiogenesis)

Angiogenesis, the formation of a vascular network supplying nutrients and oxygen and eliminating metabolic waste and carbon dioxide from newly grown organs, is a normal process during embryogenesis. Cancer cells, which require sustenance with nutrients and oxygen, have the ability to stimulate the growth of new blood vessels from those pre-existing. The two most important signalling proteins which play a significant role in promoting tumour angiogenesis are vascular endothelial growth factor-A (VEGF-A), which is an inducer, and thrombospondin-1 (TSP-1), which is an inhibitor [7, 8].

Activating invasion and metastasis (Tissue invasion & metastasis)

Under normal conditions the cells are either tightly connected to adjacent ones or to the extracellular matrix (ECM) in a tissue. Cell-cell interactions are mostly mediated by calcium-dependent cadherins (e.g. E-cadherin, N-cadherin) while the cell-ECM interactions through integrins. These adhesion molecules are downregulated in cancer cells, which can detach from neighbouring cells or ECM. Cells detached from the primary tumour mass can enter the blood stream, migrate to other sites in the human body and establish new, secondary tumours. This process of tumour spreading is called metastasis [7, 8]. Tissue invasion and metastasis is a multistep process (invasion-metastasis cascade), which comprises five stages: local invasion, intravasation into blood and lymphatic vessels, circulation through the lymphatic and vasculature system, extravasation from lumina into distant tissues, micrometastasis and colonization [8, 10]. Cancerous epithelial cells at the invasive front of the tumour can acquire a mesenchymal phenotype and develop a regulatory program, called epithelial-mesenchymal transition (EMT). These transformed epithelial cells become invasive, resist apoptosis and disseminate by migration [8, 10].

The two additional hallmarks and two enabling characteristics

Deregulating cellular energetics (reprogramming cellular metabolism)

Normal cells use glucose as a fuel source to generate energy in the form of ATP. Energy metabolism can occur under aerobic and anaerobic conditions. Glucose is metabolised by glycolysis to pyruvate, which enters mitochondria and undergoes oxidative phosphorylation in an oxygen dependent process to produce ATP. Under anaerobic conditions, ATP is preferentially produced by glycolysis in the cytoplasm. Cancer cells shift from oxidative phosphorylation (36 ATP) to glycolysis (2 ATP) despite adequate oxygen levels, while

uptake of glucose as well as secretion of lactate increase dramatically. This effect is called “aerobic glycolysis” or Warburg-effect (after Otto Warburg, who first observed this anomaly) [8, 11]. The highly proliferative cancer cells have to compensate the 18-fold lower ATP production; therefore, the increased need of glucose is associated with the upregulation of glucose transporters (GLUT1), which provide increased glucose import into the cytoplasm [8]. Furthermore, the increased level of lactate decreases the pH in the tumour microenvironment, which in turn promotes invasiveness [11].

Avoiding immune destruction

In general, the human immune system surveys, detects, destructs and eliminates pathogens, unhealthy or ailing cells, preventing the formation of incipient tumours. Some types of cancer cells evade immune surveillance, especially in immunocompromised individuals presenting virus-induced cancers. Cancer cells use inhibitory pathways to avoid detection by T cells. Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and programmed cell death ligand 1 (PD-L1) with programmed cell death protein 1 (PD-1) are essential immune checkpoints. Cancer cells can block T cell activation *via* the increased expression of PD-1, which leads to T cells becoming unresponsive [8, 12]. Immune system-mediated cancer cell destruction involves the release of tumour growth factor β (TGF- β) or other immunosuppressive factors, which negatively affect the infiltration of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells in the tumour microenvironment. Furthermore, cancer cells can recruit regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), which suppress the activity of CTLs [8].

Genome instability and mutation

Spontaneous genome mutations and other genetic changes, which occur during cell division, are generally detected and resolved by the genome surveillance and maintenance systems, whereas cancer cells with aberrant proliferation harbour increased levels of genomic changes and mutations. Defects in DNA-repair mechanisms very often affect essential genes (“caretaker genes”) and their encoded proteins through inactivating mutations or epigenetic alterations (such as DNA methylation and histone modification). These caretaker genes act like tumour suppressor genes and are responsible for detecting DNA adducts and for activating the repair machinery [8].

The causes of genome instability and mutations differ between hereditary and non-hereditary cancers. Hereditary cancers are characterized by genomic instability linked to specific mutations in DNA repair genes. One of the best-documented examples is breast cancer type 1 susceptibility protein (BRCA1), which is a tumour suppressor in breast tissues, and mutations of this protein are responsible for inherited breast and ovarian cancers [13].

Moreover, the TP53 tumour suppressor, also known as “guardian of the genome”, plays a pivotal role in inducing apoptosis/senescence to avoid the accumulation of DNA lesions. Mutations of this gene are related to inherited cancers such as the Li-Fraumeni syndrome [8, 13]. Another example of hereditary cancer is the hereditary non-polyposis colon cancer (HNPCC; also known as Lynch syndrome), in which mutations in DNA mismatch repair genes lead to microsatellite instability (MSI) [13]. In sporadic cancers there are many unknown factors which together cause malignant transformation by genetic and epigenetic events, whereby the loss of “caretaker genes” causes uncontrolled proliferation [13].

Tumour-promoting inflammation

The innate immune system is responsible for wound healing and for removing dead cells. Detection and engulfment of infectious invaders are carried out by adaptive immune responses. Immune cells (e.g. leukocytes, NK cells, T and B lymphocytes, mast cells, macrophage subtypes, neutrophils) exert diverse responses during infection and wound healing. Intriguingly, their presence has been detected in the tumour microenvironment with the evidence that the originally anti-tumour immune cells demonstrate tumour-promoting effects as well as metastasis. It was also observed that chronic inflammation can promote tumour progression. The capacity of tumour-promoting inflammation is classified as one of the enabling characteristics due to its contribution to multiple cancer hallmarks [8].

One additional hallmark and three enabling characteristics

Unlocking phenotypic plasticity

Phenotypic plasticity is the ability of organisms with given genotypes to exhibit different phenotypes in response to environmental changes. The differentiation of normal cells ultimately leads to a non-proliferative status, which is an important process during organogenesis, providing a barrier to neoplasia. Cancer cells can overcome terminal differentiation and unlock the phenotypic plasticity, leading to sustained proliferation. Unlocking plasticity can manifest in: (1) dedifferentiation from mature cells back to progenitor-like states, (2) blocked differentiation from progenitor/stem cell states and (3) transdifferentiation into alternative cell lineages [9].

Nonmutational epigenetic reprogramming

Epigenetic modifications such as DNA methylation, changes in chromatin and histone structures and non-coding RNA interactions are well-known mechanisms during early embryonic development. The whole genome is reprogrammed through epigenetic modifications during the differentiation process, but without changes on the DNA sequence

level. It is a requirement for promoting cell differentiation and ensuring DNA replication in the cell division cycle. Once epigenetic reprogramming becomes aberrant *via* global hypomethylation/hypermethylation of target genes (caused by hypoxia), altered non-coding RNA expression or disrupted histone modifications, it can promote tumour progression [9, 14].

Polymorphic microbiomes

The human microbiome (the entirety of body-resident microorganisms, such as bacteria and fungi) can vary markedly among individuals. The variability of microbiomes conferred by several factors, such as diet, environmental exposure and medication, can impact health and disease and affect cancer phenotypes. One of the most studied is the gut microbiome, which has been implicated in the pathogenesis of colon cancer through dysbiosis or the presence of tumour-promoting bacteria (*e.g.* *E. coli* with *PKS* locus, butyrate-producing bacteria). The disruption of the microbiome's ecosystem has the capability to contribute or interfere with other hallmarks of cancer [9].

Senescent cells

Cellular senescence is an important process by which cell division permanently and irreversibly stops, yet cells do not die. It has evolved as a protective mechanism to prevent neoplasia; however, senescent cells have been shown to contribute to tumour development. Senescence can be induced by various triggers such as stress in microenvironment (*e.g.* lack of nutrients, DNA damage), disruption of organelles and deregulation of signalling pathways. Senescent cells are characterized by morphological and metabolic changes and an adoption of a senescence-associated secretory phenotype (SASP) [9].

1.3. Tumour microenvironment (TME) and extracellular matrix (ECM)

The biology of solid tumours is very complex. Solid tumours present an organ-like structure comprising of tumour cells, stromal cells (fibroblasts, endothelial cells, pericytes, mesenchymal stem cells, adipocytes) and immune cells (natural killer (NK) cells, T cells, B cells, dendritic cells (DCs), macrophages) in addition to vasculature and extracellular matrix (ECM) [15, 16]. The TME, being a heterogeneous population of cells, is ever evolving and changing; therefore, it is important to investigate the contribution of each cell type and the complexity of interactions among the various cell populations to better characterise it.

Fibroblasts are abundant in normal tissue stroma, whereas in solid tumours they are one of the major cell types in the TME (up to 70–90% in breast and pancreatic cancers). Fibroblasts produce the majority of the extracellular matrix components such as collagen, fibronectin

and laminin, and secrete enzymes such as hydroxylases and metalloproteinases [16]. In normal connective tissue fibroblasts are quiescent and metabolically inactive, but activation can occur by acute or chronic inflammation. Fibroblasts activated in the TME are termed cancer-associated fibroblast (CAFs) and differ substantially from their quiescent counterparts, as they are mobile, invasive, synthetically active and promote proliferation and immune responses [15, 17]. Pericytes are located on the outer wall of the blood vessels and are responsible for (re)stabilizing the vascular network [16].

Multicellular organisms require a presence of scaffolds to support cell-to-cell connections and communication. The extracellular matrix is a network of fibrillary proteins, polysaccharide chains and other essential molecules. ECM as a fundamental component of all tissues and organs provides structural and biomechanical support for cells and protects plasma membranes. This complex network has been extensively studied in normal, non-malignant tissues, but has only lately been recognised as a critical component of the TME. Several parameters, such as the composition, organisation, amount of deposition, degradation, posttranslational modifications of the ECM, differ substantially between normal and malignant tissues. In cancer, the most ubiquitous components are produced by cancer cells or by CAFs, which can amount up to 60% of the whole tumour mass and express high levels of fibrillar collagen, fibronectin, laminin, hyaluronan and elastin in many solid tumours (Figure 3) [15-23].

Collagen is the most abundant protein of the ECM, present as a triple coiled helix of polypeptide chains. Collagen constitutes approximately 30% of the total protein amount in the human body and 90% of the ECM. To date, 28 types of collagens have been identified, encoded by 43 genes. The most important roles of collagen are to provide mechanical strength, regulate cell adhesion and support cell migration in all tissues. In the TME an increased deposition of collagen has been observed. In tumour tissues, fibrillar collagens form large macromolecules by intermolecular cross-linking with elastin, which strongly influences the behaviour of stromal and cancer cells of the TME and results in differential matrix stiffness in tumour tissues [15, 16, 18, 19]. Besides collagen, glycoproteins especially proteoglycans, have an important part in the ECM in normal and in tumour tissue. Proteoglycans (PGs) are glycosylated proteins consisting of a core protein and several covalently attached, negatively charged, sulphated side chains of glycosaminoglycans (GAGs) [15, 19]. Fibronectin is a soluble, covalently bound dimer of PGs with a high molecular weight, secreted by hepatocytes. Despite the low abundance of fibronectin, its presence is critically important for the ECM. It supports several functions such as cell adhesion, migration, proliferation and connects diverse proteins in the matrix. Fibronectin is also more strongly expressed in the TME; therefore, it can assume a crucial role in malignant transformation [16]. Laminin, a type of glycoprotein, is located in the basement

membrane of normal, healthy tissue and is involved in epithelization. Laminin is often overexpressed in malignant tissues, especially in the stromal parts of tumours, is involved in the loss of adherence of the basement membrane and the invasive behaviour of cancer cells [15, 16]. Hyaluronan also termed hyaluronic acid (HA), is a linear GAG, which conjugates gluconic acid and *N*-acetylglucosamine, resulting in large polyanionic polysaccharides. HA, with its large molecular size is a primary component of the ECM, crucial for a number of biological and structural functions, as it supports the gel-like architecture of the matrix. The long, negatively charged polymer chains have the ability to bind a high number of water molecules *via* hydrogen bonds. HA is one of the most studied GAGs in the TME due to its overexpression in cancer tissues. It can interact as a ligand with receptors, such as CD44 (cell surface adhesion receptor) or the receptor for HA-mediated motility (RHAMM), thereby regulating cancer progression and inflammatory events [15, 16, 18].

Integrins are bidirectional, heterodimeric cell-surface receptors. Currently 24 different combinations of integrin receptors are known, formed by the combination of α -subunits (18 types) and β -subunits (8 types). Integrins preferentially bind to some ECM components, such as collagen and several glycoproteins (fibronectin, laminin, *etc.*), through their extracellular domains. A high level of integrin expression has been found in many solid tumours. ECM-cell interactions are a dynamic process, which can be stimulated in two different ways by integrin receptors acting as a bridge between the ECM and the cytoskeleton. These transmembrane receptors act as reciprocal mediators in “inside-out” (from cell to ECM) and “outside-in” (from ECM to cell) signalling. Hence, some of the cellular functions (adhesion, migration, invasiveness) and homeostasis of organs and tissues are strongly affected by integrin expression levels.

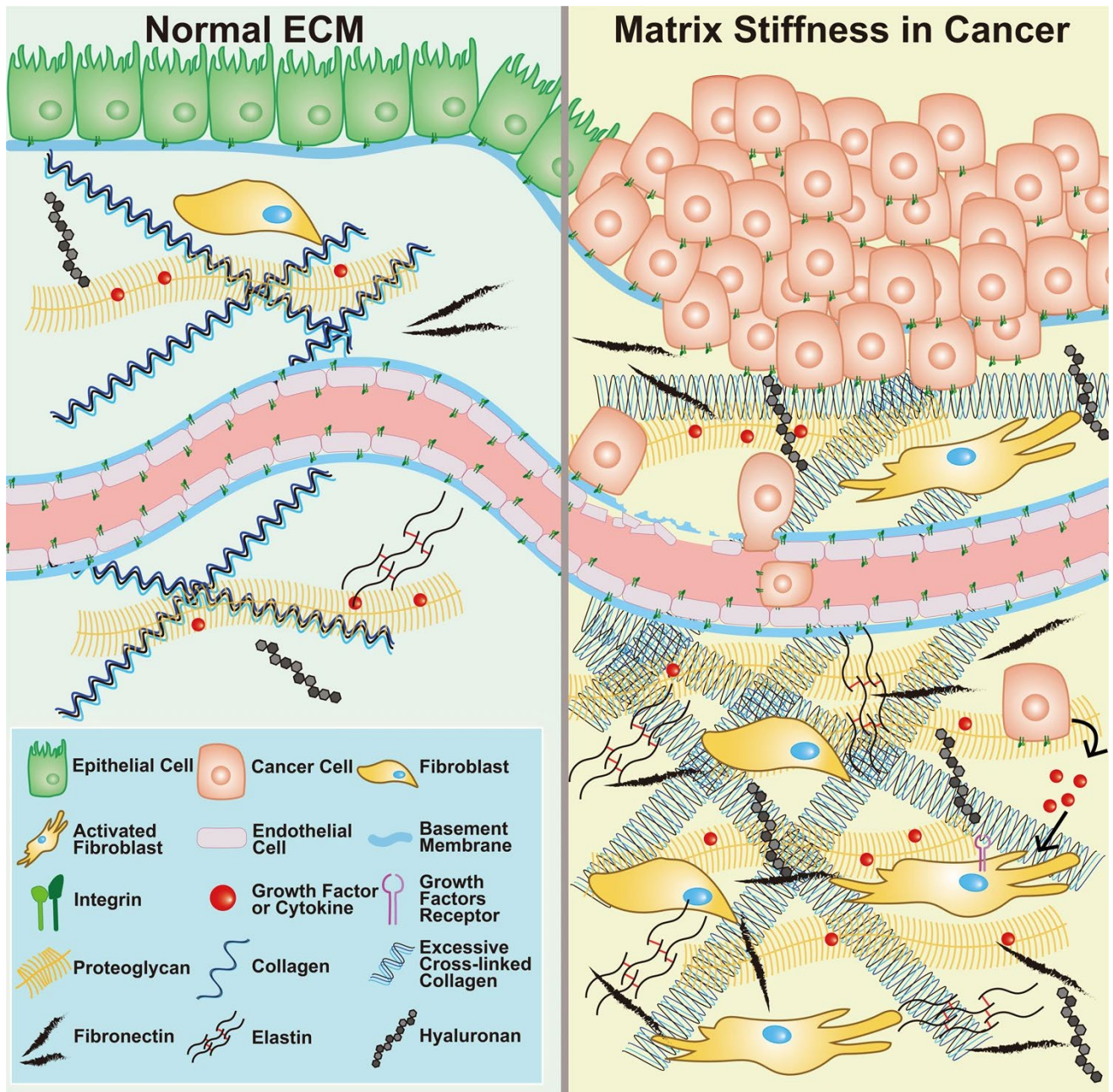


Figure 3: Differences in the components and composition between normal and malignant extracellular matrix (ECM) [16].

1.4. Three-dimensional (3D) cell culture systems

Two-dimensional (2D) cell culture models have been widely used since the early 1900s in preclinical investigations based on their low cost and easy reproducibility between experiments [24-28]. The traditional 2D monolayer cultures, with cells growing in monolayers on planar, rigid plastic or glass surfaces, are not adequate for simulating the physiological conditions in the human body due to the limited dimensionality. Approximately 50 years ago,

in the 1970s, the three-dimensional cell culture techniques were introduced by Sutherland *et al.* [29]. The 3D models, in contrast to the simpler 2D cell monolayer culture systems, more realistically recapitulate the *in vivo* cell behaviour and the complex biology of human tumours, particularly in aspects such as the presence of nutrient gradients and cell-cell as well as cell-matrix interactions. 3D cultures simulate the architectural and functional features of a tissue as well as the tissue-specific physiological and pathophysiological microenvironment [24-28].

Multicellular Tumour Spheroids

Spheroid models are the most versatile and widely used scaffold-free methods for 3D cell culture in preclinical research [25-27, 30-35]. The spherical structure is accomplished by self- or induced aggregation of cells. The spherical geometry better reflects the 3D architecture and physiological characteristics of complex tumour tissues with regard to cell-cell contacts and cell-matrix interactions [27, 30]. To achieve adequate compactness, three critical steps are necessary: (1) establishment of integrin-ECM interactions, (2) cadherin expression and accumulation on the extracellular surface and (3) cadherin-cadherin interactions [31]. The spherical architecture of a spheroid comprises different layers, such as the proliferative rim, a quiescent zone and the apoptotic/necrotic core (Figure 4). These distinct zones of heterogeneous cells are organized in (1) a peripheral layer containing proliferating and migrating cells with intact nuclei and active metabolism, constantly exposed to growth factors, nutrients and oxygen from the medium; (2) a middle layer composed of quiescent and senescent cells with shrunk nuclei and minimal metabolic activity due to the limited diffusion of growth factors; and (3) a central apoptotic/necrotic core consisting of inactive and dead cells with disintegrated nuclei and membranes due to the limited distribution of oxygen and nutrients. In the internal zone of larger spheroids (>400 μm diameter) the occurrence of hypoxia (lack of oxygen) is often detected, which induces altered gene expression mediated by HIF-1 α (hypoxia-inducible factor 1-alpha) and triggers the Warburg effect, yielding an elevated level of lactic acid from aerobic glycolysis, thereby lowering the pH of the internal layer of spheroids [30, 31, 33, 34]. Depending on the size of the spheroids, the three layers have different thickness and distribution of nutrients, oxygen and pH. The diffusion of small molecules (e.g. oxygen) becomes increasingly limited towards the core of the spheroid at around 150-200 μm depth [31, 32]. The stratified, spherical organization due to the gradient differences in nutrients and oxygen availability plays an important role in the observed reduced *in vitro* activity of anticancer drugs compared to 2D cultures. The spheroid compactness and the difference in pH mimic tumour tissues, where the penetration of the anticancer drugs is also limited [30, 33].

Multicellular Tumor Spheroid

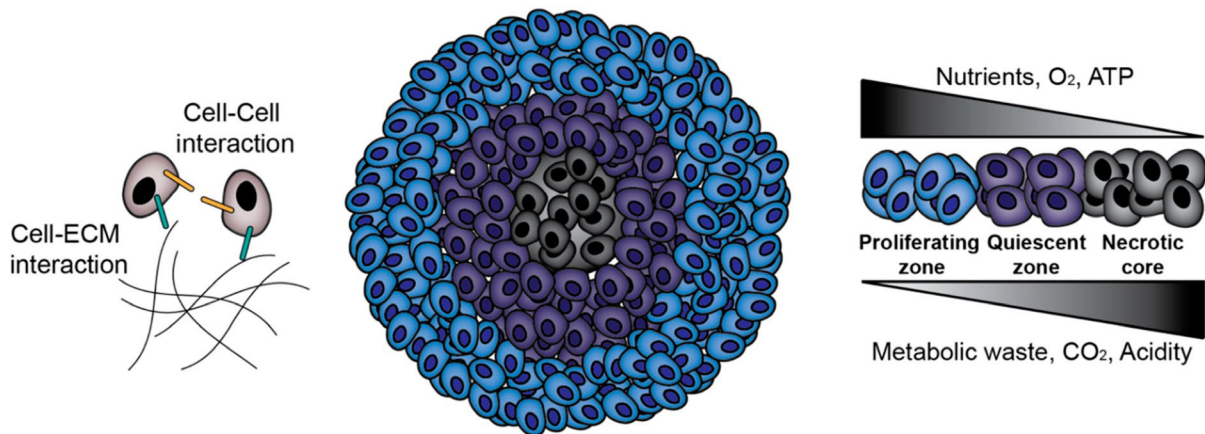


Figure 4. Structure of a multicellular tumour spheroid, including the typical three different layers (proliferating rim, quiescent zone and necrotic core), and the gradients of nutrient, oxygen, metabolic waste, ATP and pH [35].

There is a multitude of techniques, which are used for spheroid formation. Basically they can be divided into non-scaffold-based and scaffold-based ones. [25-37]

Non-scaffold-based techniques

The hanging drop culture method relies on the use of surface tension and gravity for the formation of spheroids within droplets of medium. This method takes advantage of the self-aggregation of cells at the bottom of the drop, which is hanging on the surface of an inverted lid [27, 32]. Spheroid formation can be supported by the addition of methylcellulose and/or agarose [32]. The hanging drop technique is widely applicable for many different cancer cell lines and provides simplicity of handling. Difficulties in changing media, transferring spheroids for drug treatments, and culturing for prolonged periods can be listed as disadvantages of this method [26]. A more recent 3D-printed hanging drop dripper (3d-phd) device overcomes some of these disadvantages and can achieve a robust long-term production of spheroids of a constant size [38].

Liquid overlay culture is a simple aggregate-based technique for spheroid culture. Microplates with round or conical well bottoms are used for promoting self-aggregation of cells and the formation of spheroids [27, 30]. Ultralow-adhesion cell culture plates used are coated with hydrophobic or hydrophilic agents (such as agarose, poly-2-hydroxyethyl methacrylate (poly-HEMA) or poly(N-isopropyl-acrylamide) (PNIPAAm)) to support cell-cell attachment and to minimize the interactions with the plates' surface [30, 32]. Cells spontaneously form spheroids, the size and the shape of which are less controllable. The

liquid overlay technique is applicable for many different cell lines and allows long-term experiments [30].

Magnetic levitation and 3D bioprinting are scaffold-free techniques, which use cells pre-loaded with magnetic particles/nanoparticles (e.g. magnetic iron oxide) to generate spheroids. Cells are forced to aggregate by applying an external magnetic field above (magnetic levitation) or below (magnetic 3D bioprinting) [30, 32, 34]. These are quick techniques and enable the broad use of several cell lines. However, the preparation of magnetic nanoparticles is time-consuming, and some magnetic nanoparticles give treated cells a characteristic brown colour due to iron oxide, thereby interfering with colorimetric measurements [32, 34].

Rotatory methods/microgravity bioreactors refer to techniques in which suspended cells are seeded into a rotating flask and forced to form spheroids through low shear and high mass transfer rates. In the centrifuge chamber cells are exposed to gravitational, centrifugal and Coriolis forces resulting in cell aggregation. The stirring rate must be well defined and constant, since low stirring speed can lead to cell sedimentation, whereas high speed can damage the cells and compromise spheroid formation. This is a high-cost method posing difficulties in monitoring spheroid formation [25, 30, 38].

Scaffold-based techniques

Cells are seeded onto hydrogel- or hard polymer-based supports, which promote the attachment, colonization, and formation of the three-dimensional structure. Hydrogels are 3D networks of hydrophobic polymers; therefore, they can absorb a large amount of water and recapitulate the diffusion of oxygen and nutrients. The scaffold, especially hydrogels, can provide ECM for supporting the cell growth, proliferation, and migration [27, 30, 31, 33, 36, 37].

Synthetic scaffolds are constructed by using non-natural, natural or hybrid (non-natural and natural) polymers for the development of 3D platforms with the required stiffness or porosity. The most popular synthetic polymers are polyethylene glycol (PEG), poly(lactide-co-glycolide) (PLG), poly(*N*-isopropylacrylamide) (PNIPAM), poly(vinyl alcohol) (PVA), polyglycolic acid (PGA), poly- ϵ -caprolactone (PCL) and polylactic acid (PLA), which form hydrogels *via* their polymerization. These synthetic polymers with well-defined physicochemical characteristics can be easily modified, controlled, and adjusted regarding their chemical, mechanical and biological properties depending on the requirements of the target cell populations. Furthermore, they are low-priced, inert and easily reproducible [25, 27, 30, 31, 33, 36, 37]. Alternatively, synthetic scaffolds can be created by natural polymers

such as polysaccharides or peptides of plant or animal origin. These hydrogels are well reproducible and robust for 3D structure formation. The most commonly used natural polysaccharide for hydrogel formation is hyaluronic acid (HA), which is a non-toxic, biodegradable component of the ECM, providing essential hydration for cellular mechanisms. The HA hydrogel promotes spheroid formation based on its high viscoelasticity, which resembles the stiffness of the natural environment [25, 27]. Based on their favourable physicochemical properties, such as electric charge, hydrophilicity, water solubility, biodegradability, non-toxicity and chemically active sites, mono- and polysaccharides such as chitosan, dextran, and alginate are also used for generating polysaccharide-based hydrogels [30, 38]. Peptide-based gels are an evolving area in scaffold design for 3D cell culture. Some studies report advantages of self-assembling peptide gels, such as better stiffness control and improved material properties through the substitution of amino acids, and changes in the length of peptide sequence or pH [27, 38-40].

The most common representatives for hydrogel scaffolds of biological origin are Matrigel®, collagen, fibronectin, fibrin, and gelatine, which are natural and biocompatible substances. These natural polymers exist in the ECM. Therefore, they can mirror the *in vivo* conditions and some key physiological cell functions, such as controlled proliferation, differentiation and metabolism [27, 30]. Matrigel® is a murine tumour-derived extract from the basement membrane of Engelbreth–Holm–Swarm mouse sarcoma, which contains a composite of important proteins such as collagen, fibronectin, laminin, filamin, actin, proteoglycans and different growth factors. It is currently the most widely used substrate for 3D cell culture. However, due to its animal origin the exact amount of tumour-extracted components cannot be readily estimated [30, 41]. The other widely used natural polymer is collagen, which is an abundant, structural protein in human tissues. The collagen system presents high flexibility, biodegradability and customization [25, 30, 31].

1.5. Cancer treatment

After a cancer diagnosis, patients require therapeutically effective, precise/targeted and individualised treatment. A personalised treatment plan may include a number of different treatment options such as surgery, radiotherapy, phototherapy, targeted therapy, immunotherapy, hormonal therapy, chemotherapy or a combination of the above, depending on the localization and progression of the disease (Figure 5). Standard treatments in clinical application (surgery, radiotherapy and chemotherapy) are often ineffective for treating advanced, metastatic or recurrent cancer, therefore an unmet clinical need remains for new therapies [42].



Figure 5: Cancer treatment strategies

Surgical treatments depending on the location of the cancer offer a physical way to remove tumours. Recent surgery techniques use laparoscopes (equipped with miniature cameras, therefore avoiding large incisions), lasers and/or cryosurgery (destroying cancer cells by extremely cold temperatures, using liquid nitrogen or argon gas) [43]. Robot-assisted surgery is a practice affording more precision, flexibility and control [44].

Radiotherapy (RT) is one of the oldest and most cost-effective standard techniques, used for treating about two-thirds of all cancer patients [45]. This treatment modality is based on radiation-induced DNA damage (DNA strand breaks) by high-dose radiation directed to the tumour, which leads to cell death, cell cycle arrest, and changes in the tumour microenvironment [46]. Radiation therapies are given in two different ways: as external or internal radiation. The external application employs sources of radiation external to the body (e.g. X-rays, gamma-rays, electrons), which is associated with a high risk of harming normal cells [45]. Internal radiotherapy such as brachytherapy involves sealed, high-dose radioactive sources (e.g. iridium-192, cobalt-60, iodine-125, and palladium-103) placed next to or directly into the tumour, thereby minimizing damage to normal cells [46].

Phototherapies are light-activated, photosensitizer-based approaches, such as photodynamic therapy (PDT) and photothermal therapy (PTT) [47, 48]. In PDT photosensitisers (e.g. porphyrin, chlorin and other chromophores) harvest the energy of light and cause chemical damage by reactive oxygen species (ROS) generation [47]. PTT uses photothermal agents, which absorb certain kinds of light (e.g. near-infrared light) and convert the energy from light into heat to increase the temperature of the surrounding tissue and kill cancer cells [48].

Targeted therapy involves agents which specifically target genetic components, macromolecules and biomarkers such as genes, proteins, antigens, growth factors, receptors or certain specificities of the tissue microenvironment. Targeted therapy includes monoclonal antibodies, small molecule inhibitors and antibody-drug conjugates [49, 50]. Monoclonal antibodies are designed to bind to a specific antigen presented by cancer cells and inhibit tumour growth by disrupting oncogenic signalling or by making tumour cells detectable for immune cells. In breast tumours overexpressing human epidermal growth factor receptor 2 (HER2), monoclonal antibodies such as trastuzumab and pertuzumab, targeting this receptor, have been developed as treatment [49]. Small molecule inhibitors can penetrate into cells based on their low molecular weight. The majority of them inactivate protein kinases, thereby modulating cell growth, proliferation, migration, and angiogenesis and interrupt signalling in dysregulated pathways [50]. For instance, in gastrointestinal stromal tumours the gene encoding tyrosine-protein kinase (c-KIT) is targeted by the small molecule inhibitor imatinib [49]. Antibody-drug conjugates are monoclonal antibodies covalently linked to biologically active, highly cytotoxic agents which can induce apoptosis. The antibody component allows the targeted delivery of cytotoxic chemotherapy to the cancer cells, sparing normal cells. One representative is ado-trastuzumab emtansine, which is used for metastatic breast cancer with HER2 overexpression [49, 50].

Immunotherapy is one of the more recently developed options in cancer treatment, originating from extensive studies of the molecular interactions between tumours and the immune system [51]. The underpinning premise is to stimulate, activate and alert the patient's own immune system to combat cancer cells. Cancer cells often evade immune surveillance (see the section 'Hallmarks of cancer'); therefore, it is important to enhance patients' immunity [52]. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1) are well-studied immune checkpoints, involved in adaptive immune responses against cancer cells [49, 52]. Food and Drug Administration (FDA)-approved targeted therapies for unresectable or metastatic mismatch repair deficient (dMMR) and microsatellite instability-high (MSI-H) colorectal tumours are ipilimumab (targeting CTLA-4), nivolumab and brolizumab (targeting PD-1) [50, 52]. Besides the immune checkpoint inhibitors, other immunotherapies such as cytokine therapy, adoptive cellular immunotherapy (ACT), cancer vaccines, oncolytic virus therapy and DC cell therapy are also available or are being developed [53]. The most novel immunotherapy-based approaches utilise the clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) technology, which could enable gene editing for the development of a cell-based therapy, where immune cells can recognize mutated proteins specific for a patient's tumour [53, 54].

Hormonal therapy focuses on the treatment of gender-specific tumours. The formation of breast, ovarian, cervical, endometrial, and prostate cancers are highly affected by the hormone system. Patients with oestrogen receptor-positive breast cancer receive tamoxifen, which inhibits oestrogen binding to its receptor and induces apoptosis [55]. Prostate cancer is a highly androgen-dependent disease and patients with metastases are treated with gonadal androgen deprivation therapy (ADT) agents, such as long-acting gonadotropin-releasing hormone (GnRH) agonists (e.g. goserelin) or GnRH antagonists (e.g. degarelix), slowing the tumour growth as a result of reduced luteinizing hormone (LH) and of lower testosterone levels [56].

Chemotherapy is a mainstay in cancer treatment, with the primary mechanism of action involving the interruption of the cell cycle and the induction of apoptosis of cancer cells [57]. According to the ATC (Anatomical Therapeutic Chemical) classification, chemotherapeutic (antineoplastic) agents (L01) can be divided in seven groups: alkylating agents (L01A), antimetabolites (L01B), plant alkaloids and other natural products (L01C), cytotoxic antibiotics and related substances (L01D), protein kinase inhibitors (L01E), monoclonal antibodies and antibody drug conjugates (L01F) and other antineoplastic agents (L01X) [58]. Alkylating agents (nitrogen mustards, alkyl sulphonates, ethylene imines, nitrosoureas, epoxides) are the oldest class of anticancer drugs. They are chemically reactive and covalently bind to DNA *via* C-N bonds between the carbon atom of an alkyl group and the nitrogen atom of a nucleobase. Alkylation leads to single-strand or highly deleterious double-strand DNA breaks that cause DNA replication to halt and eventually lead to cell death [59]. Most alkylating agents are monofunctional, bearing one reactive group, e.g. dacarbazine and temozolomide. Bifunctional alkylating agents, such as nitrogen mustards (chlorambucil and cyclophosphamide), have two nucleophilic sites and can generate interstrand (between two opposite strands) and/or intrastrand (on the same strand) DNA cross-links [60]. 5-Fluorouracil (5-FU), cytarabine, and gemcitabine are widely used antimetabolites, acting by interrupting DNA synthesis, based on their highly similar structure to purines and pyrimidines [57]. Plant alkaloids and other natural products include taxanes (e.g. paclitaxel), Vinca alkaloids (e.g. vinblastine and vincristine) and topoisomerase 1 (TOP1) inhibitors (e.g. irinotecan and topotecan) [57]. Paclitaxel is a microtubule-stabilizing agent, which binds to the β -tubulin subunit of the microtubule, leading to microtubule polymerisation and inhibition of mitosis [61]. Vinblastine and vincristine interact with intracellular tubulin and lead to cell cycle arrest by blocking DNA repair and RNA synthesis mechanisms [62]. Irinotecan and topotecan are derivatives of camptothecin. They inhibit the action of topoisomerase 1 by binding and stabilizing the TOP1-DNA complexes, thereby causing DNA-stand breaks, which in turn lead to cell death [63]. Cytotoxic antibiotics and related substances are mostly represented by

anthracyclines (e.g. doxorubicin and daunorubicin). Doxorubicin has several intracellular targets and modes of action: generation of reactive oxygen species (ROS), intercalation into DNA, DNA-adduct formation, TOPII activity inhibition, Ca^{2+} and iron haemostasis dysregulation, ceramide overproduction, immune cell mediated clearance of tumours, *etc.* [64]. Protein kinase inhibitors have numerous representatives (around 150 kinase-targeted drugs), classified as tyrosine, serine-threonine or nonspecific kinase inhibitors. Their main target is the ATP binding site of the kinases, which leads to DNA damage and cell death. Furthermore, kinase inhibitors more effectively discriminate between non-malignant cells and rapidly proliferating cancer cells, producing fewer side effects. Common tyrosine kinase inhibitors are gefitinib and erlotinib, targeting the epidermal growth factor receptor (EGFR) family [65].

1.6. Anticancer metal-based drugs

Platinum (Pt) metallodrugs

In the late 1960s, the serendipitous discovery of the antiproliferative effects of platinum derivatives by Rosenberg opened a new therapeutic opportunity for cancer treatment [66, 67]. The anticancer activity of the first coordination complex, known as cisplatin, was discovered after experiments with *E.coli* had revealed on inhibition of bacterial growth. Around 20 years later, in 1978, the Food and Drug Administration (FDA) approved cisplatin, cis-diamminedichloridoplatinum(II), as the first platinum-based anticancer drug for clinical application. Based on the success of cisplatin, second- and third-generation platinum(II) complexes were designed, such as carboplatin, *cis*-diammine(1,1-cyclobutanedicarboxylato)-platinum(II), and oxaliplatin, *trans*-R,R-cyclohexane-(1,2-diamine)-oxalatoplatinum(II), both widely used clinically to this day [68, 69]. There are a few platinum(II) drugs, which are only approved in specific countries: nedaplatin and miriplatin in Japan, lobaplatin and dicycloplatin in China and heptaplatin in South Korea (structures are shown in Figure 6) [70].

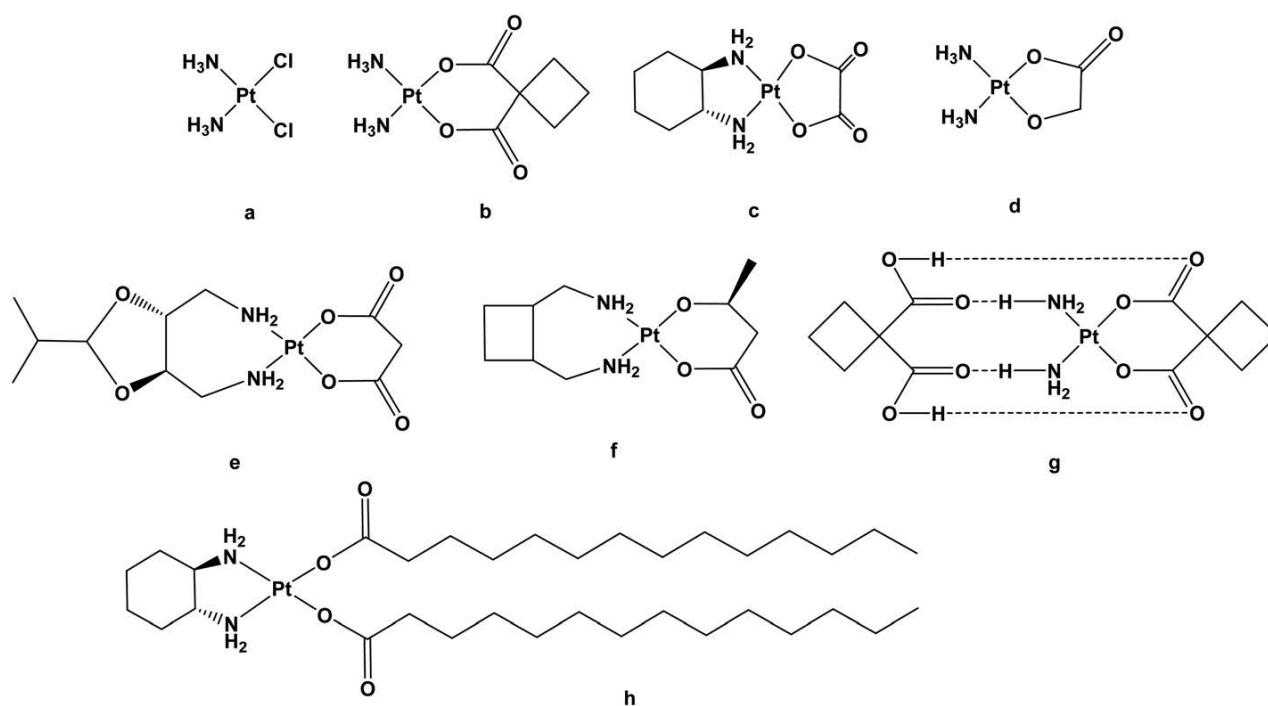


Figure 6: Structures of platinum(II)-based chemotherapeutics: (a) cisplatin, (b) carboplatin, (c) oxaliplatin, (d) nedaplatin, (e) heptaplatin, (f) lobaplatin, (g) dicycloplatin and (h) miriplatin [70].

Platinum(II) metallodrugs are employed in 25% of the treatment protocols for several different cancer types, such as urogenital cancer, non-small cell lung cancer, bladder cancer, head and neck cancer, pancreatic cancer, colorectal and other gastrointestinal cancers, various lymphomas, sarcomas, and multiple myeloma [70, 71]. The main target of the platinum drugs is DNA and their mode of action involves four key steps: (1) cellular uptake *via* passive diffusion or active transport, (2) aquation-mediated activation, (3) binding to DNA and formation of cross-links, and (4) inhibition of DNA transcription, replication and induction of apoptosis [72]. Despite their well-established cancer cell killing capacity, platinum drugs are non-selective, affecting both cancer and normal cells. This lack of selectivity causes serious systemic toxicity and provokes several undesirable side effects in patients, such as nephrotoxicity, ototoxicity (cisplatin), myelosuppression (carboplatin), and peripheral neurotoxicity (oxaliplatin) [68, 69]. Furthermore, cancer cells may become resistant against platinum drugs, compromising the therapeutic outcome. Resistance can emerge because of altered cellular drug accumulation, increased detoxification, enhanced DNA repair, decreased apoptosis, and autophagy [71]. Certain strategies have been developed for the reduction of inherent toxicity and resulting side effects, such as the liposomal encapsulation of platinum drugs and drug delivery by nanomaterial carriers [68, 69].

Ruthenium (Ru) and osmium (Os) metallodrugs

Among the transition metals, ruthenium and osmium are promising candidates for the extension of the therapeutic spectrum of metal-based drugs.

Ruthenium presents favourable chemical and biochemical properties for medicinal use such as: (1) multiple oxidation states (+II, +III and +IV), which enable activation by reduction of the inert Ru(III) prodrugs to active Ru(II) drugs by ascorbate or glutathione, or supported by hypoxic tumour microenvironment, (2) slow ligand-exchange kinetics mimicking the time scale of cell division, and (3) the ability to replace Fe(III) ions and binding to certain biological molecules such as serum transferrin or albumin. Ruthenium-based complexes have shown lower toxicity and higher water solubility. Due to their octahedral coordination geometry ruthenium complexes exert modes of action different from those of platinum-based drugs [73-75]. NAMI-A, imidazoliumtetrachlorido(dimethylsulfoxide)(imidazole)ruthenate(III) was the first intensively studied Ru(III) coordination complex evaluated clinically, progressing up to phase II trials (Figure 7) [76]. Two ruthenium coordination compounds are currently investigated in clinical trials, the ruthenium(III) drug BOLD-100 (formerly KP1339, NKP1339, IT139; the sodium salt analogue of KP1019), sodium tetrachloridobis(indazole)ruthenate(III) of Keppler's group, and the ruthenium(II) drug TLD1433 (Figure 7) [77]. Beside the coordination complexes, organometallic compounds have also been considered as anticancer drug candidates. Organometallic compounds are usually uncharged metal complexes with at least one, covalent metal-carbon bond, where the metal atom has a low oxidation state. They provide a diversity of structural variations and stereochemistry, and well-controlled, stable kinetics (such as hydrolysis) [78]. Intensively investigated organometallic ruthenium complexes are the RAPTAs of Dyson's group and RAEDs of Sadler's group (Figure 7). RAPTAs are half-sandwich arene-ruthenium(II) complexes containing two monodentate chlorido ligands and one 1,3,5-triaza-7-phosphaadamantane (PTA) ligand with a piano-stool (pseudo-octahedral) geometry. The most promising prototype is RAPTA-C (dichlorid Ru(II)-*p*-cymene-1,3,5-triaza-7-phosphaadamantane) demonstrating antimetastatic, antiangiogenic and antitumour activity. RAEDs are ruthenium(II)-arene-ethylenediamine complexes, with RM175 being the most promising candidate [78-81].

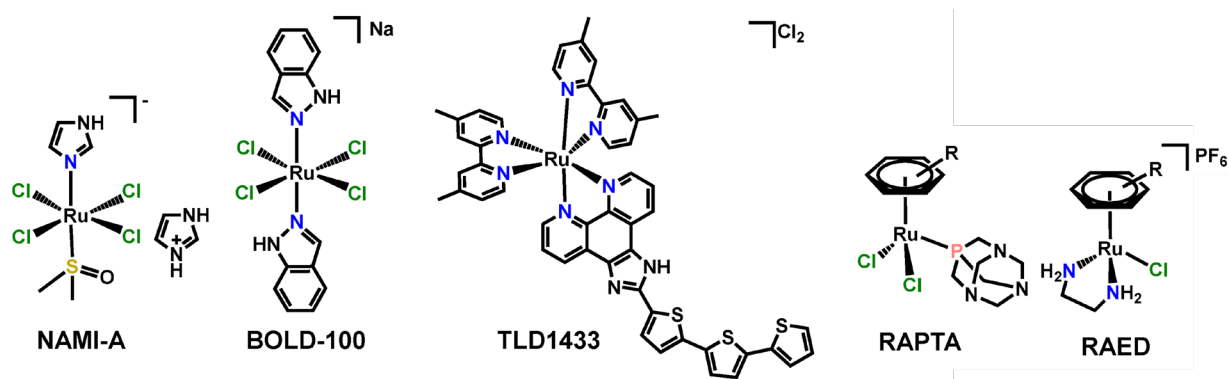


Figure 7: Chemical structure of octahedral ruthenium coordination complexes (NAMI-A, BOLD-100 and TLD1433) and half-sandwich organometallic compounds (RAPTA and RAED) [82].

Osmium as a d-block metal, located in the iron group like ruthenium, received some attention from bioinorganic chemists. Osmium complexes are very often studied together with ruthenium analogues to compare their chemical properties and biological relevance. Initial results have indicated promising anticancer activity of osmium metallodrugs, exerted by various mechanisms such as DNA interactions, redox modulation and protein inhibition [78, 83]. Osmium analogues of well-known ruthenium complexes (such as NAMI-A, KP1019, RAPTA-C and RM175) have been synthesized and studied. Interestingly, some osmium compounds (e.g. Os-RAPTA-C) exhibit less pronounced anticancer activity *in vitro* than *in vivo* compared to the ruthenium analogues, suggesting different biological behaviour. Several studies concluded that the cytotoxic activity *in vitro/in vivo* strongly depends on the ligand system of these metallodrugs. These crucial differences may rely on: (1) osmium is a softer Lewis acid, thus the coordination affinity to biomolecules is different and (2) the metal-ligand exchange processes are slower [84, 85].

Tungsten (W) metallodrugs

Tungsten and molybdenum (Mo) are transition metals of the group 6 in the d-block and have received more attention in medicinal chemistry over the last 50 years. The first spanning idea originated from Köpf-Maier and Köpf in 1979, when they reported on the antiproliferative activity of titanocene dichloride (Cp_2TiCl_2), establishing a new research field in bioorganometallic chemistry [86]. The term metallocene(s) was also introduced: on the central atom two cyclopentadienyl (Cp) ligands are bound by π -bonds. In metallocenes two main structures are distinguished, depending on the bonding angles of the Cp rings: (1) classic metallocenes with parallel bonds of two Cp ligands, resulting in a sandwich structure (e.g. ferrocene), and (2) bent metallocenes with two additional ligands (e.g. titanocene, molybdenocene, tungstenocene), shown in Figure 8 [78]. Metallocenes are mostly applied as biosensors and experimental antitumour agents [87]. Titanocene dichloride, as a bent

metallocene, has shown a promising anticancer activity in breast, colon and lung cancers. The positive results were attributed to the *cis*-dihalido motif, which is similar to the *cis*-dichlorido motif of cisplatin [78, 88]. Inspired by the initial success of titanocene dichloride (which due to its low response in metastatic cancer was abandoned after clinical phase I and II trials), molybdocene dichloride (Cp_2MoCl_2) and tungstenocene dichloride (Cp_2WCl_2) were developed as potential antitumour agents beside other metallocenes based on vanadium, niobium and rhenium, capitalising on their particular physical and chemical properties, such as electronic structure [87-91]. Cp_2MoCl_2 has received more interest, than Cp_2WCl_2 based on its higher stability of metal-Cp bonds, improved anticancer properties and favourable hydrolysis chemistry [87, 92]. Tungstenocene chemistry has been abandoned for many years in research, until the great cytotoxic activity of Cp_2WCl_2 derivatives bearing 3-hydroxy-4-pyrone ligands (one representative is shown in Figure 8) was discovered by Meléndez's group and compared to the cytotoxicities of molybdenocenes [93]. Moreover, the structure modification by replacing chlorido ligands with bidentate ligands modulates the aqueous solubility as well as stability, and the functionalisation of the bidentate ligands can provide improved anticancer potential. Another strategy for increasing anticancer activity could be the functionalization of the Cp ring with hydrophobic or hydrophilic groups and bioactive molecules [94, 95].

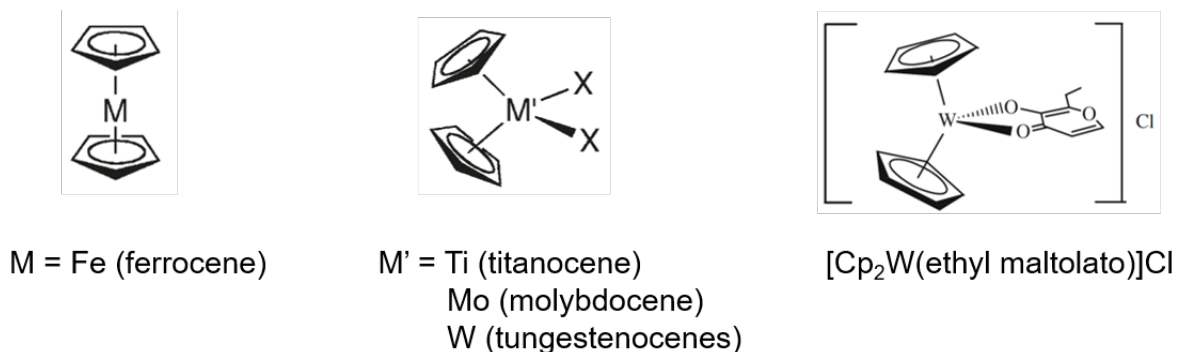


Figure 8: Structure of classic sandwich metallocenes with parallel Cp rings (left), bent metallocenes with two additional X ligands (middle) and a substituted tungstestenocene; $[\text{Cp}_2\text{W}(\text{ethyl maltolato})]\text{Cl}$ (right) [78, 93].

2. List of Publications

This doctoral thesis is based on the following three peer-reviewed publications:

2.1. Investigations on the Anticancer Potential of Benzothiazole-Based Metallacycles

Stephan Mokesch[†], Klaudia Cseh[†], Heiko Geisler, Michaela Hejl, Matthias H. M. Klose, Alexander Roller, Samuel M. Meier-Menches, Michael A. Jakupec, Wolfgang Kandioller* and Bernhard K. Keppler, *Frontiers in Chemistry*, **2020**, 8, DOI:10.3389/fchem.2020.00209

[†] These authors contributed equally to this work

2.2. Arene Variation of Highly Cytotoxic Tridentate Naphthoquinone-Based Ruthenium(II) Complexes and In-Depth In Vitro Studies

Klaudia Cseh[†], Heiko Geisler[†], Kristina Stanojkovska, Julia Westermayr, Philipp Brunmayr, Dominik Wenisch, Natalie Gajic, Michaela Hejl, Martin Schaier, Gunda Koellensperger, Michael A Jakupec, Philipp Marquetand and Wolfgang Kandioller*, *Pharmaceutics*, **2022**, 14(11), 2466, DOI: 10.3390/pharmaceutics14112466.

[†] These authors contributed equally to this work

2.3. Anticancer tungstenocenes with a diverse set of (O,O-) (O,S-) and (O,N-) chelates – a detailed biological study using an im-proved evaluation via 3D spheroid models

Klaudia Cseh, Iker Berasaluce, Valentin Fuchs, Alexandra Banc, Andreas Schweikert, Alexander Prado-Roller, Michaela Hejl, Debora Wernitznig, Gunda Koellensperger, Michael A. Jakupec, Wolfgang Kandioller, Michael S. Malarek* and Bernhard K. Keppler, *Pharmaceutics*, **2023**, 15(7), 1875, DOI: 10.3390/pharmaceutics15071875

3. Synopsis of the Publications/Results

3.1. Investigations on the Anticancer Potential of Benzothiazole-Based Metallacycles

Stephan Mokesch[†], Klaudia Cseh[†], Heiko Geisler, Michaela Hejl, Matthias H. M. Klose, Alexander Roller, Samuel M. Meier-Menches, Michael A. Jakupiec, Wolfgang Kandioller* and Bernhard K. Keppler, *Frontiers in Chemistry*, **2020**, 8

Published: 03. April 2020

Contributions:

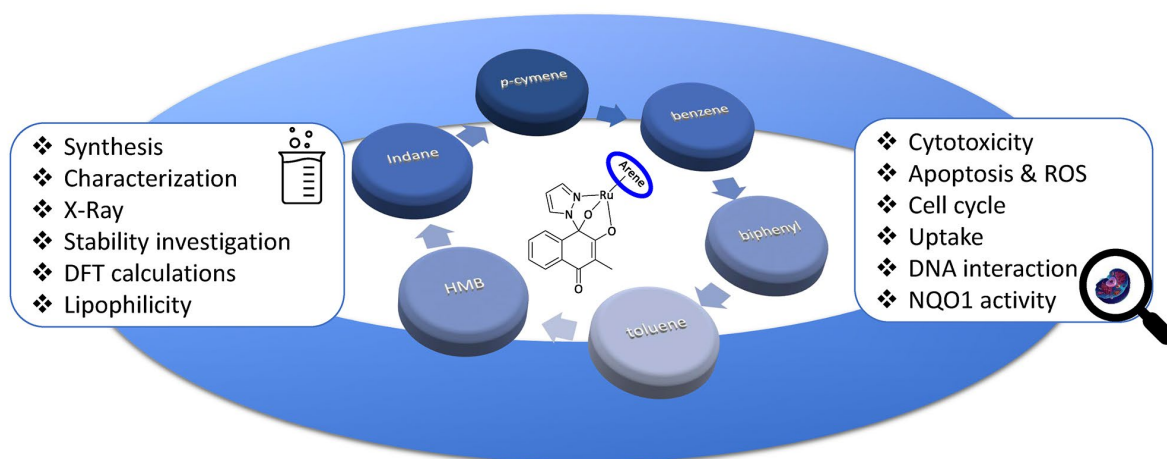
For this publication the author performed and designed the cell culture experiments such as apoptosis assay by flow cytometry and colocalization experiments with fluorescence microscopy. Further the author is contributed to formal analysis, writing—original draft preparation and visualization.

3.2. Arene Variation of Highly Cytotoxic Tridentate Naphthoquinone-Based Ruthenium(II) Complexes and In-Depth In Vitro Studies

Klaudia Cseh[†], Heiko Geisler[†], Kristina Stanojkovska, Julia Westermayr, Philipp Brunmayr, Dominik Wenisch, Natalie Gajic, Michaela Hejl, Martin Schaier, Gunda Koellensperger, Michael A Jakupiec, Philipp Marquetand and Wolfgang Kandioller*, *Pharmaceutics*, **2022**, 14(11), 2466

Published: 15. November 2022

Graphical abstract:



Contributions:

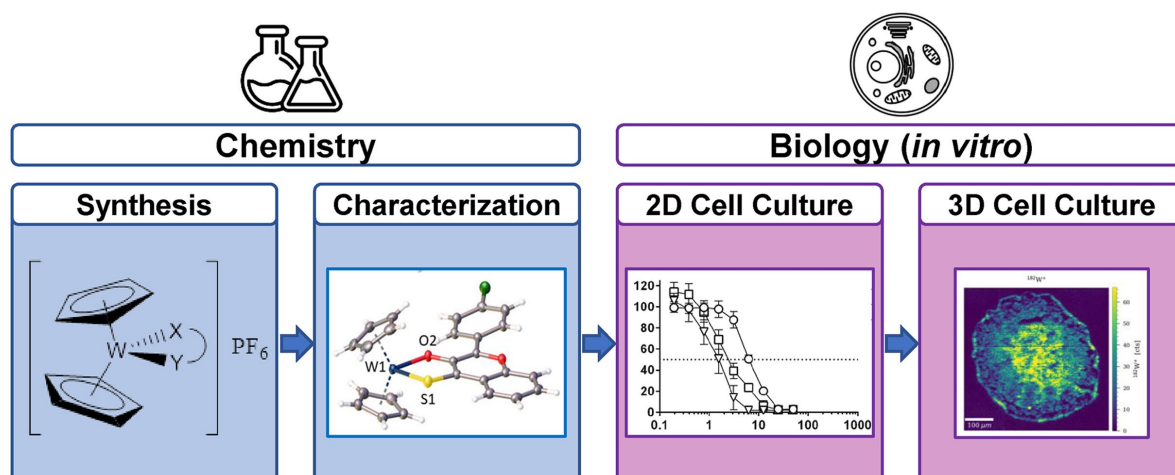
In this manuscript the author contributed to conceptualization, methodology, formal analysis, investigation, data curation, writing—original draft preparation, writing—review and editing as well as visualization. More in details the author designed and performed the cell culture experiments: colony formation assay, cellular accumulation, analysis of cell cycle distribution, detection of apoptosis induction by flowcytometry, NQO1 activity measurement by spectrometer, plasmid DNA interaction studies.

3.3. Anticancer tungstenocenes with a diverse set of (O,O–) (O,S–) and (O,N–) chelates – a detailed biological study using an im-proved evaluation via 3D spheroid models

Klaudia Cseh, Iker Berasaluce, Valentin Fuchs, Alexandra Banc, Andreas Schweikert, Alexander Prado-Roller, Michaela Hejl, Debora Wernitznig, Gunda Koellensperger, Michael A. Jakupec, Wolfgang Kandioller, Michael S. Malarek* and Bernhard K. Keppler, *Pharmaceutics*, **2023**, 15(7), 1875

Published: 03. July 2023

Graphical abstract:



Contributions:

In this manuscript the author was responsible for conceptualization, methodology, formal analysis, investigation, data curation, writing–original draft preparation, writing–review and editing as well as visualization. Furthermore, in details the author performed the following experiments in the cell culture laboratory: spheroid formation and growth curve detection, cell viability (MTT assay (partial) and AB assay), apoptosis studies by flowcytometry, reactive oxygen species (ROS) detection on spectrophotometer, sample preparation for laser ablation studies, immunofluorescence staining with a confocal laser scanning microscope, plasmid DNA interaction studies.

4. Results and Discussion



Investigations on the Anticancer Potential of Benzothiazole-Based Metallacycles

Stephan Mokesch^{††}, Klaudia Cseh^{††}, Heiko Geisler¹, Michaela Hejl¹, Matthias H. M. Klose¹, Alexander Roller¹, Samuel M. Meier-Menches^{2,3}, Michael A. Jakupiec^{1,2}, Wolfgang Kandioller^{1,2*} and Bernhard K. Keppler^{1,2}

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OPEN ACCESS

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Specialty section:

This article was submitted to
Inorganic Chemistry,
a section of the journal
Frontiers in Chemistry

Received: 29 January 2020

Accepted: 05 March 2020

Published: 03 April 2020

Citation:

Mokesch S, Cseh K, Geisler H, Hejl M,
Klose MHM, Roller A,
Meier-Menches SM, Jakupiec MA,
Kandioller W and Keppler BK (2020)
Investigations on the Anticancer
Potential of Benzothiazole-Based
Metallacycles. *Front. Chem.* 8:209.
doi: 10.3389/fchem.2020.00209

A series of 2-phenylbenzothiazole derivatives and their corresponding organometallic ruthenium(II) and osmium(II) complexes were synthesized, designed to exploit both, the attributes of the half-sandwich transition metal scaffold and the bioactivity spectrum of the applied 2-phenylbenzothiazoles. All synthesized compounds were characterized via standard analytical methods. The obtained organometallics showed antiproliferative activity in the low μM range and are thus at least an order of magnitude more potent than the free ligands. ESI-MS measurements showed that the examined compounds were stable in aqueous solution over 48 h. Additionally, their binding preferences to small biomolecules, their cellular accumulation and capacity of inducing apoptosis/necrosis were investigated. Based on the fluorescence properties of the selected ligand and the corresponding ruthenium complex, their subcellular distribution was studied by fluorescence microscopy, revealing a high degree of colocalization with acidic organelles of cancer cells.

Keywords: metallacycles, anticancer, benzothiazoles, ruthenium complexes, osmium complexes

INTRODUCTION

The first stepping stone toward the application of coordination compounds for tumor therapy was the discovery of the antiproliferative activity of cisplatin (Rosenberg, 1971, 1973). Soon, other compounds were investigated with the aim of overcoming severe limitations of cisplatin, which include a broad range of side effects, limited spectrum of responsive cancer types, and the development of tumor cell resistances. The first approach was the modification of this original Pt(II) compound (Galanski and Keppler, 2007). However, the scientific community branched out quickly to neighboring transition metals (Jakupiec et al., 2008). Promising results have been reported for the first-in-class Ru(III) complex BOLD-100 (formerly IT-139 and KP1339, see **Chart 1**) in a clinical phase I study (Trondl et al., 2014). The second intensively studied Ru(III) complex is NAMI-A; however, further clinical investigations have been canceled due to insufficient efficacy (Alessio, 2017). Another class of antitumor ruthenium coordination compounds is represented by half-sandwich complexes with piano-stool geometry. Most of these complexes also act as

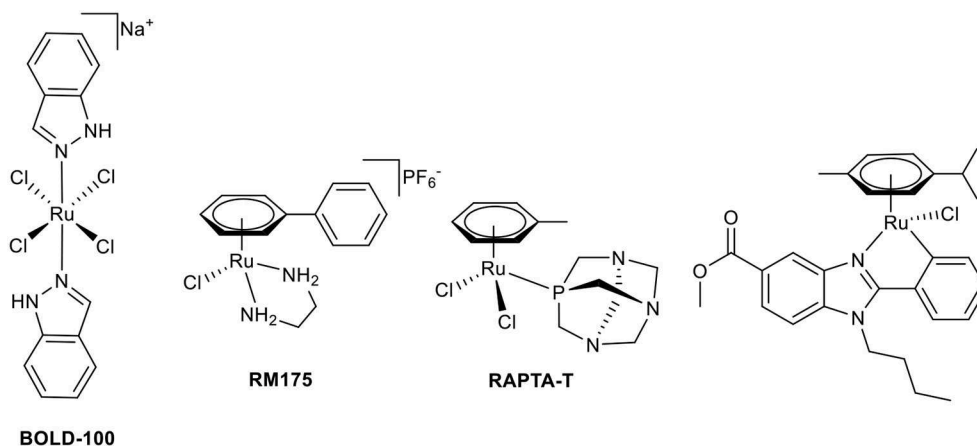


CHART 1 | Chemical structures of BOLD-100, RM175, RAPTA-T, and a biologically active C,N-chelate half-sandwich Ru(II) complex (Yellol et al., 2015).

prodrugs, where activation is achieved by hydrolysis of a labile ligand (leaving group). The formed positively charged aqua species are very attractive binding partners for biological nucleophiles, i.e., proteins or DNA. The two most prominent representatives of this compound class are Dyson's RAPTA complexes and Sadler's RAEN organometallics (Chart 1; Bergamo et al., 2012). Our group established organometallic complexes with high antitumor efficacy by utilizing O,O-, or S,O-chelating bioactive ligands, such as maltol, and S,O-chelating ligands, such as thiomaltol, and attempted structure-activity relationship assertions (Kandioller et al., 2009). The drawback of these organometallics is their lowered stability in physiological media which can be increased by exchange of the halido leaving groups, e.g., N-heterocyclic nitrogen donors (Hackl et al., 2016; Mokesch et al., 2019).

Another approach to circumvent stability issues is the synthesis of complexes with ligands chelating the transition metal via a heterocyclic nitrogen and an aromatic-carbon atom. This C,N-chelating motif has been applied to different transition metals such as Ni(II), Pt(II), Pd(II) (Chen et al., 2008; Cross et al., 2011), Ru(II) (Djukic et al., 2006; Li et al., 2015; Kondrashov et al., 2016), Rh(III), and Ir(III) (Ruiz et al., 2012). The resulting Ru complexes are mostly used as catalysts; e.g., for the stereoselective reduction of ketones (Facchetti et al., 2016), reduction of nitrile functionalities (Nirmala et al., 2016), and hydrogen transfer reactions (Ferrer Flegeau et al., 2011; Yu et al., 2015). In other cases, they are the transition states of the utilized Ru(II) catalyst (Zhang et al., 2014; Rajkumar et al., 2015; Sabater et al., 2016). More recently, Ru complexes of C,N-chelating ligands were investigated for anticancer activity (Ruiz et al., 2011; Yellol et al., 2015; Riedl et al., 2017). There is a definite trend toward the exploitation of possible synergistic effects of utilized ligand systems with inherent bioactivity and the potential affinity of the piano-stool configured transition metal complexes toward biological nucleophiles. Pérez et al. synthesized Ru complexes of benzodiazepine ligands (Pérez et al., 2002), Ruiz et al. use C,N-chelating conjugates of steroids in Ru(II) and the aforementioned Rh(III)/Ir(III) complexes (Ruiz et al., 2011, 2012). Recently

published studies report promising bioactive piano-stool Ru(II) complexes of the pharmacologically widely used benzimidazole scaffold (Chart 1; Belsa et al., 2013; Yellol et al., 2015).

The benzothiazole scaffold has been extensively investigated for non-invasive diagnosis of Alzheimer's disease (Jia et al., 2015) as well as anti-inflammatory (Ban et al., 1998; Papadopoulou et al., 2005), antimicrobial (Singh et al., 2016), anti-allergic (Ban et al., 1998), analgesic (Baell et al., 2001; Westaway et al., 2008), antineoplastic (Chua et al., 2000; Starcevic et al., 2006; Caleta et al., 2009), topoisomerase I/II (Abdel-Aziz et al., 2004; Pinar et al., 2004; Mohmed et al., 2015), and tyrosine kinase inhibiting (Okaniwa et al., 2013; Gabr et al., 2015) properties. Apart from biological applications, C,N-coordination of 2-phenylbenzothiazoles to Ir(III) are promising new candidates for the preparation of organic light-emitting diodes (OLEDs), due to their high quantum yield phosphorescence in yellow and orange (Li et al., 2014).

The goal of this study was the synthesis of 2-phenylbenzothiazole-based Ru(II) and Os(II) piano-stool complexes, investigations on their biological activity profiles, and an attempt to derive preliminary structure-activity relationships.

MATERIALS AND METHODS

All solvents were purchased from commercial sources and distilled prior use. 2-Aminothiophenol (98%, Aldrich), benzaldehyde ($\geq 98\%$, Acros), 4-fluorobenzaldehyde (98%, Aldrich), 4-chlorobenzaldehyde ($\geq 98.5\%$, Acros), 4-methylbenzaldehyde (97%, Acros), 4-methoxybenzaldehyde ($\geq 98\%$, Fluka), 2-(4-aminophenyl)benzothiazole (98%, Alfa Aesar), ruthenium(III) chloride hydrate (Johnson Matthey), osmium(VIII) tetroxide (Johnson Matthey), hydrazine hydrochloride ($> 98\%$, Aldrich), α -terpinene (90%; Acros Organics), and sodium acetate ($\geq 98.5\%$, Fluka) were purchased and used without further purification. Bis[(η^6 -p-isopropyltoluol) dichloridoruthenium(II)] (Bennett and Smith, 1974) and bis[(η^6 -p-isopropyltoluol) dichloridoosmium(II)] (Kiel et al., 1990) were synthesized as described by other authors.

Melting points were determined with a *Büchi Melting Point M-560*. The solubility was determined by dissolving the compound in DMF and subsequent dilution to a final concentration of 1% DMF/MEM (minimal essential medium). The highest concentrated dilution, where no precipitation of the compound occurred, was the determined solubility. The NMR spectra were recorded at 25°C using a *Bruker FT-NMR spectrometer Avance IIITM 500 MHz*. ¹H NMR spectra were measured at 500.10 MHz and ¹³C-NMR Spectra at 125.75 MHz in deuterated chloroform (CDCl₃). 2D-NMR spectra were measured using a gradient-enhanced mode. CHNS elemental analyses were performed with a *Eurovector EA3000 Elemental Analyzer* in the microanalytical laboratory of the University of Vienna. Single crystals of **L5**, **1a**, and **1b** suitable for X-ray diffraction analysis were grown from DCM/Et₂O at 4°C. The X-ray intensity data was measured on a Bruker D8 Venture or X8 APEX II diffractometer equipped with multilayer monochromators, Cu K α INCOATEC micro focus sealed tube and Kryoflex cooling device. The structure was solved by *direct methods* and refined by *full-matrix least-squares techniques*. Non-hydrogen atoms were refined with *anisotropic displacement parameters*. Hydrogen atoms were inserted at calculated positions and refined with a riding model, respectively, as rotating groups. The following software was used: *Bruker SAINT software package* using a narrow-frame algorithm for frame integration, *SADABS* (Sheldrick, 1996) for absorption correction, *OLEX2* (Dolomanov et al., 2009) for structure solution, refinement, molecular diagrams, and graphical user-interface, *Shelxle* (Hubschle et al., 2011) for refinement and graphical user-interface *SHELXS-2013* (Sheldrick, 2015b) for structure solution, *SHELXL-2013* (Sheldrick, 2015a) for refinement, *Platon* (Spek, 2009) for symmetry check. Experimental data and CCDC-code can be found in **Table S1**. Electrospray ionization mass spectra were recorded on a *Bruker AmaZon SL ion trap mass spectrometer* (Bruker Daltonics GmbH).

General Procedure for the Synthesis of 4'-Substituted 2-Phenylbenzothiazoles

The appropriate aldehyde (1 eq) was dissolved in ethanol and 2-aminothiophenol (1 eq) was added, followed by slow addition of hydrogen peroxide (30%, 1.8 eq) and HCl (37%, 1.1 eq). After 2.5 h of stirring at room temperature, NaOH (10%) was added until an alkaline solution was obtained. After 10 min in an ice bath, the precipitated product was collected by filtration in good yields (Guo et al., 2009).

General Complexation Procedure

The appropriate dimeric metal precursor (0.9 eq) and sodium acetate (1.1 eq) were dissolved in MeOH or MeOH:DCM (3:1) and stirred for 1 h at 40°C. After the addition of the desired benzothiazole (1 eq), the reaction mixture is either stirred at 40°C or irradiated under microwave conditions. The solvent was evaporated, the residue taken up in DCM, filtrated, concentrated and the product was then precipitated by addition of *n*-hexane.

2-Phenylbenzothiazole (L1)

The synthesis was performed according to the general procedure for the synthesis of 4'-substituted 2-phenylbenzothiazoles, using benzaldehyde (479 μ L, 4.7 mmol), 2-aminothiophenol (504 μ L, 4.7 mmol), H₂O₂ (866 μ L, 8.5 mmol), and HCl (432 μ L, 5.2 mmol), yielding an off-white solid (706 mg, 71%). Mp: 111°C, Solubility: 0.02 mg/mL = 0.09 mM (MEM, 1% DMF); ¹H-NMR (500.10 MHz, d₆-DMSO) δ : 7.47 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H5], 7.56 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H6], 7.58–7.60 (m, 3H, H3', H4'), 8.07 [d, ³J(H,H) = 8 Hz, H7], 8.09–8.12 (m, 2H, H2'), 8.15 [dd, ³J(H,H) = 8 Hz, ⁴J(H,H) = 1 Hz, 1H, H4]; ¹³C-NMR (125.75 MHz, d₆-DMSO) δ : 122.4 (C7), 122.9 (C4), 125.6 (C6), 126.7 (C5), 127.2 (C2'), 129.5 (C4'), 131.5 (C4'), 132.9 (C7a), 134.5 (C1'), 153.6 (C3a), 167.3 (C2); *m/z* 212.23, *m*_{th}: 212.05; elemental analysis calcd. for C₁₃H₉NS: C 73.90, H 4.29, N 6.63, S 15.18%; found: C 73.55, H 4.20, N 6.66, S 15.46%.

2-(4-Fluorophenyl)benzothiazole (L2)

The synthesis was performed according to the general procedure for the synthesis of 4'-substituted 2-phenylbenzothiazoles, using 4-fluorobenzaldehyde (505 μ L, 4.7 mmol), 2-aminothiophenol (504 μ L, 4.7 mmol), H₂O₂ (866 μ L, 8.5 mmol), and HCl (432 μ L, 5.2 mmol), yielding an off-white solid (802 mg, 75%). Mp: 100°C, Solubility: 0.03 mg/mL = 0.13 mM (MEM, 1% DMF); ¹H-NMR (500.10 MHz, d₆-DMSO) δ : 7.41 [ddd, ³J(H,H) = 9 Hz, ³J(H,H) = 9 Hz, ³J(H,F) = 2 Hz, 2H, H3')], 7.46 [ddd, ³J(H,H) = 8 Hz, ³J(H,F) = 8 Hz, ⁴J(H,H) = 1 Hz, 1H, H5')], 7.55 [ddd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, ⁴J(H,F) = 1 Hz, 1H, H6')], 8.06 [d, ³J(H,H) = 8 Hz, 1H, H7], 8.13–8.17 (m, 3H, H4, H2'), ¹³C-NMR (125.75 MHz, d₆-DMSO) δ : 116.5 [d, ²J(C,F) = 22 Hz, C3')], 122.4 (C7), 122.9 (C4), 125.6 (C6), 126.8 (C5), 129.5 [d, ⁴J(C,F) = 3 Hz, C1')], 129.6 [d, ³J(C,F) = 9 Hz, C2')], 134.6 (C7a), 153.5 (C3a), 163.9 [d, ¹J(C,F) = 250 Hz, C4')], 166.1 (C2); *m/z* 231.21, *m*_{th}: 230.04; elemental analysis calcd. for C₁₃H₈FNS·0.1H₂O: C 67.57, H 3.58, N 6.06, S 13.88%; found: C 67.68, H 3.56, N 6.03, S 13.92%.

2-(4-Chlorophenyl)benzothiazole (L3)

The synthesis was performed according to the general procedure for the synthesis of 4'-substituted 2-phenylbenzothiazoles, using 4-chlorobenzaldehyde (661 mg, 4.7 mmol), 2-aminothiophenol (504 μ L, 4.7 mmol), H₂O₂ (866 μ L, 8.5 mmol), and HCl (432 μ L, 5.2 mmol), yielding a purplish gray solid (955 mg, 83%). Mp: 109°C, Solubility: 0.03 mg/mL = 0.12 mM (MEM, 1% DMF); ¹H-NMR (500.10 MHz, d₆-DMSO) δ : 7.49 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H5], 7.57 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H6], 7.62–7.66 (m, 2H, H3'), 8.08 [d, ³J(H,H) = 8 Hz, 1H, H7], 8.10–8.14 (m, 2H, H2'), 8.17 [d, ³J(H,H) = 8 Hz, 1H, H4]; ¹³C-NMR (125.75 MHz, d₆-DMSO) δ : 122.5 (C7), 123.0 (C4), 125.8 (C6), 126.9 (C5), 128.9 (C3'), 129.5 (C2'), 131.7 (C1'), 134.6 (C4'), 136.1 (C7a), 153.5 (C3a), 166.0 (C2); *m/z* 246.17, *m*_{th}: 246.01; elemental analysis calcd. for C₁₃H₈ClNS·0.1H₂O: C 63.08, H 3.34, N 5.66, S 12.95%; found: C 62.99, H 3.23, N 5.72, S 13.25%.

2-(4-Methylphenyl)benzothiazole (L4)

The synthesis was performed according to the general procedure for the synthesis of 4'-substituted 2-phenylbenzothiazoles, using 4-tolualdehyde (554 μ L, 4.7 mmol), 2-aminothiophenol (504 μ L, 4.7 mmol), H₂O₂ (866 μ L, 8.5 mmol), and HCl (432 μ L, 5.2 mmol), yielding a gray solid (897 mg, 85%). Mp: 81°C, Solubility: 0.04 mg/mL = 0.18 mM (MEM, 1% DMF); ¹H-NMR (500.10 MHz, d₆-DMSO) δ : 2.37 (s, 3H, H5'), 7.36 [d, ³J(H,H) = 8 Hz, 1H, H3'), 7.44 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H5], 7.53 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H6], 7.97 [d, ³J(H,H) = 8 Hz, 1H, H2'), 8.04 [d, ³J(H,H) = 8 Hz, 1H, H7], 8.11 [d, ³J(H,H) = 8 Hz, 1H, H4]; ¹³C-NMR (125.75 MHz, d₆-DMSO) δ : 21.1 (C5'), 122.3 (C7), 122.7 (C4), 125.4 (C6), 126.6 (C5), 127.2 (C2'), 130.0 (C3'), 130.3 (C4'), 134.3 (C7a), 141.5 (C1'), 153.6 (C3a), 167.4 (C2); *m/z* 226.24, *m*_{th}: 226.07; elemental analysis calcd. for C₁₄H₁₁NS·0.1H₂O: C 74.04, H 4.97, N 6.17, S 14.12%; found: C 73.83, H 4.88, N 6.23, S 14.39%.

2-(4-Methoxyphenyl)benzothiazole (L5)

The synthesis was performed according to the general procedure for the synthesis of 4'-substituted 2-phenylbenzothiazoles, using *p*-anisaldehyde (572 μ L, 4.7 mmol), 2-aminothiophenol (504 μ L, 4.7 mmol), H₂O₂ (866 μ L, 8.5 mmol), and HCl (432 μ L, 5.2 mmol), yielding a gray solid (929 mg, 82%). Mp: 115°C, Solubility: 0.06 mg/mL = 0.25 mM (MEM, 1% DMF); ¹H-NMR (500.10 MHz, d₆-DMSO) δ : 3.85 (s, 3H, H5'), 7.12 [d, ³J(H,H) = 9 Hz, 2H, H3'), 7.42 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H5], 7.52 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H6], 8.01 [d, ³J(H,H) = 8 Hz, 1H, H7], 8.03–8.06 (m, 2H, H2'), 8.10 [d, ³J(H,H) = 8 Hz, 1H, H4]; ¹³C-NMR (125.75 MHz, d₆-DMSO) δ : 55.5 (C5'), 114.8 (C3'), 122.3 (C7), 122.5 (C4), 125.2 (C6), 125.6 (C1'), 126.6 (C5), 129.0 (C2'), 134.3 (C7a), 153.7 (C3a), 161.9 (C4'), 167.2 (C2); *m/z* 242.22, *m*_{th}: 242.06; elemental analysis calcd. for C₁₄H₁₁NOS: C 69.68, H 4.60, N 5.80, S 13.29%; found: C 69.59, H 4.50, N 5.83, S 13.49%.

[(Chlorido)(2-phenylbenzothiazolato- κ N, κ C2'))(η^6 -*p*-cymene)ruthenium(II)] (1a)

The synthesis was performed according to the general complexation procedure, using bis[dichlorido(η^6 -*p*-cymene)ruthenium(II)] (234 mg, 382 μ mol), sodium acetate (78 mg, 935 μ mol), MeOH:DCM 3:1, 4 mL), and 2-phenylbenzothiazole **L1** (177 mg, 850 μ mol). The reaction mixture was stirred at 40°C for 24 h. The product was obtained as red crystals (291 mg, 79%). Mp: >197°C (decomposition), Solubility: 0.1 mg/mL = 0.21 mM (MEM, 1% DMF); ¹H-NMR (500.10 MHz, CDCl₃) δ : 0.76 [d, ³J(H,H) = 7 Hz, 3H, Hf], 0.91 [d, ³J(H,H) = 7 Hz, 3H, Hf], 2.13 (s, 3H, Hg), 2.25 [sept, ³J(H,H) = 7 Hz, 1H, He], 5.13 [d, ³J(H,H) = 6 Hz, 1H, Hb], 5.38 [d, ³J(H,H) = 6 Hz, 1H, Hc], 8.73 [d, ³J(H,H) = 6 Hz, 1H, Hc], 5.91 [d, ³J(H,H) = 6 Hz, 1H, Hb], 7.08 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H5'), 7.23 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H4'), 7.45 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H6], 7.63 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H5], 7.68 [dd, ³J(H,H) = 8 Hz, 4J(H,H) = 1 Hz, 1H, H6'), 7.86 [d, ³J(H,H) = 8 Hz, 1H, H7], 8.26 [d, ³J(H,H) = 8 Hz, 1H, H3'), 8.38 [d, ³J(H,H) = 8 Hz, 1H, H4]; ¹³C-NMR (125.75 MHz, CDCl₃) δ : 19.1 (Cg), 21.8 (Cf),

22.8 (Cf), 31.6 (Ce), 80.6 (Cb), 82.3 (Cc), 90.1 (Cc), 90.7 (Cb), 99.3 (Cd), 102.6 (Ca), 121.3 (C4), 122.9 (C7), 123.1 (C5'), 125.5 (C6), 126.0 (C6'), 127.3 (C5), 130.3 (C4'), 132.2 (C7a), 139.6 (C1'), 140.0 (C3'), 151.2 (C3a), 174.6 (C2'), 183.4 (C2); *m/z* 504.23, *m*_{th}: 504.01; elemental analysis calcd. for C₂₃H₂₂ClNRuS: C 57.43, H 4.61, N 2.91, S 6.67%; found: C 57.19, H 4.57, N 2.94, S 6.44%.

[(Chlorido)(2-(4'-fluorophenyl)benzothiazolato- κ N, κ C2'))(η^6 -*p*-cymene)ruthenium(II)] (2a)

The synthesis was performed according to the general complexation procedure, using bis[dichlorido(η^6 -*p*-cymene)ruthenium(II)] (51 mg, 84 μ mol), sodium acetate (17 mg, 200 μ mol), MeOH:DCM (3:1, 4 mL), and 2-(4-fluorophenyl)benzothiazole **L2** (44 mg, 182 μ mol). The reaction mixture was stirred at 40°C for 24 h. The product was obtained as red crystals (68 mg, 82%). Mp: >199°C (decomposition), Solubility: 0.1 mg/mL = 0.20 mM (MEM, 1% DMF); ¹H-NMR (500.10 MHz, CDCl₃) δ : 0.77 [d, ³J(H,H) = 7 Hz, 3H, Hf], 0.91 [d, ³J(H,H) = 7 Hz, 3H, Hf], 2.14 (s, 3H, Hg), 2.25 [sept, ³J(H,H) = 7 Hz, 1H, He], 5.15 [dd, ³J(H,H) = 6 Hz, ⁴J(H,H) = 1 Hz, 1H, Hb], 5.41 [d, ³J(H,H) = 6 Hz, 1H, Hc], 5.70 [d, ³J(H,H) = 6 Hz, 1H, Hc], 5.91 [d, ³J(H,H) = 6 Hz, 1H, Hb], 6.77 [ddd, ³J(H,H) = 6 Hz, ⁴J(H,F) = 3 Hz, ⁴J(H,H) = 1 Hz, 1H, H50'), 7.45 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H6], 7.63 [dd, ³J(H,H) = 8 Hz, ³J(H,H) = 8 Hz, 1H, H5], 7.67 [dd, ³J(H,H) = 8 Hz, ⁴J(H,F) = 5 Hz, 1H, H6'), 7.85 [dd, ³J(H,H) = 8 Hz, ⁴J(H,H) = 1 Hz, 1H, H7], 7.94 [dd, ³J(H,H) = 8 Hz, ⁴J(H,F) = 2 Hz, 1H, H3'), 8.33 [d, ³J(H,H) = 8 Hz, 1H, H4]; ¹³C-NMR (125.75 MHz, CDCl₃) δ : 19.1 (Cg), 21.8 (Cf), 22.8 (Cf), 31.1 (Ce), 80.8 (Cb), 82.8 (Cc), 90.1 (Cc), 90.7 (Cb), 99.5 (Cd), 103.1 (Ca), 110.9 [d, ³J(C,F) = 24 Hz, C5'), 121.1 (C4), 123.0 (C7), 125.5 (C6), 125.9 [d, ³J(C,F) = 18 Hz, C3'), 127.4 (C5), 127.4 [³J(C,F) = 9 Hz, C6'), 132.0 (C7a), 135.9 (C1'), 151.1 (C3a), 162.8 [d, ³J(C,F) = 257 Hz, C4'), 173.6 (C2), 186.6 [d, ⁴J(C,F) = 5 Hz, C2'); *m/z* 464.21, *m*_{th}: 464.04; elemental analysis calcd. for C₂₃H₂₁ClFNRuS: C 55.36, H 4.24, N 2.81, S 6.43%; found: C 55.14, H 4.26, N 2.83, S 6.28%.

[(Chlorido)(2-(4'-chlorophenyl)benzothiazolato- κ N, κ C2'))(η^6 -*p*-cymene)ruthenium(II)] (3a)

The synthesis was performed according to the general complexation procedure, using bis[dichlorido(η^6 -*p*-cymene)ruthenium(II)] (100 mg, 160 μ mol), sodium acetate (33 mg, 400 μ mol), and MeOH (4 mL). Before the addition of the ligand, the mixture was irradiated under microwave conditions for 5 min at 40°C. After addition of 2-(4-chlorophenyl)benzothiazole **L3** (89 mg, 360 μ mol), the reaction mixture was again irradiated for 10 min at 80°C. The product was obtained as dark red crystals (91 mg, 54%). Mp: >162°C (decomposition), Solubility: 0.04 mg/mL = 0.08 mM (MEM, 1% DMF); ¹H-NMR (500.10 MHz, CDCl₃) δ : 0.76 [d, ³J(H,H) = 7 Hz, 3H, Hf], 0.91 [d, ³J(H,H) = 7 Hz, 3H, Hf], 2.15 (s, 3H, Hg), 2.24 [sept, ³J(H,H) = 7 Hz, 1H, He], 5.16 [dd, ³J(H,H) = 6 Hz, ⁴J(H,H) = 1 Hz, 1H, Hb], 5.43 [d, ³J(H,H) = 6 Hz, 1H, Hc], 5.72 [dd, ³J(H,H) = 6 Hz, ⁴J(H,H) = 1 Hz, 1H, Hc], 5.93 [d, ³J(H,H) = 6 Hz, 1H, Hb], 7.06 [dd, ³J(H,H) = 8 Hz, ⁴J(H,H) = 2 Hz,

1H, H5'), 7.46 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H6], 7.59 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H6'), 7.64 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H5], 7.86 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H7], 8.22 [d, $^4J(\text{H,H}) = 2 \text{ Hz}$, 1H, H3'), 8.35 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H4]; ^{13}C -NMR (125.75 MHz, CDCl_3) δ : 19.2 (Cg), 21.9 (Cf), 22.8 (Cf), 31.1 (Ce), 80.7 (Cb), 83.0 (Cc), 90.0 (Cc), 90.8 (Cb), 99.5 (Cd), 103.2 (Ca), 122.3 (C4), 123.0 (C7), 123.6 (C5'), 125.7 (C6), 126.6 (C6'), 127.5 (C5), 132.1 (C7a), 135.9 (C1'), 138.1 (C4'), 139.1 (C3'), 151.0 (C3a), 173.6 (C2'), 184.9 (C2); m/z 538.16, m_{th} : 537.97; elemental analysis calcd. for $\text{C}_{23}\text{H}_{21}\text{Cl}_2\text{NRuS}$: C 53.59, H 4.11, N 2.72, S 6.22%; found: C 53.32, H 4.08, N 2.77, S 6.11%.

[(Chlorido)(2-(4'-methylphenyl)benzothiazolato- κN , $\kappa\text{C2}'$)(η^6 -*p*-cymene)ruthenium(II)] (4a)

The synthesis was performed according to the general complexation procedure, using bis[dichlorido(η^6 -*p*-cymene)ruthenium(II)] (202 mg, 320 μmol), sodium acetate (70 mg, 800 μmol), and MeOH (4 mL). Before the addition of the ligand, the mixture was irradiated under microwave conditions for 5 min at 40°C. After addition of 2-(4-methylphenyl)benzothiazole **L4** (164 mg, 720 μmol), the reaction mixture was again irradiated for 10 min at 80°C. The product was obtained as green needles (134 mg, 41%). Mp: >187°C (decomposition), Solubility: 0.05 mg/mL = 0.10 mM (MEM, 1% DMF); ^1H -NMR (500.10 MHz, CDCl_3) δ : 0.75 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 0.90 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 2.13 (s, 3H, Hg), 2.23 [sept, $^3J(\text{H,H}) = 7 \text{ Hz}$, 1H, He], 2.44 (s, 3H, H7'), 5.13 [dd, $^3J(\text{H,H}) = 6 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, Hb], 5.40 [dd, $^3J(\text{H,H}) = 6 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, Hc], 5.71 [dd, $^3J(\text{H,H}) = 6 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, Hc], 5.91 [d, $^3J(\text{H,H}) = 6 \text{ Hz}$, 1H, Hb], 6.88 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H5'), 7.42 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H6], 7.57 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H6'), 7.61 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H5], 7.83 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H7], 8.08 (s, 1H, H3'), 8.35 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H4]; ^{13}C -NMR (125.75 MHz, CDCl_3) δ : 19.1 (Cg), 21.9 (Cf), 22.3 (C7'), 22.8 (Cf), 31.0 (Ce), 80.3 (Cb), 82.5 (Cc), 89.9 (Cc), 90.7 (Cb), 98.8 (Cd), 102.4 (Ca), 121.1 (C4), 122.9 (C7), 124.4 (C5'), 125.2 (C6), 125.8 (C6'), 127.2 (C5), 132.1 (C7a), 137.0 (C1'), 140.4 (C3'), 140.5 (C4'), 151.2 (C3a), 174.5 (C2'), 183.4 (C2); m/z 518.20, m_{th} : 518.03; elemental analysis calcd. for $\text{C}_{24}\text{H}_{24}\text{ClNRuS} \cdot 0.75\text{H}_2\text{O}$: C 56.68, H 5.05, N 2.75, S 6.31%; found: C 56.65, H 4.89, N 2.76, S 6.19%.

[(Chlorido)(2-(4'-methoxyphenyl)benzothiazolato- κN , $\kappa\text{C2}'$)(η^6 -*p*-cymene)ruthenium(II)] (5a)

The synthesis was performed according to the general complexation procedure, using bis[dichlorido(η^6 -*p*-cymene)ruthenium(II)] (117 mg, 187 μmol), sodium acetate (38 mg, 460 μmol), MeOH:DCM (3:1, 4 mL), and 2-(4-methoxyphenyl)benzothiazole **L5** (100 mg, 416 μmol). The reaction mixture stirred at 40°C for 24 h. The product was obtained as red crystals (120 mg, 63%). Mp: >148°C (decomposition), Solubility: 0.08 mg/mL = 0.16 mM (MEM, 1% DMF); ^1H -NMR (500.10 MHz, CDCl_3) δ : 0.77 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 0.92 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 2.13 (s, 3H,

Hg), 2.25 [sept, $^3J(\text{H,H}) = 7 \text{ Hz}$, 1H, He], 3.94 (s, 3H, H7'), 5.12 [d, $^3J(\text{H,H}) = 6 \text{ Hz}$, 1H, Hb], 5.38 [d, $^3J(\text{H,H}) = 6 \text{ Hz}$, 1H, Hc], 5.70 [d, $^3J(\text{H,H}) = 6 \text{ Hz}$, 1H, Hc], 5.88 [d, $^3J(\text{H,H}) = 6 \text{ Hz}$, 1H, Hb], 6.63 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 2 \text{ Hz}$, 1H, H5'], 7.40 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H6], 7.59 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H5], 7.62 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H6'], 7.77 [d, $^4J(\text{H,H}) = 2 \text{ Hz}$, 1H, H3'], 7.81 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H7], 8.30 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H4]; ^{13}C -NMR (125.75 MHz, CDCl_3) δ : 19.1 (Cg), 21.8 (Cf), 22.9 (Cf), 31.0 (Ce), 55.4 (C7'), 80.6 (Cb), 82.5 (Cc), 90.0 (Cc), 90.5 (Cb), 98.8 (Cd), 102.5 (Ca), 109.8 (C5'), 120.8 (C4), 122.8 (C7), 124.1 (C3'), 124.9 (C6), 127.1 (C6'), 127.3 (C5), 131.9 (C7a), 132.9 (C1'), 151.2 (C3a), 160.3 (C4'), 173.9 (C2'), 185.7 (C2); m/z 534.20, m_{th} : 534.02; elemental analysis calcd. for $\text{C}_{24}\text{H}_{24}\text{ClNRuS}$: C 56.41, H 4.73, N 2.74, S 6.27%; found: C 56.27, H 4.63, N 2.78, S 6.14%.

[(Chlorido)(2-(4'-aminophenyl)benzothiazolato- κN , $\kappa\text{C2}'$)(η^6 -*p*-cymene)ruthenium(II)] (6a)

The synthesis was performed according to the general complexation procedure, using bis[dichlorido(η^6 -*p*-cymene)ruthenium(II)] (451 mg, 737 μmol), sodium acetate (152 mg, 1,810 μmol), MeOH:DCM (3:1, 4 mL), and 2-(4-aminophenyl)benzothiazole **L6** (370 mg, 1,640 μmol). The reaction mixture stirred at 40°C for 24 h under argon. The product was obtained as a green powder (73 mg, 10%). Mp: >222°C (decomposition), Solubility: 0.08 mg/mL = 0.16 mM (MEM, 1% DMF); ^1H -NMR (500.10 MHz, CDCl_3) δ : 0.76 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 0.91 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 2.12 (s, 3H, Hg), 2.24 [sept, $^3J(\text{H,H}) = 7 \text{ Hz}$, 1H, He], 5.06 [d, $^3J(\text{H,H}) = 6 \text{ Hz}$, 1H, Hb], 5.37 [d, $^3J(\text{H,H}) = 6 \text{ Hz}$, 1H, Hc], 5.67 [d, $^3J(\text{H,H}) = 6 \text{ Hz}$, 1H, Hc], 5.88 [d, $^3J(\text{H,H}) = 6 \text{ Hz}$, 1H, Hb], 6.37 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 2 \text{ Hz}$, 1H, H5'], 7.35 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H6], 7.48 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H6'], 7.52–7.59 (m, 2H, H3', H5), 7.77 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H7], 8.25 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H4]; ^{13}C -NMR (125.75 MHz, CDCl_3) δ : 19.1 (Cg), 21.9 (Cf), 22.8 (Cf), 31.0 (Ce), 80.2 (Cb), 82.6 (Cc), 89.7 (Cc), 90.5 (Cb), 98.4 (Cd), 102.4 (Ca), 110.8 (C5'), 120.4 (C4), 122.7 (C7), 124.5 (C6), 124.8 (C3'), 127.0 (C5), 127.5 (C6'), 130.6 (C1'), 131.7 (C7a), 148.2 (C4'), 151.3 (C3a), 173.9 (C2'), 185.6 (C2); m/z 461.24, m_{th} : 461.06; elemental analysis calcd. for $\text{C}_{23}\text{H}_{23}\text{ClN}_2\text{RuS} \cdot 0.25\text{H}_2\text{O}$: C 55.19, H 4.73, N 5.60, S 6.41%; found: C 55.11, H 4.74, N 5.47, S 6.42%.

[(Chlorido)(2-phenylbenzothiazolato- κN , $\kappa\text{C2}'$)(η^6 -*p*-cymene)osmium(II)] (1b)

The synthesis was performed according to the general complexation procedure, using bis[dichlorido(η^6 -*p*-cymene)osmium(II)] (100 mg, 130 μmol), sodium acetate (26 mg, 310 μmol), MeOH:DCM (3:1, 4 mL), and 2-phenylbenzothiazole **L1** (60 mg, 280 μmol). The reaction mixture was irradiated with microwaves for 1.5 h at 70°C. The product was obtained after a second precipitation as red crystals (91 mg, 63%). Mp: >228°C (decomposition), Solubility: 0.09 mg/mL = 0.16 mM (MEM, 1% DMF); ^1H -NMR (500.10 MHz, CDCl_3) δ : 0.71 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 0.87 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 2.16 [sept, $^3J(\text{H,H}) = 7 \text{ Hz}$, 1H, He],

2.27 (s, 3H, Hg), 5.37 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hb], 5.66 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hc], 5.75 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hc], 5.89 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hb], 7.06 [ddd, d, $^3J(\text{H,H}) = 7 \text{ Hz}$, $^3J(\text{H,H}) = 7 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H5'], 7.15 [ddd, $^3J(\text{H,H}) = 7 \text{ Hz}$, $^3J(\text{H,H}) = 7 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H4'], 7.45 [ddd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H6], 7.60 [ddd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H5], 7.75 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H6'], 7.86 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H7], 8.12 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H3'], 8.17 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H4]; ^{13}C -NMR (125.75 MHz, CDCl_3) δ : 19.0 (Cg), 22.1 (Cf), 23.2 (Cf), 31.4 (Ce), 69.7 (Cb), 73.2 (Cc), 80.7 (Cc), 80.8 (Cb), 89.6 (Cd), 96.8 (Ca), 121.7 (C4), 123.0 (C5'), 123.0 (C7), 125.6 (C6), 125.8 (C6'), 127.4 (C5), 131.3 (C3'), 132.0 (C7a), 139.7 (C4'), 139.9 (C1'), 150.9 (C3a), 169.0 (C2'), 178.1 (C2); m/z 594.26, m_{th} : 594.07; elemental analysis calcd. for $\text{C}_{23}\text{H}_{22}\text{ClNO}_5\text{S}$: C 48.45, H 3.89, N 2.46, S 5.62%; found: C 48.25, H 3.86, N 2.52, S 5.55%.

[(Chlorido)(2-(4'-fluorophenyl)benzothiazolato- κN , $\kappa\text{C}2'$)(η^6 -*p*-cymene)osmium(II)] (2b)

The synthesis was performed according to the general complexation procedure, using bis[dichlorido(η^6 -*p*-cymene)osmium(II)] (200 mg, 225 μmol), sodium acetate (51 mg, 620 μmol), and MeOH (4 mL). Before the addition of the ligand, the mixture was irradiated under microwave conditions for 5 min at 40°C. After addition of 2-(4-fluorophenyl)benzothiazole **L2** (130 mg, 562 μmol), the reaction mixture was again irradiated for 20 min at 90°C. The product was obtained as a gray powder (124 mg, 42%). Mp: >227°C (decomposition), Solubility: 0.10 mg/mL = 0.17 mM (MEM, 1% DMF); ^1H -NMR (500.10 MHz, CDCl_3) δ : 0.72 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 0.88 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 2.17 [sept, $^3J(\text{H,H}) = 7 \text{ Hz}$, 1H, He], 2.27 (s, 3H, Hg), 5.37 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hb], 5.68 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hc], 5.72 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hc], 5.88 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hb], 6.77 [ddd, d, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 2 \text{ Hz}$, $^4J(\text{H,F}) = 2 \text{ Hz}$, 1H, H5'], 7.45 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H6], 7.61 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H5], 7.71–7.80 (m, 2H, H3', H6'), 7.85 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H7], 8.13 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H4]; ^{13}C -NMR (125.75 MHz, CDCl_3) δ : 19.0 (Cg), 22.1 (Cf), 23.2 (Cf), 31.4 (Ce), 70.0 (Cb), 73.8 (Cc), 80.7 (Cc), 80.8 (Cb), 90.1 (Cd), 97.3 (Ca), 110.8 [d, $^3J(\text{C,F}) = 24 \text{ Hz}$, 1C, C5'], 121.6 (C4), 123.0 (C7), 125.4 [d, $^3J(\text{C,F}) = 17 \text{ Hz}$, 1C, C3'], 125.6 (C6), 127.5 [d, $^3J(\text{C,F}) = 9 \text{ Hz}$, 1C, C6'], 127.5 (C5), 131.8 (C7a), 136.4 [d, $^4J(\text{C,F}) = 2 \text{ Hz}$, 1C, C1'], 150.9 (C3a), 164.0 [d, $^1J(\text{C,F}) = 256 \text{ Hz}$, 1C, C4'], 171.8 [d, $^3J(\text{C,F}) = 6 \text{ Hz}$, 1C, C2'], 176.9 (C2); m/z 612.25, m_{th} : 612.06; elemental analysis calcd. for $\text{C}_{23}\text{H}_{21}\text{ClFNO}_5\text{S} \cdot 0.1\text{H}_2\text{O}$: C 46.82, H 3.62, N 2.37, S 5.44%; found: C 46.49, H 3.70, N 2.31, S 5.15%.

[(Chlorido)(2-(4'-chlorophenyl)benzothiazolato- κN , $\kappa\text{C}2'$)(η^6 -*p*-cymene)osmium(II)] (3b)

The synthesis was performed according to the general complexation procedure, using bis[dichlorido(η^6 -*p*-cymene)osmium(II)] (250 mg, 316 μmol), sodium acetate (63 mg, 772 μmol), and MeOH (4 mL). Before the addition of the ligand, the mixture was irradiated under microwave

conditions for 5 min at 40°C. After addition of 2-(4-chlorophenyl)benzothiazole **L3** (172 mg, 702 μmol), the reaction mixture was again irradiated for 20 min at 90°C. The product was obtained as orange crystals (87 mg, 23%). Mp: >202°C (decomposition), Solubility: 0.04 mg/mL = 0.07 mM (MEM, 1% DMF); ^1H -NMR (500.10 MHz, CDCl_3) δ : 0.71 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 0.88 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 2.16 [sept, $^3J(\text{H,H}) = 7 \text{ Hz}$, 1H, He], 2.28 (s, 3H, Hg), 5.39 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hb], 5.70 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hc], 5.74 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hc], 5.89 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hb], 7.04 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 2 \text{ Hz}$, 1H, H5'], 7.46 [ddd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H6], 7.61 [ddd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H5], 7.67 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H6'], 7.86 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^4J(\text{H,H}) = 1 \text{ Hz}$, 1H, H7], 8.08 [d, $^4J(\text{H,H}) = 2 \text{ Hz}$, 1H, H3'], 8.17 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H4]; ^{13}C -NMR (125.75 MHz, CDCl_3) δ : 19.0 (Cg), 22.1 (Cf), 23.2 (Cf), 31.4 (Ce), 69.8 (Cb), 73.9 (Cc), 80.7 (Cc), 80.8 (Cb), 90.1 (Cd), 97.4 (Ca), 121.7 (C4), 123.1 (C7), 123.4 (C5'), 125.8 (C6), 126.6 (C6'), 127.6 (C5), 131.9 (C7a), 137.1 (C1'), 138.5 (C4'), 139.0 (C3'), 150.9 (C3a), 170.3 (C2'), 177.0 (C2); m/z 628.20, m_{th} : 628.03; elemental analysis calcd. for $\text{C}_{23}\text{H}_{21}\text{Cl}_2\text{NO}_5\text{S}$: C 45.69, H 3.50, N 2.32, S 5.30%; found: C 45.75, H 3.46, N 2.29, S 5.27%.

[(Chlorido)(2-(4'-methylphenyl)benzothiazolato- κN , $\kappa\text{C}2'$)(η^6 -*p*-cymene)osmium(II)] (4b)

The synthesis was performed according to the general complexation procedure, using bis[dichlorido(η^6 -*p*-cymene)osmium(II)] (120 mg, 150 μmol), sodium acetate (30 mg, 370 μmol), and MeOH (4 mL). Before the addition of the ligand, the mixture was irradiated under microwave conditions for 5 min at 40°C. After addition of 2-(4-methylphenyl)benzothiazole **L4** (76 mg, 340 μmol), the reaction mixture was again irradiated for 25 min at 90°C. The product was obtained as yellow needles (109 mg, 62%). Mp: >192°C (decomposition), Solubility: 0.03 mg/mL = 0.05 mM (MEM, 1% DMF); ^1H -NMR (500.10 MHz, CDCl_3) δ : 0.71 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 0.87 [d, $^3J(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 2.15 [sept, $^3J(\text{H,H}) = 7 \text{ Hz}$, 1H, He], 2.27 (s, 3H, Hg), 2.43 (s, 3H, H7'), 5.36 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hb], 5.67 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hc], 5.73 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hc], 5.87 [d, $^3J(\text{H,H}) = 5 \text{ Hz}$, 1H, Hb], 6.88 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H5'], 7.42 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H6], 7.58 [dd, $^3J(\text{H,H}) = 8 \text{ Hz}$, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H5], 7.64 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H6'], 7.83 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H7], 7.93 (s, 1H, H3'), 8.13 [d, $^3J(\text{H,H}) = 8 \text{ Hz}$, 1H, H4]; ^{13}C -NMR (125.75 MHz, CDCl_3) δ : 19.0 (Cg), 22.1 (Cf), 22.2 (C7'), 23.2 (Cf), 31.4 (Ce), 69.4 (Cb), 73.4 (Cc), 80.5 (Cc), 80.6 (Cb), 89.3 (Cd), 96.7 (Ca), 121.5 (C4), 122.9 (C7), 124.3 (C5'), 125.3 (C6), 125.6 (C6'), 127.3 (C5), 131.8 (C7a), 137.4 (C1'), 140.1 (C3'), 141.6 (C4'), 150.9 (C3a), 169.1 (C2'), 178.0 (C2); m/z 608.26, m_{th} : 608.08; elemental analysis calcd. for $\text{C}_{24}\text{H}_{24}\text{ClNO}_5\text{S} \cdot 0.5\text{H}_2\text{O}$: C 48.59, H 4.25, N 2.36, S 5.41%; found: C 48.38, H 4.11, N 2.26, S 5.10%.

[(Chlorido)(2-(4'-methoxyphenyl)benzothiazolato- κN , $\kappa\text{C}2'$)(η^6 -*p*-cymene)osmium(II)] (5b)

The synthesis was performed according to the general complexation procedure, using

bis[dichlorido(η^6 -*p*-cymene)osmium(II)] (150 mg, 190 μ mol), sodium acetate (38 mg, 465 μ mol), and MeOH (4 mL). Before the addition of the ligand, the mixture was irradiated under microwave conditions for 5 min at 40°C. After addition of 2-(4-methoxyphenyl)benzothiazole **L5** (102 mg, 422 μ mol), the reaction mixture was again irradiated for 30 min at 90°C. The product was obtained as brown needles (74 mg, 32%). Mp: >137°C (decomposition), Solubility: 0.02 mg/mL = 0.03 mM (MEM, 1% DMF); $^1\text{H-NMR}$ (500.10 MHz, CDCl_3) δ : 0.72 [d, $^3\text{J}(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 0.88 [d, $^3\text{J}(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 2.16 [sept, $^3\text{J}(\text{H,H}) = 7 \text{ Hz}$, 1H, He], 2.27 (s, 3H, Hg), 3.93 (s, 3H, H7'), 5.35 [d, $^3\text{J}(\text{H,H}) = 5 \text{ Hz}$, 1H, Hb], 5.66 [d, $^3\text{J}(\text{H,H}) = 5 \text{ Hz}$, 1H, Hc], 5.71 [d, $^3\text{J}(\text{H,H}) = 5 \text{ Hz}$, 1H, Hc], 5.86 [d, $^3\text{J}(\text{H,H}) = 5 \text{ Hz}$, 1H, Hb], 6.63 [dd, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, $^4\text{J}(\text{H,H}) = 2 \text{ Hz}$, 1H, H5'], 7.40 [ddd, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, $^4\text{J}(\text{H,H}) = 1 \text{ Hz}$, 1H, H6], 7.56 [ddd, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, $^4\text{J}(\text{H,H}) = 1 \text{ Hz}$, 1H, H5], 7.61 [d, $^4\text{J}(\text{H,H}) = 2 \text{ Hz}$, 1H, H3'], 7.70 [d, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, 1H, H6'], 7.81 [d, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, 1H, H7'], 8.09 [d, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, 1H, H4]; $^{13}\text{C-NMR}$ (125.75 MHz, CDCl_3) δ : 18.9 (Cg), 22.1 (Cf), 23.2 (Cf), 31.4 (Ce), 55.4 (C7'), 69.7 (Cb), 73.5 (Cc), 80.4 (Cc), 80.5 (Cb), 89.4 (Cd), 96.8 (Ca), 109.9 (C4'), 121.2 (C4), 122.9 (C7), 123.5 (C5'), 125.0 (C6), 127.2 (C6'), 127.3 (C5), 131.7 (C7a), 133.4 (C1'), 151.0 (C3a), 161.5 (C4'), 171.2 (C2'), 177.2 (C2); m/z 624.24, m_{th} : 624.08; elemental analysis calcd. for $\text{C}_{24}\text{H}_{24}\text{ClNOOsS}\cdot\text{H}_2\text{O}$: C 46.63, H 4.24, N 2.27, S 5.19%; found: C 46.66, H 3.92, N 2.29, S 5.06%.

[(Chlorido)(2-(4'-aminophenyl)benzothiazolato- κN , $\kappa\text{C2'$)(η^6 -*p*-cymene)osmium(II)] (**6b**)

The synthesis was performed according to the general complexation procedure, using bis[dichlorido(η^6 -*p*-cymene)osmium(II)] (100 mg, 130 μ mol), sodium acetate (26 mg, 310 μ mol), and MeOH (4 mL). Before the addition of the ligand, the mixture was irradiated under microwave conditions for 5 min at 40°C. After addition of 2-(4-aminophenyl)benzothiazole **L6** (64 mg, 280 μ mol), the reaction mixture was again irradiated for 45 min at 90°C. The product was obtained as a green powder (114 mg, 77%). Mp: >134°C (decomposition), Solubility: 0.06 mg/mL = 0.10 mM (MEM, 1% DMF); $^1\text{H-NMR}$ (500.10 MHz, CDCl_3) δ : 0.72 [d, $^3\text{J}(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 0.88 [d, $^3\text{J}(\text{H,H}) = 7 \text{ Hz}$, 3H, Hf], 2.14 [sept, $^3\text{J}(\text{H,H}) = 7 \text{ Hz}$, 1H, He], 2.26 (s, 3H, Hg), 4.00 (s, 2H, H7'), 5.30 [d, $^3\text{J}(\text{H,H}) = 5 \text{ Hz}$, 1H, Hb], 5.64 [d, $^3\text{J}(\text{H,H}) = 5 \text{ Hz}$, 1H, Hc], 5.68 [d, $^3\text{J}(\text{H,H}) = 5 \text{ Hz}$, 1H, Hc], 5.85 [d, $^3\text{J}(\text{H,H}) = 5 \text{ Hz}$, 1H, Hb], 6.38 [dd, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, $^4\text{J}(\text{H,H}) = 2 \text{ Hz}$, 1H, H5'], 7.37 [d, $^4\text{J}(\text{H,H}) = 2 \text{ Hz}$, 1H, H3'], 7.54 [dd, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, 1H, H5], 7.76 [d, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, 1H, H6'], 7.89 [d, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, 1H, H7], 8.03 [d, $^3\text{J}(\text{H,H}) = 8 \text{ Hz}$, 1H, H4]; $^{13}\text{C-NMR}$ (125.75 MHz, CDCl_3) δ : 18.9 (Cg), 22.1 (Cf), 23.2 (Cf), 31.4 (Ce), 69.3 (Cb), 73.6 (Cc), 80.2 (Cc), 80.4 (Cb), 88.8 (Cd), 96.6 (Ca), 110.8 (C5'), 120.8 (C4), 122.7 (C7), 124.3 (C3'), 124.6 (C6), 127.1 (C5), 127.5 (C6'), 131.1 (C1'), 131.4 (C7a), 149.5 (C4'), 151.0 (C3a), 171.1 (C2'), 177.2 (C2); m/z 609.25, m_{th} : 609.08; elemental analysis calcd. for $\text{C}_{23}\text{H}_{23}\text{ClN}_2\text{OsS}\cdot 0.5\text{H}_2\text{O}$: C 46.49, H 4.07, N 4.71, S 5.40%; found: C 46.30, H 4.07, N 4.48, S 5.02%.

ESI-MS Experiments

Electrospray ionization mass spectra were recorded on a *Bruker AmaZon SL ion trap mass spectrometer* (Bruker Daltonics GmbH). The data was acquired and processed using Compass 1.3 and Data Analysis 4.0 software packages (Bruker Daltonics GmbH). Protein spectra were deconvoluted by applying the maximum entropy algorithm with a m/z 0.2 mass step and a m/z 0.5 instrument peak width. The mass spectra were recorded by direct infusion with a sample concentration of 5 μM and a flow rate of 240 $\mu\text{L/h}$. Amino acids and nucleoside triphosphates were measured with the following settings: dry temperature 180°C, nebulizer 8.00 psi, dry gas 6 L/min, and the high voltage capillary was set to $\pm 4.5 \text{ kV}$ for positive and negative modes and the target mass was set to m/z 600. Proteins were measured with the following settings: dry temperature 180°C, nebulizer 6.00 psi, dry gas 6.00 L/min, the high voltage capillary was set to -3.5 kV and the target mass was set to m/z 1,000. Amino acids, nucleoside triphosphates and stability measurement samples were diluted with water: methanol mixture (50: 50), whereas protein samples were diluted with water: methanol: formic acid (49.9: 49.9: 0.2). The stock solutions of the ligands and complexes were prepared in DMF and diluted to a final concentration of 1% DMF in H_2O . The molar ratios used for the interaction studies were ligand/complex: L-histidine: L-cysteine: L-methionine (1: 1: 1: 1), ligand/complex: N-Ac-L-cysteine: N-Ac-L-histidine: N-Ac-L-methionine: and Se-methyl-L-cysteine (1: 1: 1: 1: 1), ligand/complex: Se-L-cystine: DTT (1: 1: 1), ligand/complex: 5'-ATP: 5'-GTP (1: 1: 1), ligand/complex: amino acids: nucleoside triphosphates (1: 1: 1) and ligand/complex: cytochrome-c: ubiquitin (1: 1: 1). The mass spectra were recorded after 1, 3, 6, 24, and 48 h of incubation at 37°C. The relative intensities described in the discussion section refer to the percent peak area of all visible ruthenium and osmium adducts in the spectrum.

Cell Culture

CH1/PA-1 (ovarian teratocarcinoma) cells were a generous gift from Lloyd R. Kelland (CRC Center for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK). A549 and SW480 cells were kindly provided by the Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Austria. All cell lines were grown as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with L-glutamine (4 mM), sodium pyruvate (1 mM), and 1% (v/v) non-essential amino acid solution (all from Sigma-Aldrich) and 10% (v/v) heat-inactivated fetal calf serum (FCS, from BioWest) in 75 cm^2 flasks (Starlab) at 37°C under a humidified atmosphere containing 5% CO_2 in air.

MTT Assay

Antiproliferative activity of the compounds was determined with the colorimetric MTT assay (MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). 1×10^3 CH1/PA-1, 2×10^3 SW480 and 3×10^3 A549 cells were seeded in 100 μL per well into 96-well microculture plates (Starlab). After 24 h, test compounds were dissolved in DMF (Acros), serially diluted in complete MEM not to exceed a

final DMF content of 0.5% v/v and added in 100 μ L per well. After 96 h, the drug-containing medium was replaced with 100 μ L of RPMI 1640/MTT mixture [6 parts of RPMI 1640 medium (Sigma-Aldrich; supplemented with 10% heat-inactivated fetal bovine serum and 4 mM L-glutamine), 1 part of MTT solution in phosphate-buffered saline (5 mg/mL; both from Sigma-Aldrich)]. After incubation for 4 h, the MTT-containing medium was replaced with 150 μ L DMSO per well to dissolve the formazan product formed by viable cells. Optical densities at 550 nm (and at the reference wavelength of 690 nm) were measured with a microplate reader (ELx808, Bio-Tek). The 50% inhibitory concentrations (IC₅₀) relative to untreated controls were interpolated from concentration-effect curves. At least three independent experiments were performed, each with triplicates per concentration level.

Flow Cytometry

Compounds were studied for their capacity of inducing programmed cell death (apoptosis) or necrosis by the flow-cytometric annexin V/propidium iodide (PI) assay. SW480 cells were harvested by using trypsin and 7×10^4 cells were placed into 24-well culture plates (Starlab) in 600 μ L of complete MEM per well. After 24 h preincubation, cells were treated with different concentrations of the test compounds. The 20 mM stock solutions of the test compounds in DMF were diluted with MEM so that the final DMF concentration never exceeded 0.5% v/v of the treatment solution to avoid DMF toxicity. After incubation for 24 h at 37°C under 5% CO₂, the media were aspirated and collected; cells were washed once with 37°C warm PBS, trypsinised with TrypLE™ Express (Thermo Fisher) and mixed with the corresponding supernatant. Cell pellets were obtained by centrifugation (300 g, 3 min) and the supernatants were discarded. Then, cells were resuspended with FITC-conjugated annexin V (0.25 μ g/mL; BioVision) in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂), incubated at 37°C for 15 min and stained with PI (1 μ g/mL; Fluka) shortly before measurement with a Millipore Guava easyCyte 8HT flow cytometer and InCyte software. The resulting dot plots were analyzed with FlowJo software (TreeStar). All experiments were performed at least three times independently using the same conditions, and each comprised of three replicates.

Cellular Accumulation

Accumulation of selected compounds in SW480 cells was determined analogous to a method recently developed for the quantification of Os in biological samples (Klose et al., 2017), which was applied here to Ru as well for reasons of comparability. 1.8×10^5 cells per well were seeded in 1 mL of complete MEM into 12-well plates (Starlab) and incubated at 37°C for 24 h. Test compounds were dissolved in DMF, diluted to 50 μ M (0.5% DMF) with complete MEM and added in 500 μ L per well upon removal of the medium that had been used for seeding. After exposure for 2 h at 37°C, the medium was removed and cells were washed three times with phosphate buffered saline (PBS; Sigma-Aldrich) and lysed with 400 μ L of an adjusted radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1.0% Triton X-100, 0.1% SDS, and 50 mM Tris, pH 8.0) on a

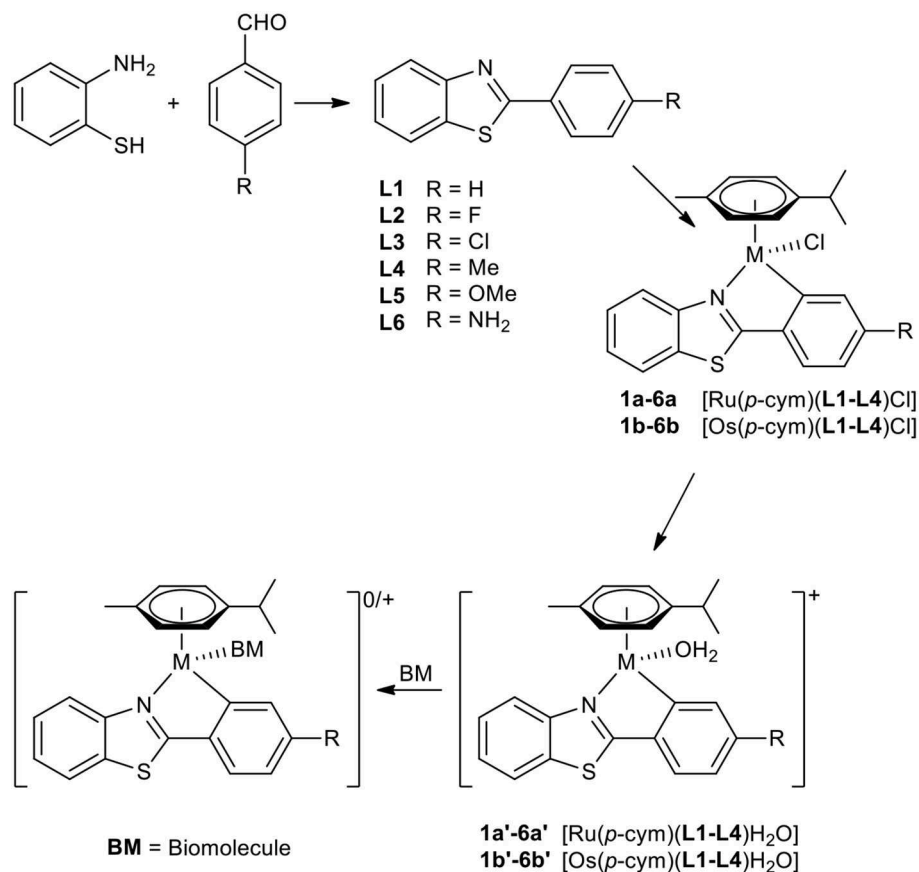
shaker for 2 h at 4°C. Residues were scraped off the wells, and 350 μ L of lysates were transferred into 1.5 mL microtubes and centrifuged at 14,000 rpm for 10 min at 4°C. Three hundred microliter of lysates were diluted with a stabilization solution (ascorbic acid, thiourea, EDTA, each 0.5 mM in water, and HNO₃ as appropriate to yield a final content of 3%) to final volumes of 8 mL. Ru and Os were quantified by ICP-MS with an Agilent 7500ce instrument equipped with a CETAC ASX-520 autosampler and a MicroMist nebulizer at a sample uptake rate of ~ 0.25 mL/min. The Agilent MassHunter software package (Workstation Software, version B.01.01, Build 123.11, Patch 4, 2012) was used for data processing. Nickel was used as cone material. The experimental parameters were as follows: 1,560 W RF power, 0.92–0.97 L/min carrier gas, 0.22–0.27 L/min make up gas, 15 L/min plasma gas, 0.3 s dwell time and 10 replicates each with 100 sweeps. Results are the means of at least three independent experiments. The ICP-MS was tuned on a daily basis to achieve maximum sensitivity. Additional instrument parameters can be found in **Table S11**.

Fluorescence Microscopy

SW480 cells were seeded on coverslips in 6-well plates (1.5×10^5 cells per well in 2 mL of MEM) and allowed to attach overnight. Cells were incubated with **L6** and **6a** (50 μ M, 60–120 min) in MEM and stained with LysoTracker Red (Invitrogen) (1 μ M, 5 min). After removing the medium and washing with PBS (1 $\times$), the cells were treated with fixing solution (2 % paraformaldehyde in PBS) for 5 min and washed with PBS (1 $\times$). A BX40 fluorescence microscope with an F-View CCD camera, Cell $\wedge$ F fluorescence imaging software, and a 60 $\times$ magnification oil immersion objective lens (all from Olympus) were used for the visualization of compound distribution. The green fluorescence signal of compounds **L6** and **6a** upon excitation at wavelengths of 330–395 nm as well as the red fluorescence signal of LysoTracker Red upon excitation at wavelengths of 510–550 nm were recorded. The exposure time (100 ms) for both channels was kept as short as possible to minimize bleaching. The recorded images were colocalized by means of ImageJ 1.52a and GIMP 2.10.12 software. The correlation analysis of fluorescence images was performed by using Fiji ImageJ software (<https://imagej.net/Fiji/Downloads>). To evaluate the degree of colocalization of the green (drug) and red (LysoTracker) signals, Pearson's correlation coefficients were calculated (ImageJ, Coloc2 plugin). For this purpose, regions of interest (ROIs) were selected to delineate single cells for analysis ($n = 15$ for each sample).

RESULTS AND DISCUSSION

A set of six 4'-substituted 2-phenylbenzothiazoles and their respective Ru(II) and Os(II) complexes was synthesized (**Scheme 1**). The ligands were prepared by facilitating an acid catalyzed nucleophilic attack of the amino group of 2-aminothiophenol on the carbonyl carbon of the appropriate 4-substituted benzaldehyde, followed by oxidative ring closure to form the desired product in good yields. Their respective complexes were synthesized by initial activation of the dimeric precursor [M(cym)Cl₂]₂ by sodium acetate, where



SCHEME 1 | Synthesis of compounds **1a-6b** and the supposed behavior in biological media.

M is either Ru(II) or Os(II). Addition of the appropriate 2-phenylbenzothiazole lead to the formation of the desired C,N-metallacycle. The reactions were performed either at 40°C (ruthenium complexes **1a-6a** and **1b**) or under microwave conditions (osmium complexes **2b-6b**). After work-up the pure products were obtained by precipitation from dichloromethane (DCM) and *n*-hexane.

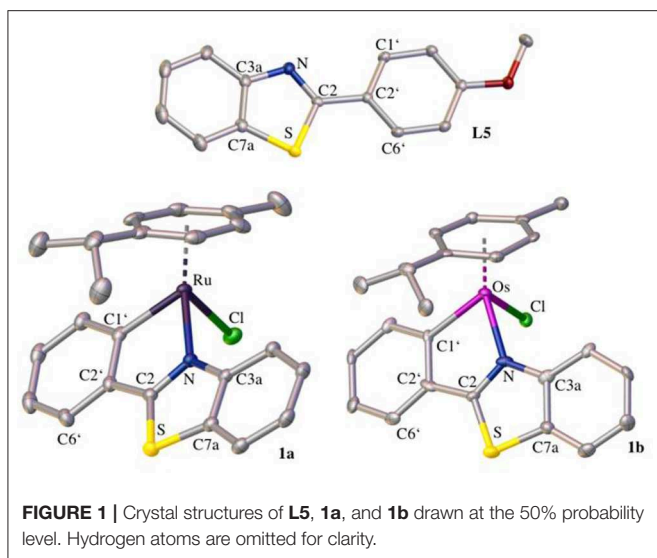
Characterization

All ligands and complexes were characterized by means of ¹H-NMR, ¹³C-NMR in DMSO-*d*₆ (ligands) and CDCl₃ (complexes), elemental analysis, and ESI-MS. ¹H-¹H-COSY, ¹H-¹³C-HMBC, and ¹H-¹³C-HSQC were recorded to unambiguously assign the detected NMR signals. Furthermore, their melting points and solubilities were determined. ¹H and ¹³C-NMR spectroscopy confirmed the formation of the desired metallacycles. The ligand spectra showed two doublets for the 4-substituted phenyl ring (see **Figures S1-S3**). Upon C,N-coordination the symmetry is broken, yielding separate signals for the phenyl protons. Additionally, the coordinated benzothiazole ligand hinders inversion at the metal center in aprotic solvents such as CDCl₃. As a consequence, the arene protons of *p*-cymene were found as four distinct doublets (see **Figures S3-S9**). At first glance complex spectra of **6a,b** look impure; however, it was found that

partial protonation of the aniline took place due to common acid traces in CDCl₃, leading to two sets of signals.

The crystal structure of **L5** and the ruthenium(II) and osmium(II) complex of 2-phenylbenzothiazole (**1a** and **1b**, respectively) was determined via X-ray diffraction analysis (**Tables S2-S8**).

The elemental cell of **L5** was composed of four molecules with slightly different bond lengths. **L5** crystallized in the monoclinic crystal system and in the crystal structure (**Figure 1**) the distortion of the thiazole ring due to the heteroatoms is clearly visible. The bond length between C3a and C7a ranges from 1.4072 to 1.4104 Å, whereas the carbon-nitrogen bonds are slightly shorter (C3a-N: 1.3858–1.3944 Å, C2-N: 1.3026–1.3113 Å) and the carbon-sulfur bonds are significantly longer (C7a-S: 1.7225–1.7341 Å, C2-S: 1.7566–1.7625 Å). This distortion is caused by the difference in size between the carbon atoms and the sulfur and nitrogen atoms. Further bond lengths and angles are listed in **Table S1**. Both the ruthenium and the osmium complex crystallized in an orthorhombic crystal system in the centrosymmetric space group P2₁2₁2₁. The crystal structures (**Figure 1**) illustrate the typical piano-stool configuration, with the facial coordinated cym as the seat of the piano-stool and the chlorido ligand and the bidentate 2-phenylbenzothiazole ligand acting as the three legs. In the pseudo-octahedral geometry of the



complex, a new distorted five-membered ring is formed by the chelating ligand and the metal center. The coordination bonds are very similar for both metal analogs, which was to be expected as the atomic radii for both metals differ only by 0.01 Å (Ru: 1.34 Å, Os: 1.35 Å). In the ruthenium complex, the bond length is 2.1115(19) Å and 2.047(2) Å for the coordination bond between the metal and the nitrogen and carbon atom, respectively. The analogous bond lengths in the osmium complex are 2.109(7) and 2.073(19) Å. The M–Cl distance is in the expected range, with 2.4133(6) and 2.411(2) Å in the ruthenium and osmium complex, respectively. Moreover, the coordination ring is not planar and features a torsion angle of 5.1(2)° (between M–N–C2–C1') in the ruthenium complex. In the osmium complex this twist is less pronounced with a torsion angle of 2.4(10)°. Due to this torsion, the thiazole ring loses its planarity as well and shows a slight torsion of 1.4(2)° in complex **1a** and 2.1(10)° in complex **1b** (between C3a–N–C2–S). When comparing the structure of the free ligand and the ligand in the complex, it is evident that the coordination to the metal atom causes the bond between the benzothiazole ring system and the phenyl ring as well as the bond C1'–C6' to shorten, while the bond C1'–C2' is elongated. Although this effect is apparent in both metal complexes, it is slightly more prominent in the osmium complex. In the benzothiazole ring system, more differences between the two complexes become apparent. For instance, in the ruthenium complex the bond C2–N is stretched minimally upon coordination (for the bond C3a–N no difference in bond length can be observed), whereas in the osmium complex both bonds C2–N and C3a–N appear shortened. In general, complexation with the osmium center seems to have a more pronounced effect on bond lengths and angles than coordination to ruthenium.

Stability Investigations

The stability of **1a**, **6a**, **1b**, and **6b** in aqueous solution over a period of 48 h at 37°C was investigated to confirm sufficient

stability for further biological experiments. All complexes form immediately the corresponding aqua species after dissolution in aqueous systems. Mass spectra were recorded after 1, 3, 6, 24, and 48 h. **L1** and **L6** were examined as well and were completely stable. As expected no solvent adducts or decomposition products were detected for the investigated organometallics over 48 h.

1a proved to be nearly stable over the entire period, with a characteristic $[M-Cl]^+$ peak (m/z 446.07 $\pm$ 0.01, m_{th} : 446.05), with only minor traces of a dimeric hydrolysis product starting to form after 6 h (m/z 550.06 $\pm$ 0.03), until it reached a total of 7% after 48 h. Complex **6a** showed the $[M-Cl]^+$ peak (m/z 461.08 $\pm$ 0.01, m_{th} : 461.07) and no solvent adduct peaks were found. Osmium complexes **1b** and **6b** were stable over the entire period and only the peaks $[M-Cl]^+$ were found (m/z 536.11 $\pm$ 0.01, m_{th} : 536.11) and (m/z 551.11 $\pm$ 0.01, m_{th} : 551.12), respectively.

Understanding the affinity of the selected compounds (**1a**, **6a**, **1b**, and **6b**) toward small biomolecules like amino acids, proteins and nucleoside triphosphates gives valuable information about possible modes of action *in vivo*. In order to gain this understanding, the compounds were incubated with the respective biomolecule-containing solution for 48 h at 37°C. Again, the mass spectra were recorded after 1, 3, 6, 24, and 48 h. Solution 1 (equimolar mixture of L-cysteine [Cys], L-histidine [His] and L-methionine [Met] in 1% DMF) was used to point out preferences toward the typical donor amino acids. Solution 2 (equimolar mixture of N-Ac-L-cysteine [N-Ac-Cys], N-Ac-L-histidine [N-Ac-His], N-Ac-L-methionine [N-Ac-Met] and Se-methyl-L-cysteine [Cys-Se^{Me}] in 1% DMF) was applied as an adapted amino acid model closer to proteins, where bidentate binding is not accessible. Solution 3 (equimolar mixture of Se-L-cysteine and DTT in 1% DMF) was tested with regards to selenium binding affinity of the complexes. Solution 4 (equimolar mixture of 5'-ATP [ATP] and 5'-GTP [GTP] in 1% DMF) is a mixture used to determine possible binding preferences toward the typical DNA targets for metal-based anticancer agents. Solution 5 (equimolar mixture of solution 2 and solution 4) was employed for competitive observations and solution 6 is equimolar mixture of ubiquitin and cytochrome c as potential binding partners in biological systems.

When administered into the blood stream, the first biological nucleophiles able to interact with the compounds are serum proteins. To gain insight into preferences of the investigated complexes, they were incubated with different mixtures of biologically relevant amino acids and model proteins. When incubated with the amino acid containing solutions 1–3 and 5 all complexes showed the characteristic $[M-Cl]^+$ peak $[M(cym)(L)]^+$ (see Table S9). Additionally, all complexes formed a thermodynamically favored adduct with L-methionine with an amount of 49–62% of all assignable adducts with solution 1: $[M(cym)(L)(Met)]^+$ (Ru, **L1**, 56%; Ru, **L6**, 49%; Os, **L1**, 56%; Os, **L6**, 62%; see Table S9). Complex **1a** also formed an additional methionine adduct lacking the ligand $[Ru(cym)(Met)]^+$ (m/z 384.09 $\pm$ 0.02, m_{th} : 384.06, 9%). When incubated with solution 2 all complexes formed a thermodynamically stable $[M(cym)(L)(N-Ac-Met)]^+$ adduct in the range of 59–83% (Ru, **L1**, 59%; Ru, **L6**, 61%; Os, **L1**, 74%; Os, **L6**, 83%; see Table S9).

TABLE 1 | Inhibition of cancer cell growth in three human cancer cell lines; 50% inhibitory concentrations (μM ; means $\pm$ standard deviations), obtained by the MTT assay (exposure time: 96 h).

Compound	A549	SW480	CH1/PA-1
L1	>40	>40	>40
L2	>50	>50	>50
L3	>50	>50	>50
L4	>100	>100	>100
L5	76 $\pm$ 8	>100	44 $\pm$ 7
L6	78 $\pm$ 8	143 $\pm$ 8	27 $\pm$ 4
1a	12 $\pm$ 1	6.8 $\pm$ 0.9	4.1 $\pm$ 0.3
2a	8.4 $\pm$ 0.4	4.0 $\pm$ 0.3	2.7 $\pm$ 0.3
3a	8.2 $\pm$ 0.6	4.1 $\pm$ 0.2	4.0 $\pm$ 0.4
4a	14 $\pm$ 2	8.5 $\pm$ 0.7	7.8 $\pm$ 0.7
5a	10.5 $\pm$ 0.2	5.3 $\pm$ 0.7	4.2 $\pm$ 0.6
6a	16 $\pm$ 2	13 $\pm$ 1	6.4 $\pm$ 1.5
1b	4.0 $\pm$ 0.6	4.8 $\pm$ 0.9	1.2 $\pm$ 0.2
2b	4.0 $\pm$ 0.7	4.4 $\pm$ 0.1	2.1 $\pm$ 0.3
3b	4.9 $\pm$ 1.0	5.5 $\pm$ 1.1	2.0 $\pm$ 0.3
4b	5.6 $\pm$ 1.3	6.1 $\pm$ 1.5	1.7 $\pm$ 0.1
5b	3.9 $\pm$ 0.6	5.0 $\pm$ 1.0	1.3 $\pm$ 0.2
6b	8.7 $\pm$ 0.7	7.2 $\pm$ 1.6	2.5 $\pm$ 0.5

The ruthenium complexes also formed several cysteine adducts: $[\text{Ru}(\text{cym})(\text{L})(\text{N-Ac-Cys})]^+$ (**L** = **L1**, m/z 607.04 $\pm$ 0.02, m_{th} : 607.09, 7%; **L** = **L6**, m/z 624.07 $\pm$ 0.01, m_{th} : 623.77, 11%) and $[\text{Ru}(\text{cym})(\text{Cys-Se}^{\text{Me}})]^+$ (m/z 417.99 $\pm$ 0.02, m_{th} : 417.99, 5–9%). Complex **1a** also formed two additional L-cysteine adducts upon treatment with solution 2: $[\text{Ru}(\text{cym})_2(\text{N-Ac-Cys})_3]^+$ (m/z 958.01 $\pm$ 0.01, m_{th} : 958.12, 8%) and an adduct with N-acetylated L-cysteine: $[\text{Ru}(\text{cym})_2(\text{N-Ac-Cys-Cys-N-Ac})]^+$ (m/z 795.04 $\pm$ 0.03, m_{th} : 795.07, 3%). Apart from the characteristic $[\text{M-Cl}]^+$, all complexes formed an adduct with DTT, rather than with Se-L-cysteine when incubated with solution 3: $[\text{M}(\text{cym})(\text{L})(\text{DTT})]^+$ (Ru, **L1**, 74%, Ru, **L6**, 55%; Os, **L1**, 63%; Os, **L6**, 52%; see Table S9). Overall, L-methionine was the preferred binding partner for all compounds, with the osmium complexes **1b** and **6b** exclusively forming the methionine adduct described above. The ruthenium complexes **1a** and **6a** also formed L-cysteine adducts in low amounts, especially **1a**. None of the complexes showed any affinity toward L-histidine. The selective DTT adduct formation over Se-L-cysteine allows the assumption that the mode of action of the investigated complexes does not depend on interference with selenium dependent pathways (relevant cell functions based on glutathione peroxidase, glutathione reductase and γ -glutamyl transpeptidase).

The reactivity of the complexes toward the proteins ubiquitin (Ub) and cytochrome c (Cyt) were investigated to gain better knowledge of their *in vivo* behavior. Cytochrome c has three possible binding sites (His26, His23, and Met65) and represents a possible biological target, while Ub has two possible binding sites (Met1 and His68) and was chosen as a model protein in order to understand the reactivity of the complexes to proteins in general. The solutions were diluted with 0.2% formic acid (v/v)

prior to recording of the spectra, so the proteins occurred in their highest possible charged states (Ub: 7–14 and Cyt: 11–19). This convoluted the spectra for obvious reasons, hence the recorded mass spectra needed to be deconvoluted. In the following paragraph all percent values are percent of the assignable protein peaks, rather than percent of the assignable metal peaks.

When incubated with Cyt, none of the complexes showed any interaction and Cyt was only found in its unreacted form [Cyt] (m/z 12358.54 $\pm$ 0.42, m_{th} : 12365), even though Met65 would be a possible binding site according to the amino acid experiments. In contrast, all complexes showed a mediocre reactivity toward Ub: $[\text{M}(\text{cym})(\text{L})(\text{Ub})]^+$ (Ru, **L1**, 12%; Ru, **L6**, 17%, Os, **L1**; 28%; Os, **L6**, 3%; see Table S9). Both ruthenium complexes also formed an adduct with Ub where the ligand was cleaved off: $[\text{Ru}(\text{cym})(\text{Ub})]^+$ (m/z 8797.31 $\pm$ 0.66, m_{th} : 8799.64, 9–11%). These results suggest that the reaction with cytochrome c is not a likely pathway for possible biological activity. On the other hand interactions with ubiquitin were observed even though the affinity was not very high.

The reactivity of all complexes toward the nucleoside-triphosphates ATP and GTP revealed a selectivity of the compounds toward guanine. The kinetically favored adduct was $[\text{M}(\text{cym})(\text{L})(\text{GTP})]^{2-}$ (Ru, **L1**, ~20–30%; Ru, **L6**, ~60%; Os, **L1**, ~74%; Os, **L6**, ~70%; see Table S9). Complexes **1a** and **6a** also formed other kinetically favored products: $[\text{Ru}(\text{cym})(\text{GTP})(\text{DMF})]^{2-}$ (m/z 415.95 $\pm$ 0.02, m_{th} : 416.03, ~30–40%, only **1a**) and $[(\text{Ru}(\text{cym})(\text{GTP}))]^-$ (m/z 757.95 $\pm$ 0.01, m_{th} : 757.45, 26–60%). The thermodynamically stable products of ruthenium complexes **1a** and **6a** were $[\text{Ru}(\text{cym})(\text{GTP})]^{2-}$ (m/z 377.49 $\pm$ 0.01, m_{th} : 377.50, 36–48%) and an adduct with ATP $[\text{Ru}(\text{cym})(\text{ATP})]^-$ (m/z 739.96 $\pm$ 0.01, m_{th} : 740.01, 27–39%). Complex **6b** also formed a second guanine adduct: $[(\text{Os}(\text{cym})(\text{DMF})_2(\text{GTP}))]^-$ (m/z 992.02 $\pm$ 0.02, m_{th} : 992.16, ~30% during the entire period). The osmium complex **1b** also showed reactivity with adenine: $[(\text{Os}(\text{cym})(\text{DMF})_2(\text{ATP}))]^-$ (m/z 977.02 $\pm$ 0.02, m_{th} : 977.17, ~26% during the entire period). These results suggest that all complexes have at least twice the reactivity toward guanine than to adenine.

Incubation with the mixture of amino acids and nucleotides (solution 5) revealed that all complexes showed the same distribution of amino acid adducts as described for incubation with solution 2, and none with the nucleotides. These results suggest that biological activity can be primarily attributed to protein interactions, whereas DNA as the biological target is rather unlikely.

Antiproliferative Activity in Cancer Cells

The antiproliferative activity of ligands **L1–L6** and the respective ruthenium (**1a–6a**) and osmium (**1b–6b**) complexes was evaluated in human ovarian teratocarcinoma (CH1/PA-1), colon (SW480) and non-small cell lung carcinoma (A549) cell lines (Table 1, Figures S10–S12). In general, the free ligands hardly show relevant antiproliferative effects in the tested cell lines. Overall coordination of the benzothiazole ligand scaffold to organometallic fragments resulted in highly cytotoxic compounds with IC_{50} values in the low micromolar range. Based on the obtained data most complexes were at least about one

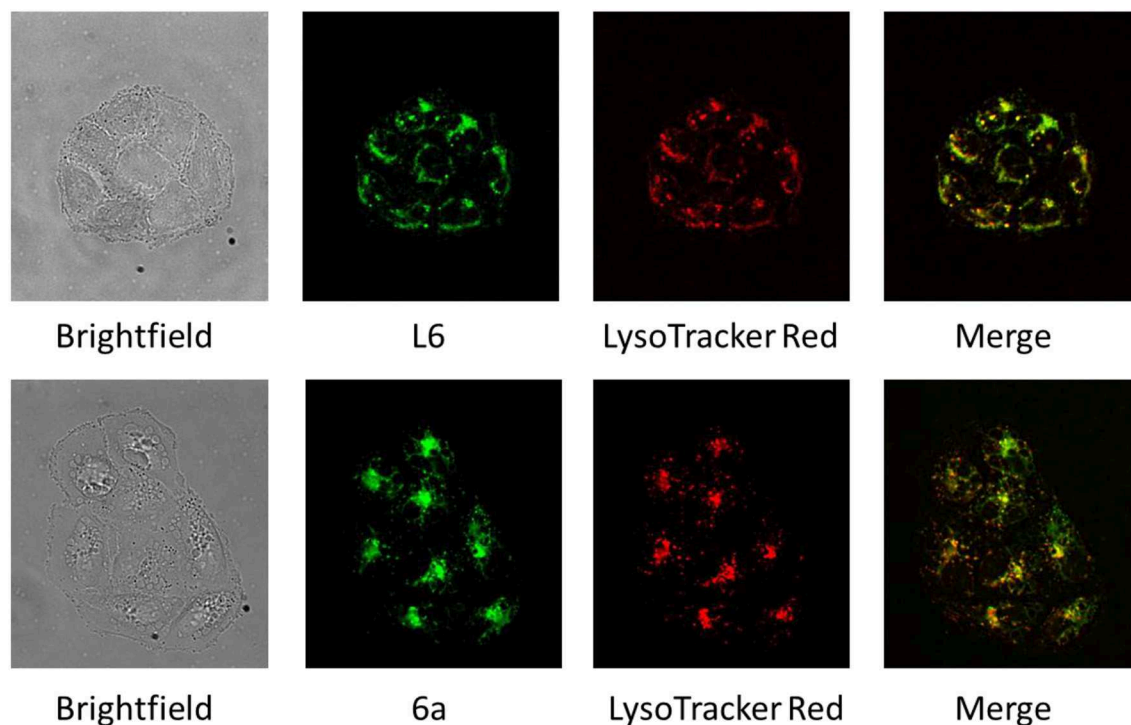


FIGURE 2 | Brightfield and fluorescence microscopy images of SW480 cells treated with **L6** and **6a** (green), stained with LysoTracker Red (red), and merged images revealing an extensive colocalization of fluorescence (yellow).

order of magnitude more active than the free ligand. Although CH1/PA-1 cells were found to be the most sensitive of the cell lines used, the difference in efficacy compared to the more chemo-resistant A549 and SW480 cells is remarkably small. The antiproliferative potency of the investigated compounds, hence, seems to depend on the metal center and increases in the following order: ligands << ruthenium(II) compounds < osmium(II) compounds; with differences in cytotoxicity of the complexes being far less pronounced. The obtained trends are in good agreement with recently reported triazole-based metallacycles, where also a pronounced increase of cytotoxicity upon *C,N*-coordination to organoruthenium and -osmium moieties was observed (Riedl et al., 2018). With regard to variation of the substituent on the phenylbenzothiazole ligand, consistent structure-activity relationships are difficult to derive, as their impact on cytotoxic potency is rather small. For further biological experiments we have chosen ruthenium complexes **2a**, **6a** and osmium derivatives **2b**, **6b** to cover the whole range of determined IC_{50} values.

Apoptosis Assay

Flow-cytometric analysis of SW480 cells treated with **2a**, **2b**, **6a**, and **6b** for 24 h and then double-stained with an annexin V-FITC conjugate (AV) and propidium iodide (PI) revealed strong apoptotic effects of the Ru compound **2a** (Figure S13), comparable to the positive control (50 μ M of Pt complex KP1998; Scaffidi-Domianello et al., 2012). Surprisingly, the Os analog **2b**, which is as potent as **2a** according to the 96-h MTT assay, does

not induce apoptosis within 24 h (Table S10). Compound **6a**, the least potent of the Ru series according to the MTT assay, induces apoptosis to a similar extent as **2a** at 20 μ M, but is by far surpassed by the latter at 40 μ M (18% vs. 88% of apoptotic cells in total). The Os analog **6b** is less active than both of them, but induces apoptosis noticeably (14%) at 40 μ M.

Cellular Accumulation

Cellular accumulation of these compounds in SW480 cells after 2 h exposure is generally high with rather small differences: **6b** (382 ± 86 fg Os/cell), **2a** (301 ± 86 fg Ru/cell), **6a** (254 ± 58 fg Ru/cell), **2b** (243 ± 83 fg Os/cell). Since this rank order does neither correlate with antiproliferative nor with apoptotic potency, the high initial uptake alone cannot account for the outstanding effects of compound **2a** in the apoptosis assay.

Colocalization of L6 and 6a With Lysosomes

The fluorescence properties of **L6** and **6a** were exploited to visualize their intracellular distribution in SW480 colon carcinoma cells by fluorescence microscopy in a live cell setting (Figure 2). Staining with LysoTracker Red was used to study the localization of both compounds with regard to lysosomal compartments, and a high degree of overlap between the fluorescence images strongly suggests a pronounced colocalization with lysosomes (see Figure 2). To statistically confirm the visual colocalization of the drug with the acidic compartments of the cell (Figure 2), correlation analysis was

conducted. Pearson's coefficients of 0.77 ± 0.08 and 0.81 ± 0.06 between red fluorescence (LysoTracker Red) and green fluorescence (corresponding to compounds **L6** and **6a**, respectively) were obtained, indicating a substantial extent of correlation. Lysosomes might represent a potential target for the novel Ru complexes with alkaline and lipophilic physiochemical properties. Trapping of the compounds in acidic compartments due to changes in charge based on the weakly basic properties of the aniline group is conceivable, but remains speculative at this stage. Furthermore, morphological differences between the samples treated with two different compounds (**L6** and **6a**) can be observed. In comparison to cells treated with **L6**, the cells exposed to **6a** show a pronounced cytoplasmic disparity due to beginning vacuolation.

CONCLUSIONS

A series of 2-phenylbenzothiazoles and their corresponding ruthenium(II) and osmium(II) half-sandwich complexes were synthesized. The obtained compounds were characterized by standard analytical methods and their aqueous stability was investigated. Additionally, ESI-MS studies showed general binding preferences with regards to possible binding partners, after administration. Surprisingly, no complex showed any affinity toward Se- and N-donor amino acids. Almost only sulfur-donor amino acid adducts were found, especially with thioether moieties over thiols; a preference also observed with 5'-GTP adducts, which were almost exclusively formed, leaving present 5'-ATP untouched. However, competition experiments revealed a pronounced affinity toward amino acids over NTPs. Furthermore, the IC₅₀ values were determined in human cancer cell lines, and proved to be in the low μM range, with comparatively small differences between the most sensitive and the most resistant cell line. Although osmium complexes showed a stronger overall effect on cancer cell proliferation over 96 h than their ruthenium counterparts, the ruthenium complexes

more potently induced apoptosis within 24 h. For one selected ligand and its ruthenium complex, fluorescence microscopic images suggest a high accumulation in lysosomes and other subcellular acidic compartments. The cellular accumulation of the investigated complexes was in a very similar range for all tested organometallics and could hence not provide an explanation for the different cytotoxic properties of the ruthenium and osmium metallacycles.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary material.

AUTHOR CONTRIBUTIONS

WK and BK: conceptualization and methodology. MJ, AR, SM-M, WK, and BK: validation. SM, HG, KS, MK, AR, and MH: formal analysis. SM, KC, HG, MK, AR, and MH: investigation. AR, SM-M, MJ, WK, and BK: resources and data curation. SM, HG, MK, MJ, and WK: writing—original draft preparation. SM, HG, and WK: visualization. WK and BK: supervision, project administration, and funding acquisition.

ACKNOWLEDGMENTS

We thank the University of Vienna and the Research Cluster Translational Cancer Therapy Research for financial support. Furthermore, the authors thank Anton A. Legin for assistance in quantitative image analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchem.2020.00209/full#supplementary-material>

REFERENCES

- Abdel-Aziz, M., Matsuda, K., Otsuka, M., Uyeda, M., Okawara, T., and Suzuki, K. (2004). Inhibitory activities against topoisomerase I and II by polyhydroxybenzoyl amide derivatives and their structure-activity relationship. *Bioorg. Med. Chem. Lett.* 14, 1669–1672. doi: 10.1016/j.bmcl.2004.01.060
- Alessio, E. (2017). Thirty years of the drug candidate NAMI-A and the myths in the field of ruthenium anticancer compounds: a personal perspective. *Eur. J. Inorg. Chem.* 2017, 1549–1560. doi: 10.1002/ejic.201600986
- Baell, J. B., Forsyth, S. A., Gable, R. W., Norton, R. S., and Mulder, R. J. (2001). Design and synthesis of type-III mimetics of ω -conotoxin GVIA. *J. Comput. Aided Mol. Des.* 15, 1119–1136. doi: 10.1023/A:1015930031890
- Ban, M., Taguchi, H., Katsushima, T., Takahashi, M., Shinoda, K., Watanabe, A., et al. (1998). Novel antiallergic and antiinflammatory agents. Part I: synthesis and pharmacology of glycolic amide derivatives. *Bioorg. Med. Chem.* 6, 1069–1076. doi: 10.1016/S0968-0896(98)00065-0
- Belsa, L., López, C., González, A., Font-Bardia, M., Calvet, T., Calvis, C., et al. (2013). Neutral and ionic cycloruthenated 2-phenylindoles as cytotoxic agents. *Organometallics* 32, 7264–7267. doi: 10.1021/om400941b
- Bennett, M. A., and Smith, A. K. (1974). Arene ruthenium(II) complexes formed by dehydrogenation of cyclohexadienes with ruthenium(III) trichloride. *J. Chem. Soc. Dalton Transac.* 1974, 233–241. doi: 10.1039/dt9740000233
- Bergamo, A., Gaiddon, C., Schellens, J. H. M., Beijnen, J. H., and Sava, G. (2012). Approaching tumour therapy beyond platinum drugs: Status of the art and perspectives of ruthenium drug candidates. *J. Inorg. Biochem.* 106, 90–99. doi: 10.1016/j.jinorgbio.2011.09.030
- Caleta, I., Kralj, M., Marjanović, M., Bertoša, B., Tomić, S., Pavlović, G., et al. (2009). Novel cyano- and amidinobenzothiazole derivatives: synthesis, antitumor evaluation, and X-ray and Quantitative Structure-Activity Relationship (QSAR) analysis. *J. Med. Chem.* 52, 1744–1756. doi: 10.1021/jm801566q
- Chen, C., Qiu, H., Chen, W., and Wang, D. (2008). Structural variations in nickel, palladium, and platinum complexes containing pyrimidyl N-heterocyclic carbene ligand. *J. Organomet. Chem.* 693, 3273–3280. doi: 10.1016/j.jorganchem.2008.07.026
- Chua, M.-S., Kashiya, E., Bradshaw, T. D., Stinson, S. F., Brantley, E., Sausville, E. A., et al. (2000). Role of CYP1A1 in modulation of antitumor properties of the novel agent 2-(4-Amino-3-methylphenyl)benzothiazole (DF 203, NSC 674495) in human breast cancer cells. *Cancer Res.* 60, 5196–5203.

- Cross, W. B., Daly, C. G., Ackerman, R. L., George, I. R., and Singh, K. (2011). N-heterocyclic carbene tethered amido complexes of palladium and platinum. *Dalton Transac.* 40, 495–505. doi: 10.1039/C0DT01149E
- Djukic, J.-P., Duquenne, M., Berger, A., and Pfeffer, M. (2006). The epimerization of chiral half sandwich 2-phenylpyridine-based ruthenacycle. *Inorg. Chim. Acta* 359, 1754–1760. doi: 10.1016/j.ica.2005.06.026
- Dolomanov, O. V., Bourhis, L. J., Gildea, R. J., Howard, J. A. K., and Puschmann, H. (2009). OLEX2: a complete structure solution, refinement and analysis program. *J. Appl. Crystallogr.* 42, 339–341. doi: 10.1107/S0021889808042726
- Facchetti, S., Jurcik, V., Baldino, S., Giboulot, S., Nedden, H. G., Zanotti-Gerosa, A., et al. (2016). Preparation of Pincer 4-functionalized 2-aminomethylbenzo[h]quinoline ruthenium catalysts for ketone reduction. *Organometallics* 35, 277–287. doi: 10.1021/acs.organomet.5b00978
- Ferrer Flegeau, E., Bruneau, C., Dixneuf, P. H., and Jutand, A. (2011). Autocatalysis for C–H bond activation by ruthenium(II) complexes in catalytic arylation of functional arenes. *J. Am. Chem. Soc.* 133, 10161–10170. doi: 10.1021/ja201462n
- Gabr, M. T., El-Gohary, N. S., El-Bendary, E. R., and El-Kerdawy, M. M. (2015). New series of benzothiazole and pyrimido[2,1-b]benzothiazole derivatives: synthesis, antitumor activity, EGFR tyrosine kinase inhibitory activity and molecular modeling studies. *Med. Chem. Res.* 24, 860–878. doi: 10.1007/s00044-014-1114-x
- Galanski, M., and Keppler, B. K. (2007). Searching for the magic bullet: anticancer platinum drugs which can be accumulated or activated in the tumor tissue. *Anticancer. Agents Med. Chem.* 7, 55–73. doi: 10.2174/187152007779314017
- Guo, H. Y., Li, J. C., and Shang, Y. L. (2009). A simple and efficient synthesis of 2-substituted benzothiazoles catalyzed by H₂O₂/HCl. *Chin. Chem. Lett.* 20, 1408–1410. doi: 10.1016/j.ccl.2009.06.037
- Hackl, C. M., Legina, M. S., Pichler, V., Schmidlehner, M., Roller, A., Dömötör, O., et al. (2016). Thiomaltol-based organometallic complexes with 1-methylimidazole as leaving group: synthesis, stability, and biological behavior. *Chem. Eur. J.* 22, 17269–17281. doi: 10.1002/chem.201603206
- Hubschle, C. B., Sheldrick, G. M., and Dittrich, B. (2011). ShelXle: a Qt graphical user interface for SHELXL. *J. Appl. Crystallogr.* 44, 1281–1284. doi: 10.1107/S0021889811043202
- Jakupec, M. A., Galanski, M., Arion, V. B., Hartinger, C. G., and Keppler, B. K. (2008). Antitumor metal compounds: more than theme and variations. *Dalton Transac.* 2008, 183–194. doi: 10.1039/B712656P
- Jia, J., Cui, M., Dai, J., and Liu, B. (2015). 2-Phenylbenzothiazole conjugated with cyclopentadienyl tricarbonyl [CpM(CO)₃] (M = Re, 99mTc) complexes as potential imaging probes for β -amyloid plaques. *Dalton Transac.* 44, 6406–6415. doi: 10.1039/C5DT00023H
- Kandioller, W., Hartinger, C. G., Nazarov, A. A., Kasser, J., John, R., Jakupec, M. A., et al. (2009). Tuning the anticancer activity of maltol-derived ruthenium complexes by derivatization of the 3-hydroxy-4-pyrone moiety. *J. Organomet. Chem.* 694, 922–929. doi: 10.1016/j.jorganchem.2008.10.016
- Kiel, W. A., Ball, R. G., and Graham, W. A. G. (1990). Carbonyl- η -hexamethylbenzene complexes of osmium. Carbon-hydrogen activation by (η -C₆Me₆)Os(CO)(H)₂. *J. Organometal. Chem.* 383, 481–496. doi: 10.1016/0022-328X(90)85149-S
- Klose, M. H. M., Hejl, M., Heffeter, P., Jakupec, M. A., Meier-Menches, S. M., Berger, W., et al. (2017). Post-digestion stabilization of osmium enables quantification by ICP-MS in cell culture and tissue. *Analyst* 142, 2327–2332. doi: 10.1039/C7AN00350A
- Kondrashov, M., Provost, D., and Wendt, O. F. (2016). Regioselectivity in C–H activation: reagent control in cyclometallation of 2-(1-naphthyl)-pyridine. *Dalton Transac.* 45, 525–531. doi: 10.1039/C5DT04068J
- Li, B., Darcel, C., Roisnel, T., and Dixneuf, P. H. (2015). Cycloruthenation of aryl imines and N-heteroaryl benzenes via C–H bond activation with Ru(II) and acetate partners. *J. Organomet. Chem.* 793, 200–209. doi: 10.1016/j.jorganchem.2015.02.050
- Li, X., Zang, T.-N., Chi, H.-J., Dong, Y., Xiao, G.-Y., and Zhang, D.-Y. (2014). Multifunctional phosphorescent iridium (III) complexes based on 2-phenylbenzothiazole derivative for highly efficient organic light-emitting diodes. *Dyes Pigments* 106, 51–57. doi: 10.1016/j.dyepig.2014.02.025
- Mohmed, J., Palreddy, R. R., Gunda, S. K., and Ch, S. D. (2015). Homology modeling and molecular docking studies of novel quinazolinone and benzothiazole derivatives as DNA topoisomerase II inhibitors. *Int. J. Pharm. Sci. Rev. Res.* 35, 84–89.
- Mokesch, S., Schwarz, D., Hejl, M., Klose, M. H. M., Roller, A., Jakupec, M. A., et al. (2019). Fine-tuning the activation mode of an 1,3-indandione-based ruthenium(II)-cymene half-sandwich complex by variation of its leaving group. *Molecules* 24:2373. doi: 10.3390/molecules24132373
- Nirmala, M., Saranya, G., and Viswanathamurthi, P. (2016). Ruthenium(II) complexes incorporating salicylaldiminato-functionalized N-heterocyclic carbene ligands as efficient and versatile catalysts for hydration of organonitriles. *Inorgan. Chim. Acta* 442, 134–144. doi: 10.1016/j.ica.2015.11.033
- Okaniwa, M., Hirose, M., Arita, T., Yabuki, M., Nakamura, A., Takagi, T., et al. (2013). Discovery of a selective kinase inhibitor (TAK-632) targeting pan-RAF inhibition: design, synthesis, and biological evaluation of C-7-substituted 1,3-benzothiazole derivatives. *J. Med. Chem.* 56, 6478–6494. doi: 10.1021/jm400778d
- Papadopoulou, C., Geronikaki, A., and Hadjipavlou-Litina, D. (2005). Synthesis and biological evaluation of new thiazolyl/benzothiazolyl-amides, derivatives of 4-phenyl-piperazine. *Il Farmaco* 60, 969–973. doi: 10.1016/j.farmac.2005.06.014
- Pérez, J., Riera, V., Rodríguez, A., and Miguel, D. (2002). Synthesis and structure of the first ruthenated benzodiazepines. *Organometallics* 21, 5437–5438. doi: 10.1021/om0205288
- Pinar, A., Yurdakul, P., Yildiz, I., Temiz-Arpaci, O., Acan, N. L., Aki-Sener, E., et al. (2004). Some fused heterocyclic compounds as eukaryotic topoisomerase II inhibitors. *Biochem. Biophys. Res. Commun.* 317, 670–674. doi: 10.1016/j.bbrc.2004.03.093
- Rajkumar, S., Karthik, S., and Gandhi, T. (2015). Ru(II)-catalyzed β -carboline directed C–H arylation and isolation of its cycloruthenated intermediates. *J. Org. Chem.* 80, 5532–5545. doi: 10.1021/acs.joc.5b00411
- Riedl, C. A., Flocke, L. S., Hejl, M., Roller, A., Klose, M. H. M., Jakupec, M. A., et al. (2017). Introducing the 4-phenyl-1,2,3-triazole moiety as a versatile scaffold for the development of cytotoxic ruthenium(II) and osmium(II) arene cyclometalates. *Inorg. Chem.* 56, 528–541. doi: 10.1021/acs.inorgchem.6b02430
- Riedl, C. A., Hejl, M., Klose, M. H. M., Roller, A., Jakupec, M. A., Kandioller, W., et al. (2018). N- and S-donor leaving groups in triazole-based ruthena(ii)cycles: potent anticancer activity, selective activation, and mode of action studies. *Dalton Transac.* 47, 4625–4638. doi: 10.1039/C8DT00449H
- Rosenberg, B. (1971). Some biological effects of platinum compounds. *Platin. Met. Rev.* 15, 42–51.
- Rosenberg, B. (1973). Platinum coordination complexes in cancer chemotherapy. *Naturwissenschaften* 60, 399–406. doi: 10.1007/BF00623551
- Ruiz, J., Rodríguez, V., Cutillas, N., Espinosa, A., and Hannon, M. J. (2011). A Potent ruthenium(II) antitumor complex bearing a lipophilic levonorgestrel group. *Inorg. Chem.* 50, 9164–9171. doi: 10.1021/ic201388n
- Ruiz, J., Rodríguez, V., Cutillas, N., Samper, K. G., Capdevila, M., Palacios, O., et al. (2012). Novel C,N-chelate rhodium(iii) and iridium(iii) antitumor complexes incorporating a lipophilic steroidal conjugate and their interaction with DNA. *Dalton Transac.* 41, 12847–12856. doi: 10.1039/c2dt31654d
- Sabater, S., Müller-Bunz, H., and Albrecht, M. (2016). Carboxylate-functionalized mesoionic carbene precursors: decarboxylation, ruthenium bonding, and catalytic activity in hydrogen transfer reactions. *Organometallics* 35, 2256–2266. doi: 10.1021/acs.organomet.6b00322
- Scaffidi-Domianello, Y. Y., Legina, A. A., Jakupec, M. A., Roller, A., Kukushkin, V. Y., Galanski, M., et al. (2012). Novel oximate-bridged platinum(II) Di- and trimer(s): synthetic, structural, and *in vitro* anticancer activity studies. *Inorg. Chem.* 51, 7153–7163. doi: 10.1021/ic300148e
- Sheldrick, G. M. (1996). “SADABS”. Göttingen: University of Göttingen.
- Sheldrick, G. M. (2015a). “SHELXL”. Göttingen: University of Göttingen.
- Sheldrick, G. M. (2015b). “SHELXS”. Göttingen: University of Göttingen.
- Singh, M., Kumar Singh, S., Gangwar, M., Sellamuthu, S., Nath, G., and K., et al. (2016). Design, synthesis and mode of action of some new 2-(4'-aminophenyl) benzothiazole derivatives as potent antimicrobial agents. *Lett. Drug Design Discov.* 13, 429–437. doi: 10.2174/1570180812666150821003220
- Spek, A. (2009). Structure validation in chemical crystallography. *Acta Crystallograph. Sect. D* 65, 148–155. doi: 10.1107/S090744490804362X
- Starcevic, K., Caleta, I., Cincic, D., Kaitner, B., Kralj, M., Ester, K., et al. (2006). Synthesis, crystal structure determination and antiproliferative evaluation of novel benzazoyl benzamides. *Heterocycles* 68, 2285–99. doi: 10.3987/COM-06-10844

- Trondl, R., Heffeter, P., Kowol, C. R., Jakupec, M. A., Berger, W., and Keppler, B. K. (2014). NKP-1339, the first ruthenium-based anticancer drug on the edge to clinical application. *Chem. Sci.* 5, 2925–2932. doi: 10.1039/C3SC53243G
- Westaway, S. M., Thompson, M., Rami, H. K., Stemp, G., Trouw, L. S., Mitchell, D. J., et al. (2008). Design and synthesis of 6-phenylnicotinamide derivatives as antagonists of TRPV1. *Bioorg. Med. Chem. Lett.* 18, 5609–5613. doi: 10.1016/j.bmcl.2008.08.105
- Yellol, J., Pérez, S. A., Buceta, A., Yellol, G., Donaire, A., Szumlas, P., et al. (2015). Novel C,N-cyclometalated benzimidazole ruthenium(II) and iridium(III) complexes as antitumor and antiangiogenic agents: a structure–activity relationship study. *J. Med. Chem.* 58, 7310–7327. doi: 10.1021/acs.jmedchem.5b01194
- Yu, Q., Hu, L. A., Wang, Y., Zheng, S., and Huang, J. (2015). Directed meta-selective bromination of arenes with ruthenium catalysts. *Angew. Chem. Int. Ed.* 54, 15284–15288. doi: 10.1002/anie.201507100
- Zhang, L. L., Li, L.-H., Wang, Y.-Q., Yang, Y.-F., Liu, X.-Y., and Liang, Y.-M. (2014). Ruthenium-catalyzed direct C–H amidation of arenes: a mechanistic study. *Organometallics* 33, 1905–1908. doi: 10.1021/om500080z

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Investigations on the Anticancer Potential of Benzothiazole-Based Metallacycles

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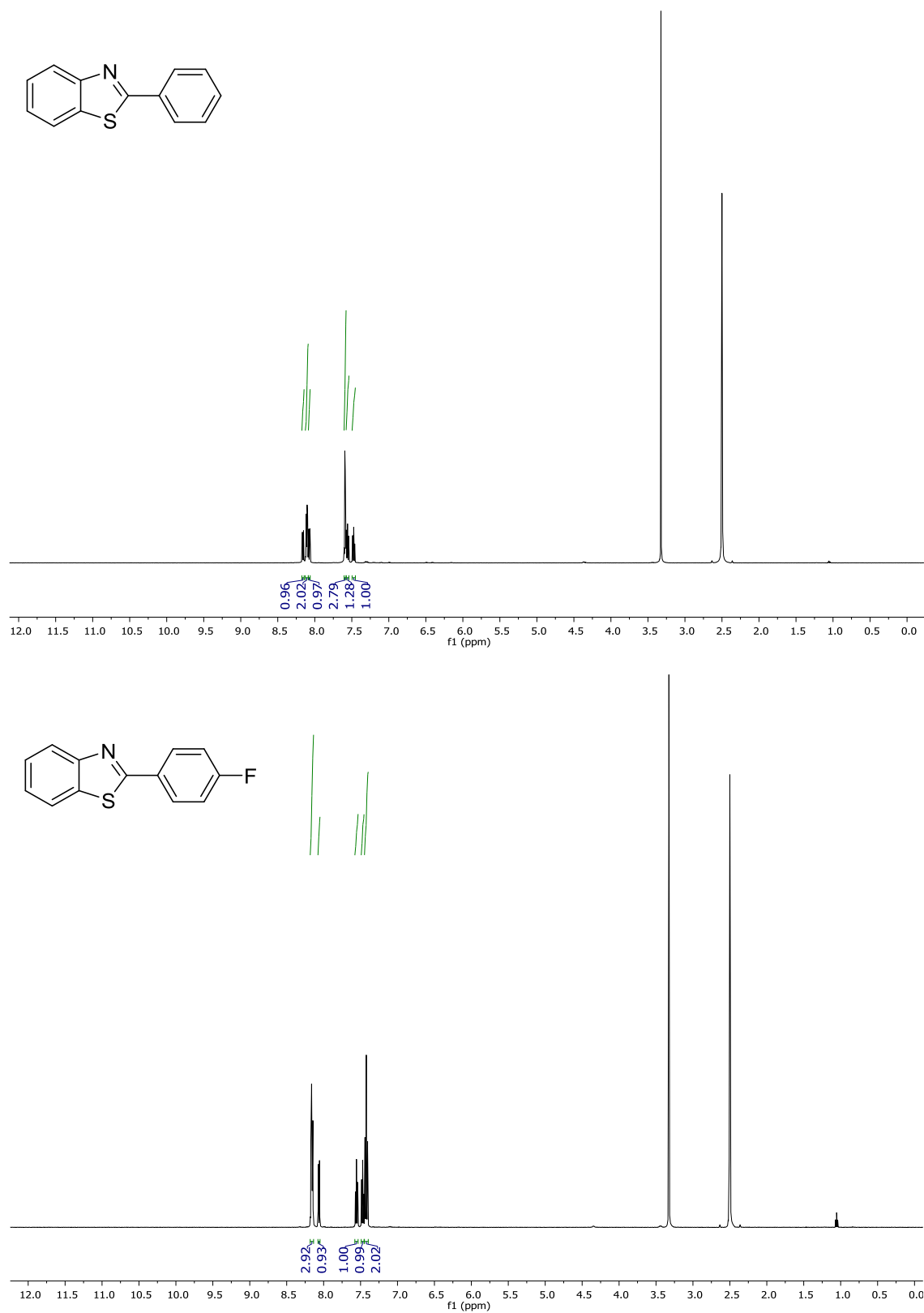
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1 ^1H NMR spectraFigure S1: ^1H -NMR spectra of compound L1 (top) and L2 (bottom)

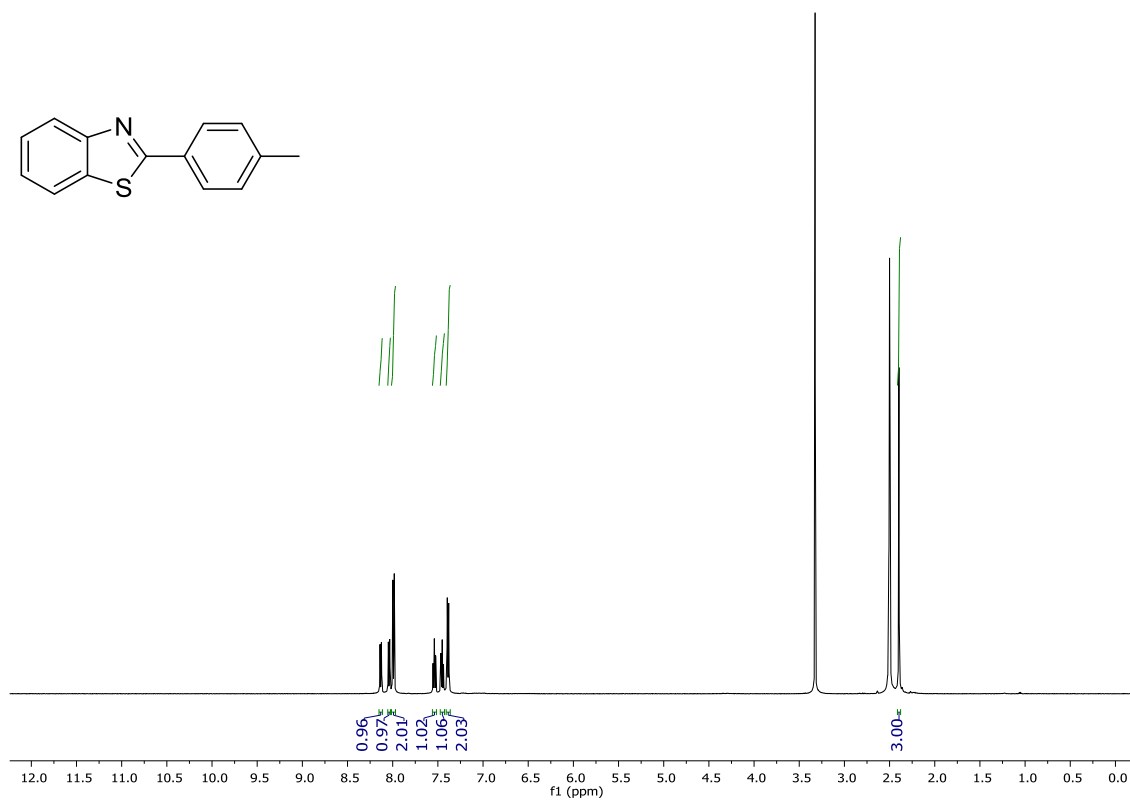
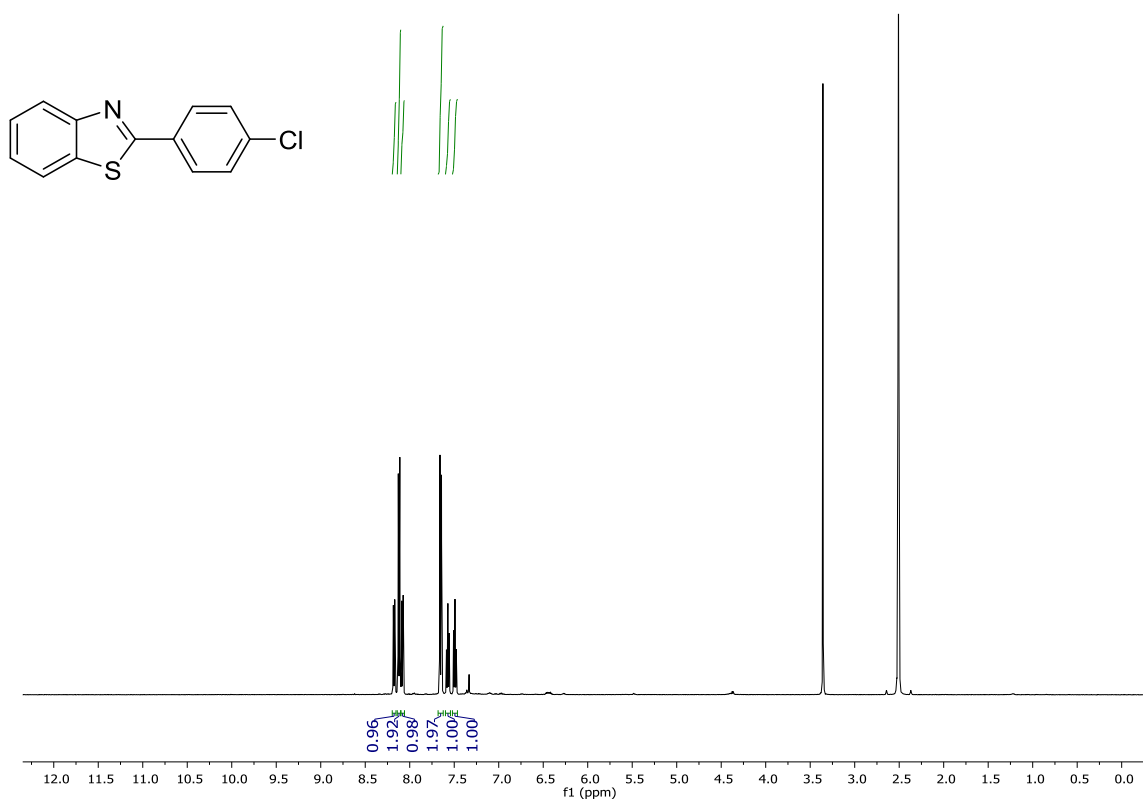


Figure S2: ^1H -spectra of compound L3 (top) and L4 (bottom)

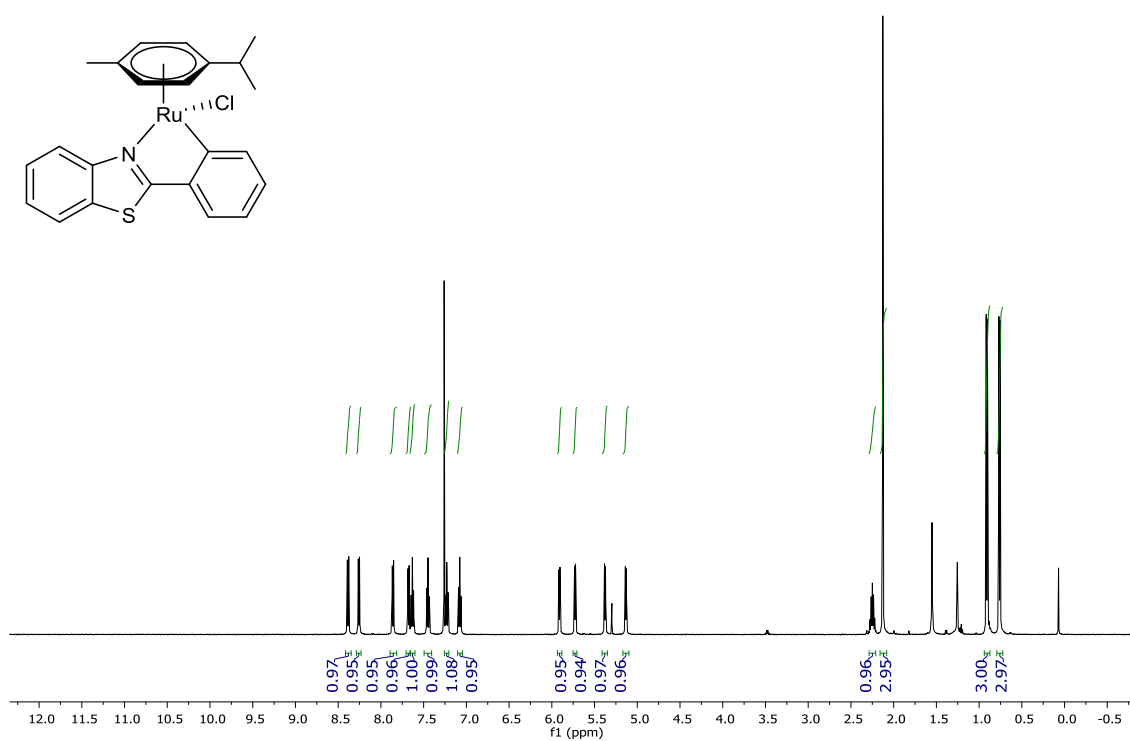
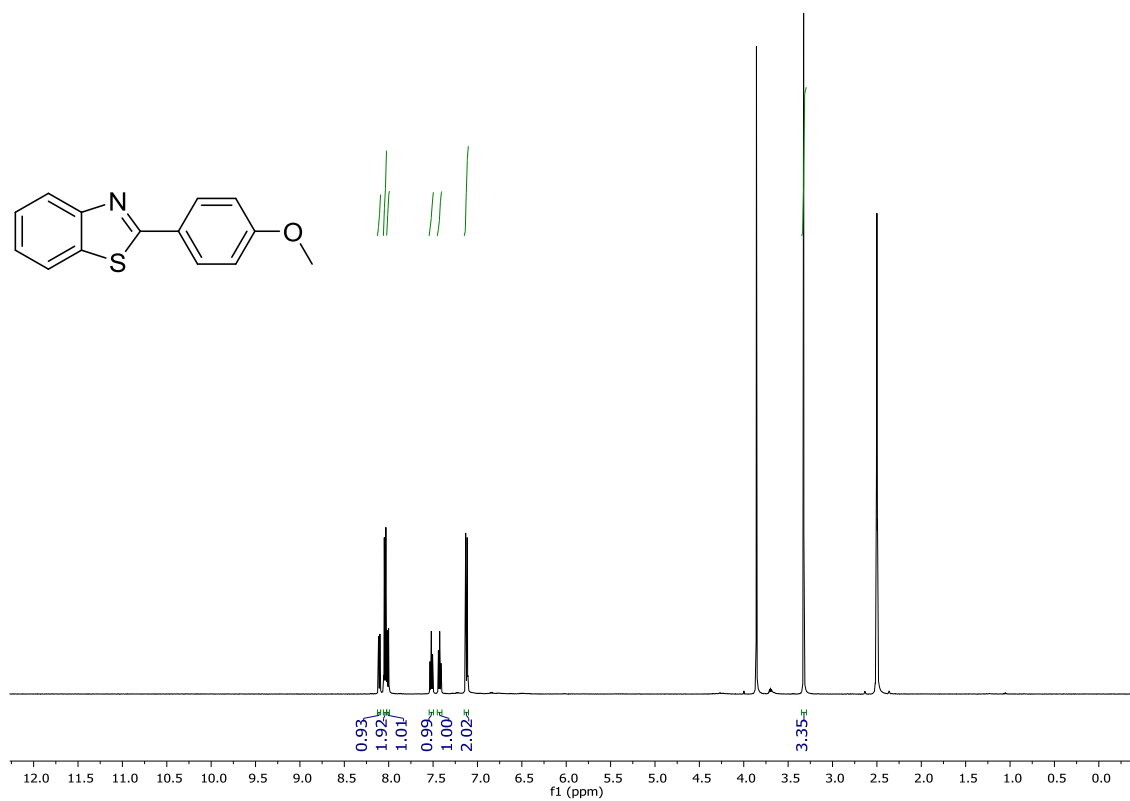


Figure S3: ¹H-spectra of compound L5 (top) and 1a (bottom)

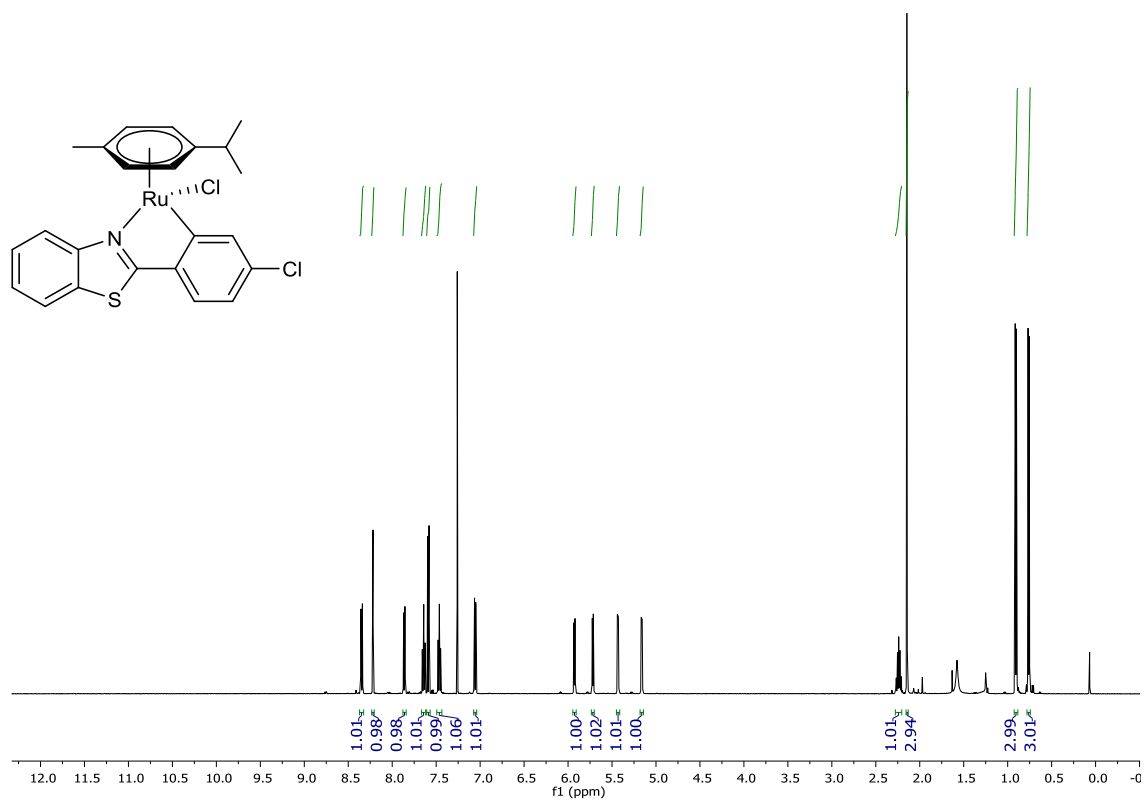
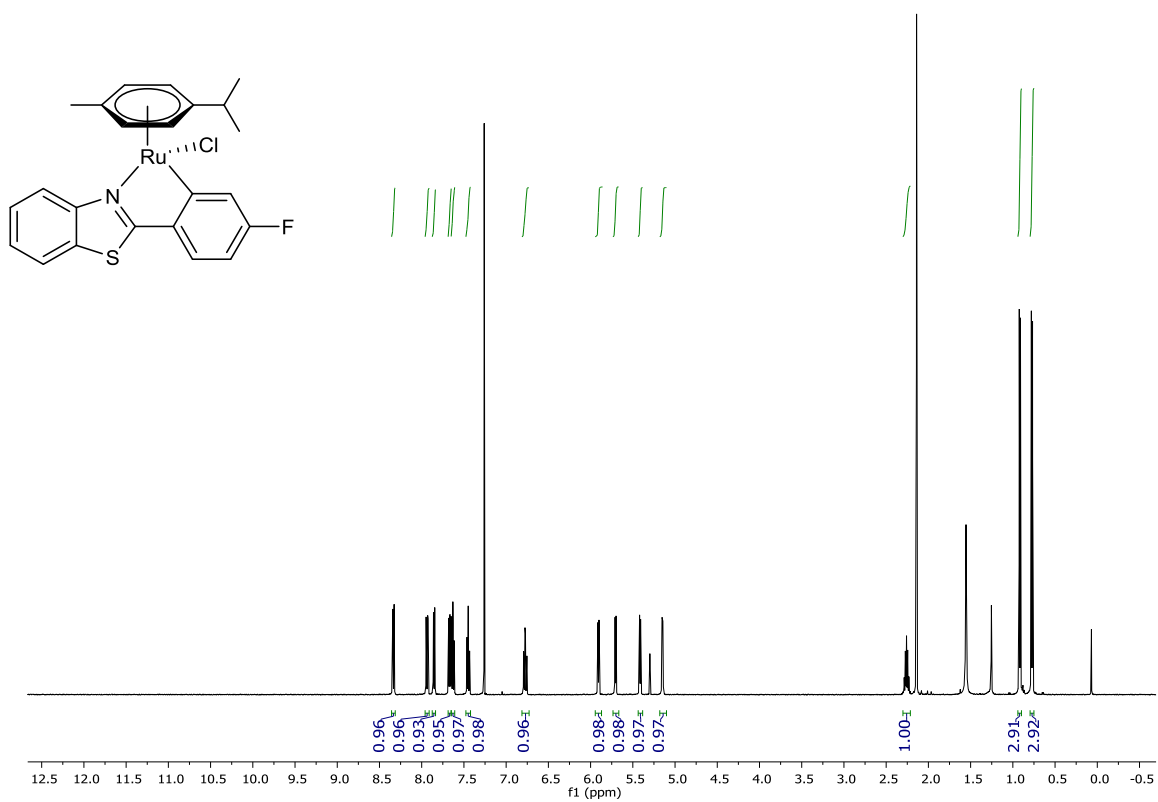


Figure S4: ¹H-spectra of compound 2a (top) and 3a (bottom)

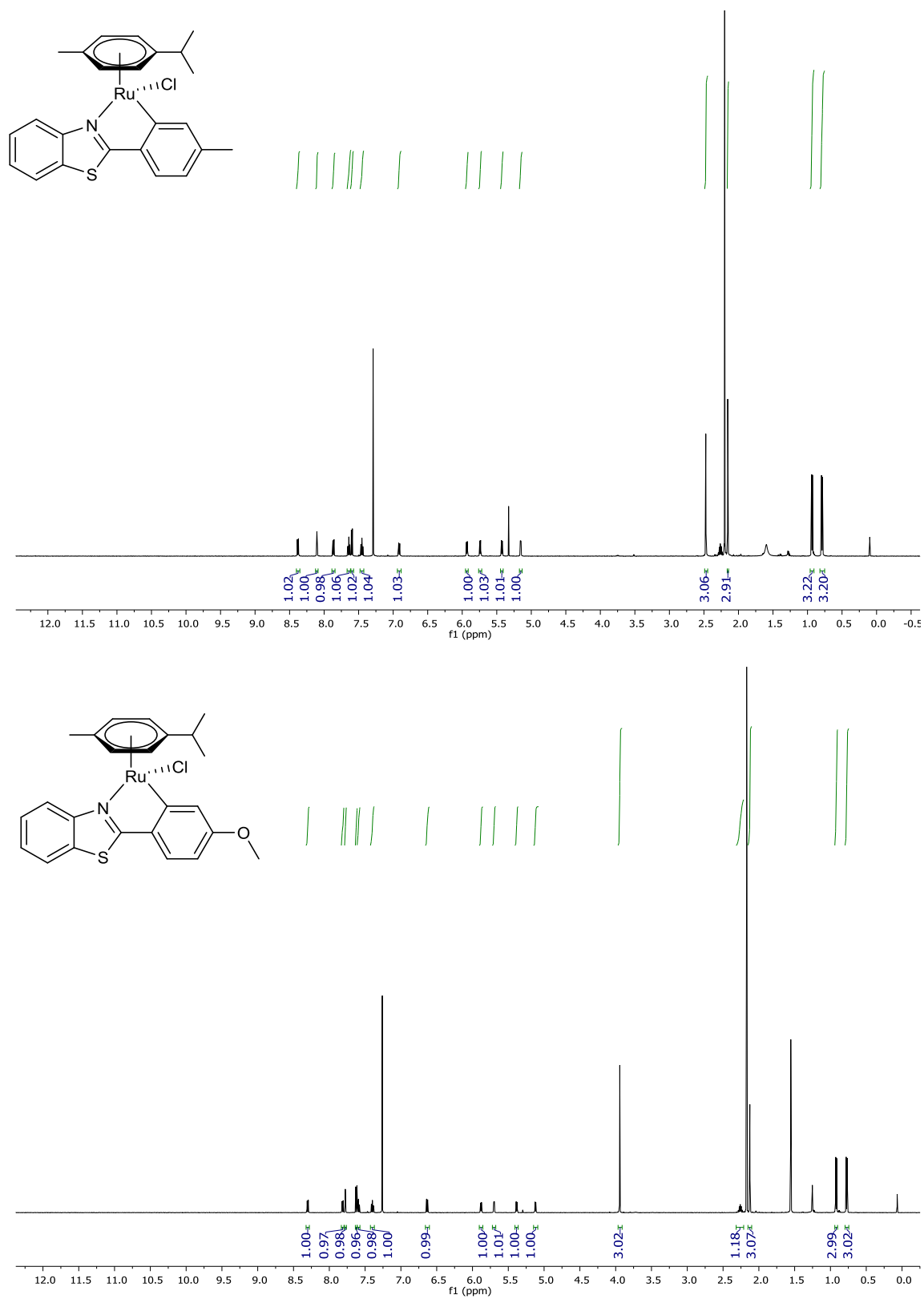


Figure S5: ^1H -spectra of compound 4a (top) and 5a (bottom)

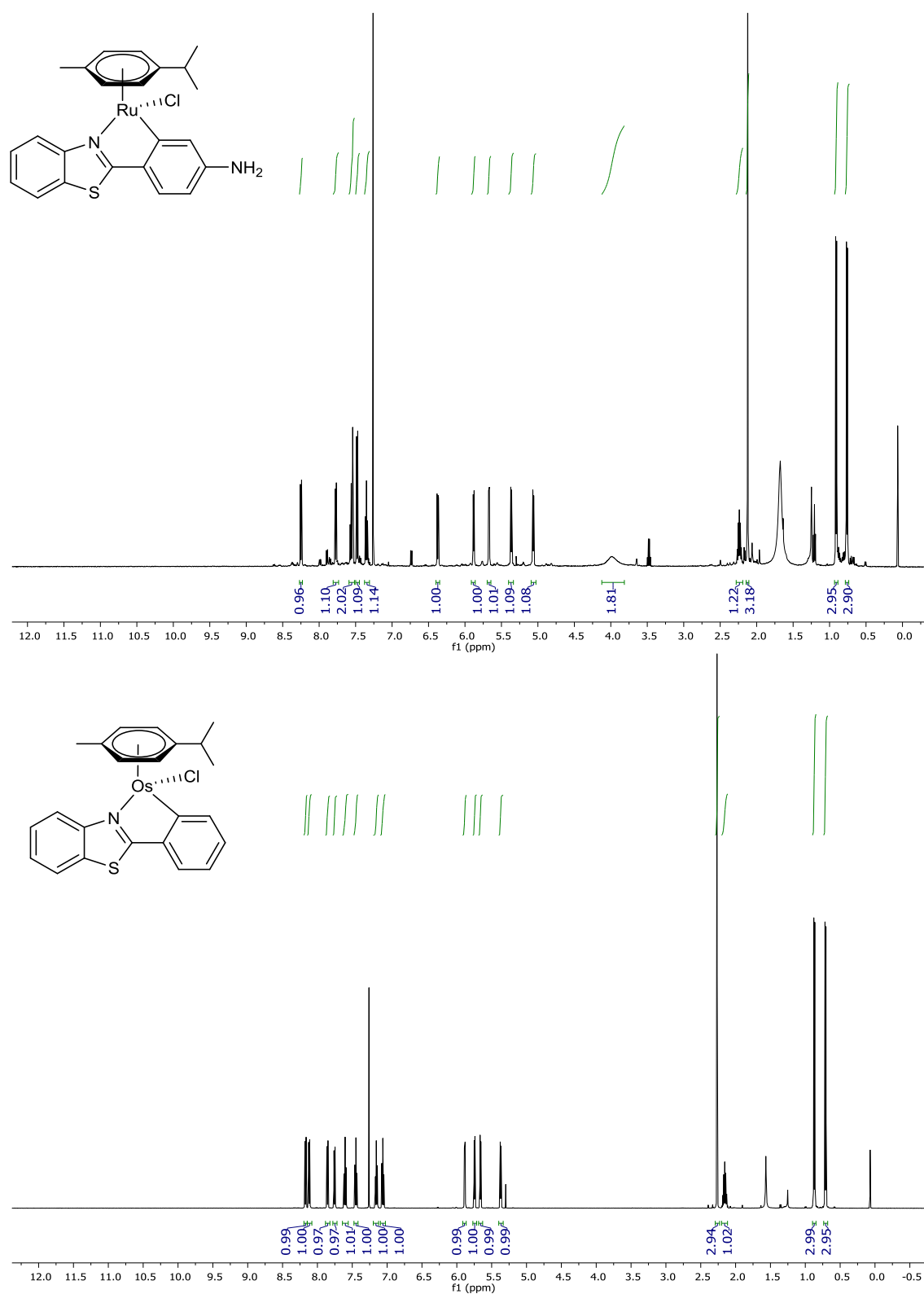


Figure S6: ^1H -spectra of compound 6a (top) and 1b (bottom)

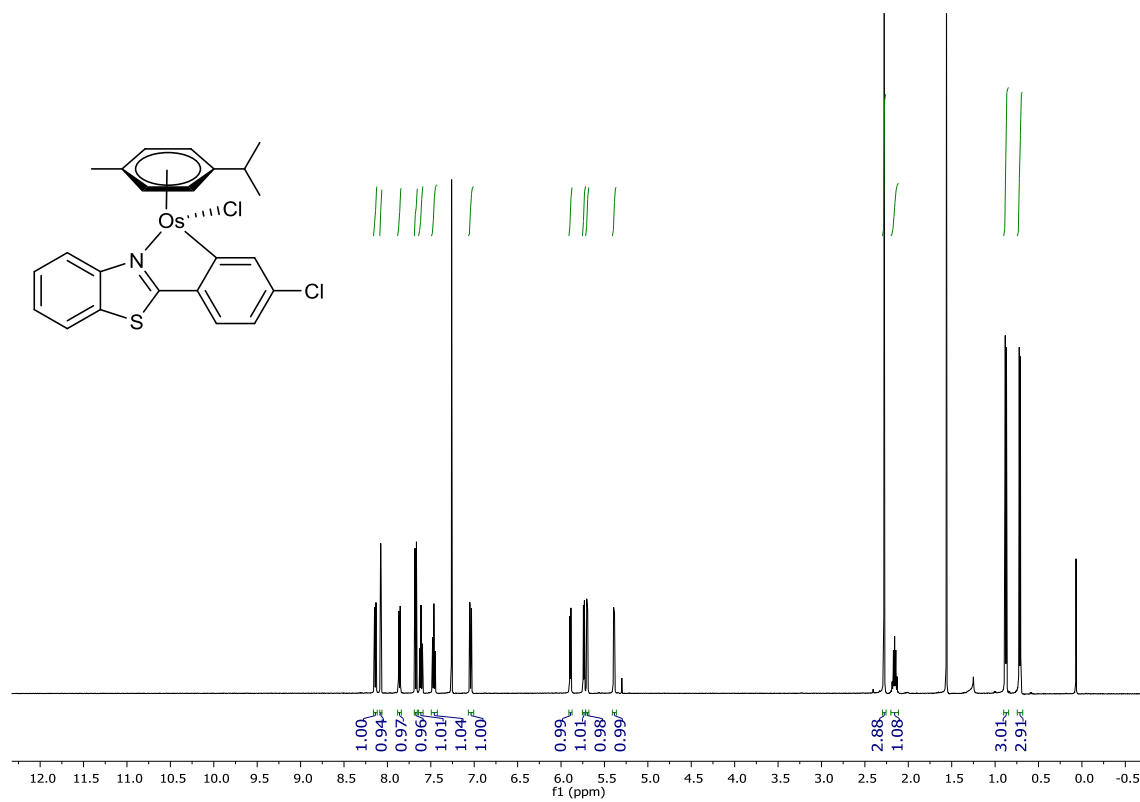
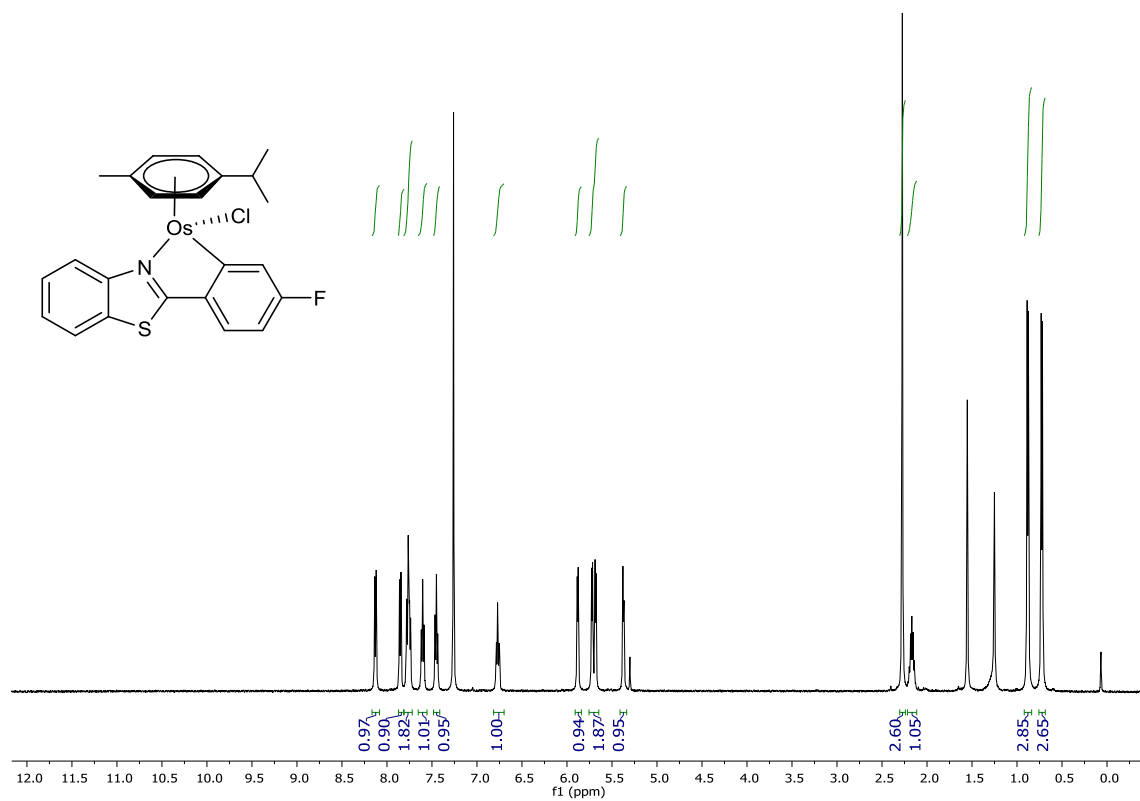


Figure S7: ¹H-spectra of compound 2b (top) and 3b (bottom)

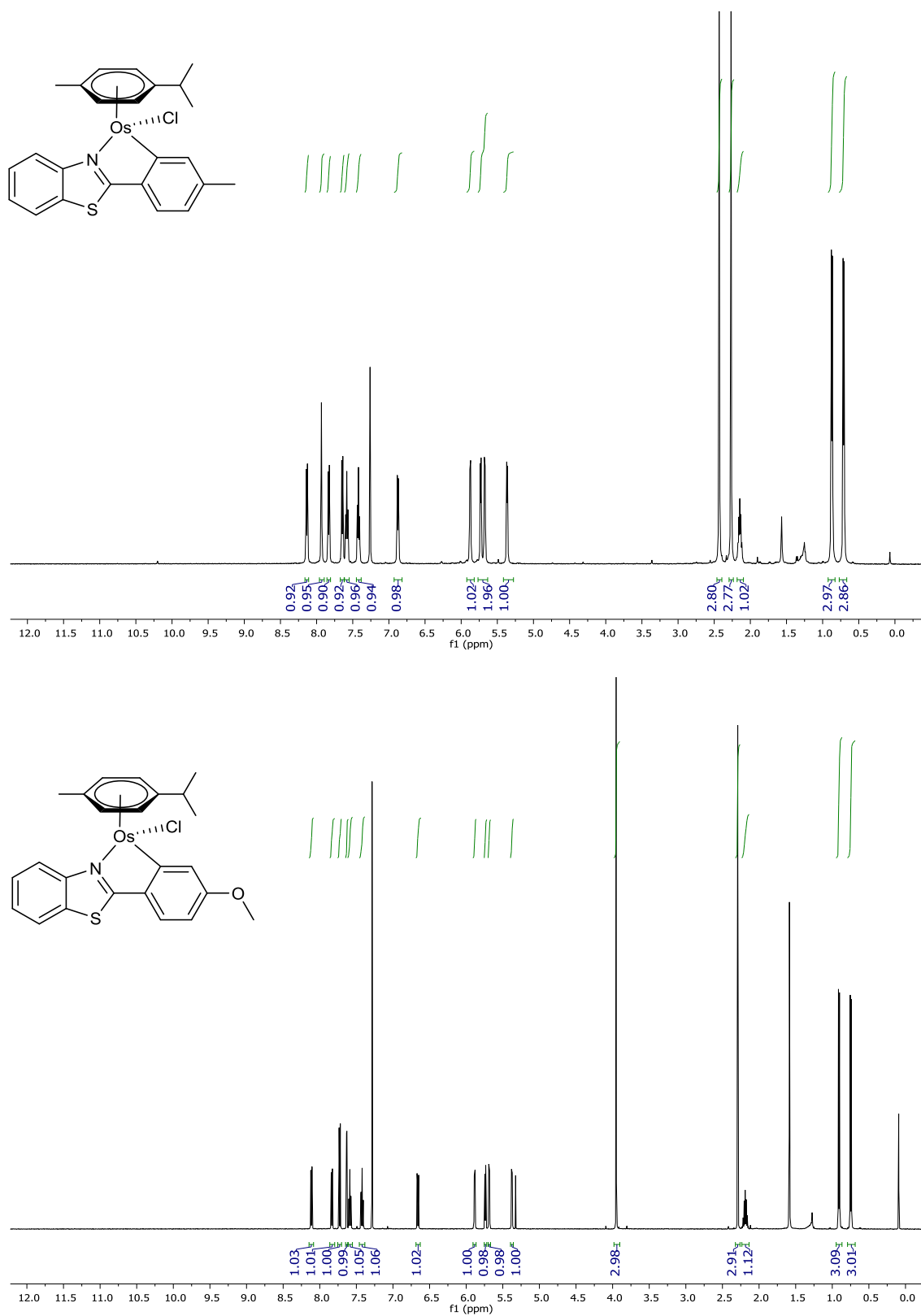


Figure S8. ^1H -spectra of compound 4b (top) and 5b (bottom)

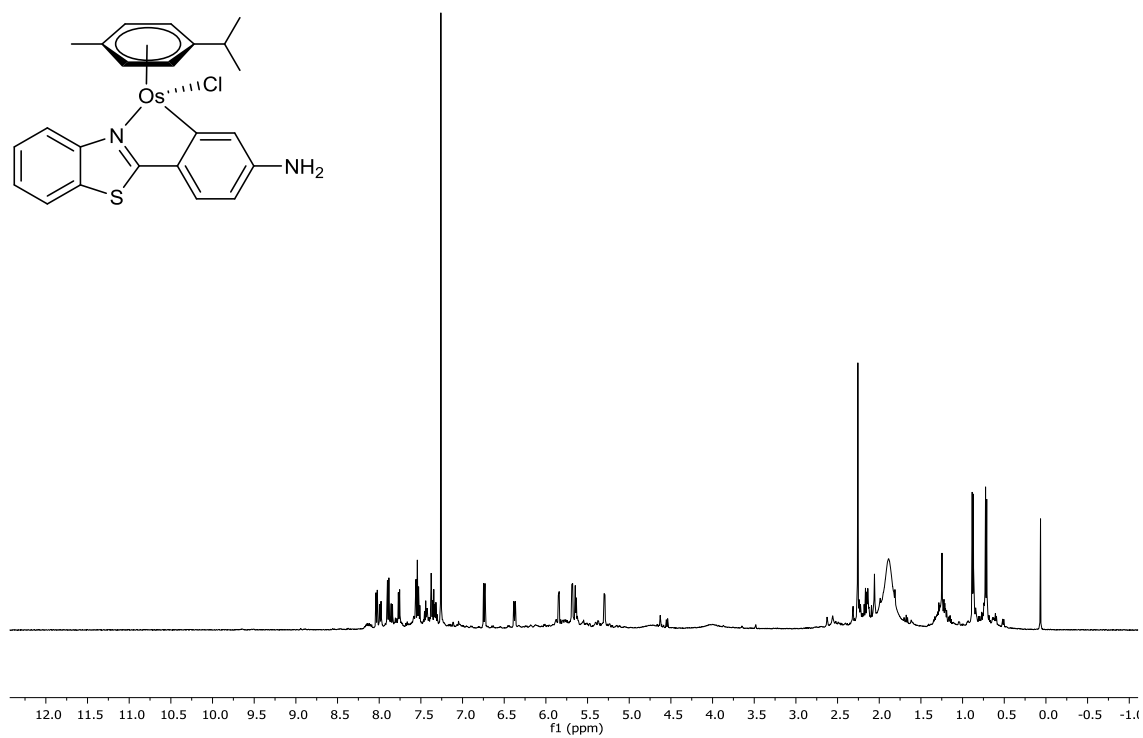


Figure S9: ^1H -spectrum of compound 6b

2 Crystallographic Data

Table S1. Experimental parameters and CCDC-Codes

Short	Machine	Source	Temp.	Detector Distance	Time/Frame	#Frames	Frame width	CCDC
	Bruker		[K]	[mm]	[s]		[°]	
1a	D8	Mo	100	35	16	2954	0.4	1978569
1b	X8	Mo	100	35	5	440	0.5	1978570
L5	D8	Mo	100	34	18	2326	0.5	1978571

Table S2. Relevant bond lengths, angles and torsion angles in compounds 5, 1a, and 1b

Compound	5	1a	1b
M-Cl [Å]	-	2.4133(6)	2.411(2)
M-N [Å]	-	2.1115(19)	2.109(7)
M-C2' [Å]	-	2.047(2)	2.073(10)
M-Cym _{centr}	-	1.712	1.704
C2-N [Å]	1.3026-1.3113	1.318(3)	1.301(12)
C3a-N [Å]	1.3858-1.3944	1.392(3)	1.374(14)
C2-S [Å]	1.7566-1.7625	1.732(2)	1.733(8)
C7a-S [Å]	1.7225-1.7341	1.747(3)	1.746(11)
C3a-C7a [Å]	1.4072-1.4104	1.400(3)	1.427(13)
C2-C1' [Å]	1.4637-1.4662	1.444(3)	1.439(15)
C1'-C2' [Å]	1.3942-1.3979	1.411(3)	1.425(13)
C1'-C6' [Å]	1.4056-1.4108	1.396(4)	1.389(14)
N-M-C2' [°]	-	77.52(8)	76.6(4)

Cl-M-N [°]	-	88.93(5)	83.9(2)
Cl-M-C2' [°]	-	84.93(6)	86.4(2)
C2-N-M [°]	-	115.00(15)	116.4(7)
C1'-C2'-M [°]	-	116.84(15)	116.4(8)
C2-C1'-C2' [°]	119.25-119.83	112.25(19)	111.7(9)
C3a-N-C2 [°]	109.73-110.76	111.49(19)	112.8(8)
N-C2-S [°]	115.43-115.87	115.29(16)	114.8(8)
C2-S-C7a [°]	89.14-89.25	89.37(10)	90.1(5)
C2'-C1'-C6' [°]	118.59-118.72	123.2(2)	123.3(11)
M-N-C2-C1' [°]	-	5.1(2)	2.4(10)
C3a-N-C2-S [°]	-	1.4(2)	2.1(10)

Table S3. Sample and crystal data of L5

Chemical formula	C ₁₄ H ₁₁ NOS	Crystal system	monoclinic	
Formula weight [g/mol]	241.3	Space group	C2/c	
Temperature [K]	100	Z	32	
Measurement method	\Phi and \omega scans	Volume [Å³]	9182.1(7)	
Radiation (Wavelength [Å])	MoK α (λ = 0.71073)	Unit cell dimensions [Å] and [°]	38.449(2)	90
Crystal size [mm³]	0.63 × 0.181 × 0.1		9.6018(3)	103.666(3)
Crystal habit	clear yellowblock		25.5966(10)	90
Density (calculated) [g/cm³]	1.396	Absorption coeff. / [mm⁻¹]	0.262	
Abs. correction Tmin	0.6055	Abs. correction Tmax	0.7460	
Abs. correction type	multiscan	F(000) [e⁻]	4032	

Table S4. Data collection and structure refinement of L5

Index ranges	$-54 \leq h \leq 54, -13 \leq k \leq 13, -36 \leq l \leq 36$	Theta range for data collection [°]	4.586 to 60.242	
Reflections number	227554	Data / restraints / parameters	13526/0/617	
Refinement method	Least squares	Final R indices	all data	R1 = 0.0526. wR2 = 0.1134
Function minimized	$\Sigma w(F_o^2 - F_c^2)^2$		$I > 2\sigma(I)$	R1 = 0.0383. wR2 = 0.1030
Goodness-of-fit on F^2	1.013	Weighting scheme	$w = 1/[\sigma^2(F_o^2) + (0.0588P)^2 + 9.5500P]$	
Largest diff. peak and hole [e Å ⁻³]	0.91/-0.32		where $P = (F_o^2 + 2F_c^2)/3$	

Table S5. Sample and crystal data of 1a

Chemical formula	C23H22ClNRuS	Crystal system	orthorhombic	
Formula weight [g/mol]	480.99	Space group	<i>Pna21</i>	
Temperature [K]	100	Z	4	
Measurement method	$\backslash \Phi$ and $\backslash \omega$ scans	Volume [Å ³]	1956.05(16)	
Radiation (Wavelength [Å])	MoK α ($\lambda = 0.71073$)	Unit cell dimensions [Å] and [°]	7.9904(4)	90
Crystal size [mm ³]	$0.183 \times 0.173 \times 0.099$		14.5823(7)	90
Crystal habit	clear orange block		16.7875(8)	90
Density (calculated) [g/cm ³]	1.633	Absorption coeff. / [mm ⁻¹]	1.053	
Abs. correction Tmin	0.6927	Abs. correction Tmax	0.7460	
Abs. correction type	multiscan	F(000) [e ⁻]	976	

Table S6. Data collection and structure refinement of 1a

Index ranges	$-11 \leq h \leq 11, -20 \leq k \leq 20, -23 \leq l \leq 23$	Theta range for data collection [°]	3.7 to 60.198	
Reflections number	96508	Data / restraints / parameters	5762/1/247	
Refinement method	Least squares	Final R indices	all data	R1 = 0.0184. wR2 = 0.0465
Function minimized	$\Sigma w(F_o^2 - F_c^2)^2$		$I > 2\sigma(I)$	R1 = 0.0180. wR2 = 0.0461
Goodness-of-fit on F^2	1.1	Weighting scheme	$w = 1/[\sigma^2(F_o^2) + (0.0258P)^2 + 0.8970P]$	
Largest diff. peak and hole [e Å ⁻³]	0.98/-0.58		where $P = (F_o^2 + 2F_c^2)/3$	

Table S7. Sample and crystal data of 1b

Chemical formula	C ₂₃ H ₂₂ ClNO ₅ S	Crystal system	orthorhombic	
Formula weight [g/mol]	570.12	Space group	<i>P</i> 212121	
Temperature [K]	100	Z	4	
Measurement method	\Phi and \omega scans	Volume [Å ³]	1941.52(13)	
Radiation (Wavelength [Å])	MoK α (λ = 0.71073)	Unit cell dimensions [Å] and [°]	7.6690(3)	90
Crystal size [mm ³]	0.160 × 0.150 × 0.050		10.4181(3)	90
Crystal habit	clear orange block		24.3005(11)	90
Density (calculated) [g/cm ³]	1.95	Absorption coeff. / [mm ⁻¹]	6.821	
Abs. correction Tmin	0.4696	Abs. correction Tmax	0.7460	
Abs. correction type	multiscan	F(000) [e ⁻]	1104	

Table S8. Data collection and structure refinement of 1b

Index ranges	-9 ≤ h ≤ 8, -12 ≤ k ≤ 12, -29 ≤ l ≤ 24	Theta range for data collection [°]	5.15 to 50.69	
Reflections number	7981	Data / restraints / parameters	3562/18/247	
Refinement method	Least squares	Final R indices	all data	R1 = 0.0352. wR2 = 0.0660
Function minimized	$\Sigma w(F_o^2 - F_c^2)^2$		I > 2σ(I)	R1 = 0.0320. wR2 = 0.0643
Goodness-of-fit on F ²	1.055	Weighting scheme	$w = 1 / [\sigma^2(F_o^2) + (0.0235P)^2 + 3.1903P]$	
Largest diff. peak and hole [e Å ⁻³]	2.55/-1.18		where $P = (F_o^2 + 2F_c^2) / 3$	

3 Abundant adducts found in different ESI-mass spectra

Table S9. Common adducts and their measured and theoretical m/z ratios

adduct	m/z		
	found	st dev	calc
[1a -Cl] ⁺	446.07	0.01	446.05
[6a -Cl] ⁺	461.08	0.01	461.07
[1b -Cl] ⁺	536.11	0.01	536.11
[6b -Cl] ⁺	551.12	0.01	551.12
[Ru(cym)(L1)(Met)] ⁺	595.07	0.01	595.11
[Ru(cym)(L6)(Met)] ⁺	610.08	0.01	610.12
[Os(cym)(L1)(Met)] ⁺	685.12	0.01	685.17
[Os(cym)(L6)(Met)] ⁺	700.13	0.01	700.18
[Ru(cym)(L1)(N-Ac-Met)] ⁺	637.08	0.01	637.12
[Ru(cym)(L6)(N-Ac-Met)] ⁺	652.09	0.01	652.13
[Os(cym)(L1)(N-Ac-Met)] ⁺	727.11	0.01	727.18
[Os(cym)(L6)(N-Ac-Met)] ⁺	742.11	0.01	742.19
[Ru(cym)(L1)(DDT)] ⁺	598.00	0.01	598.07
[Ru(cym)(L6)(DDT)] ⁺	613.02	0.01	613.08
[Os(cym)(L1)(DDT)] ⁺	688.05	0.01	688.13
[Os(cym)(L6)(DDT)] ⁺	703.07	0.01	703.14
[Ru(cym)(L1)(Ub)] ⁺	9008.3	1.3	9009.69
[Ru(cym)(L6)(Ub)] ⁺	9023.46	0.42	9024.70
[Os(cym)(L1)(Ub)] ⁺	9103.25	8.13	9100.75
[Os(cym)(L6)(Ub)] ⁺	9113.42	1.73	9114.75
[Ru(cym)(L1)(GTP)] ²⁻	483.00	0.02	483.02
[Ru(cym)(L6)(GTP)] ²⁻	490.51	0.01	490.53
[Os(cym)(L1)(GTP)] ²⁻	528.00	0.01	528.05
[Os(cym)(L6)(GTP)] ²⁻	535.98	0.02	536.06

4 Biological data

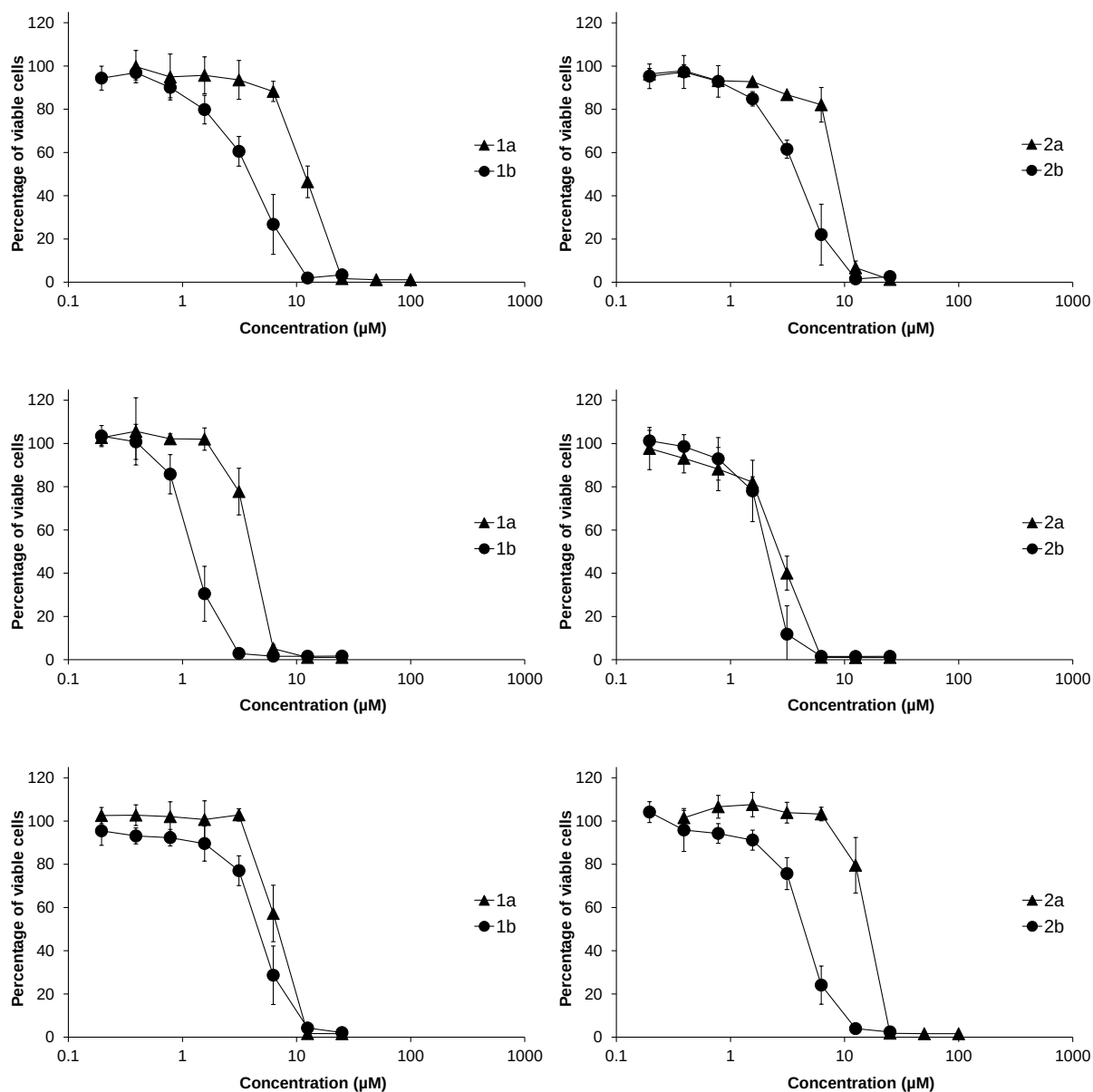


Figure S10: Concentration-effect curves of compounds 1a and 1b (left), 2a and 2b (right) in A549 (top), CH1/PA-1 (middle) and SW480 (bottom) cells, obtained by the MTT assay (exposure: 96 h). Values are means $\pm$ standard deviations from at least three independent experiments.

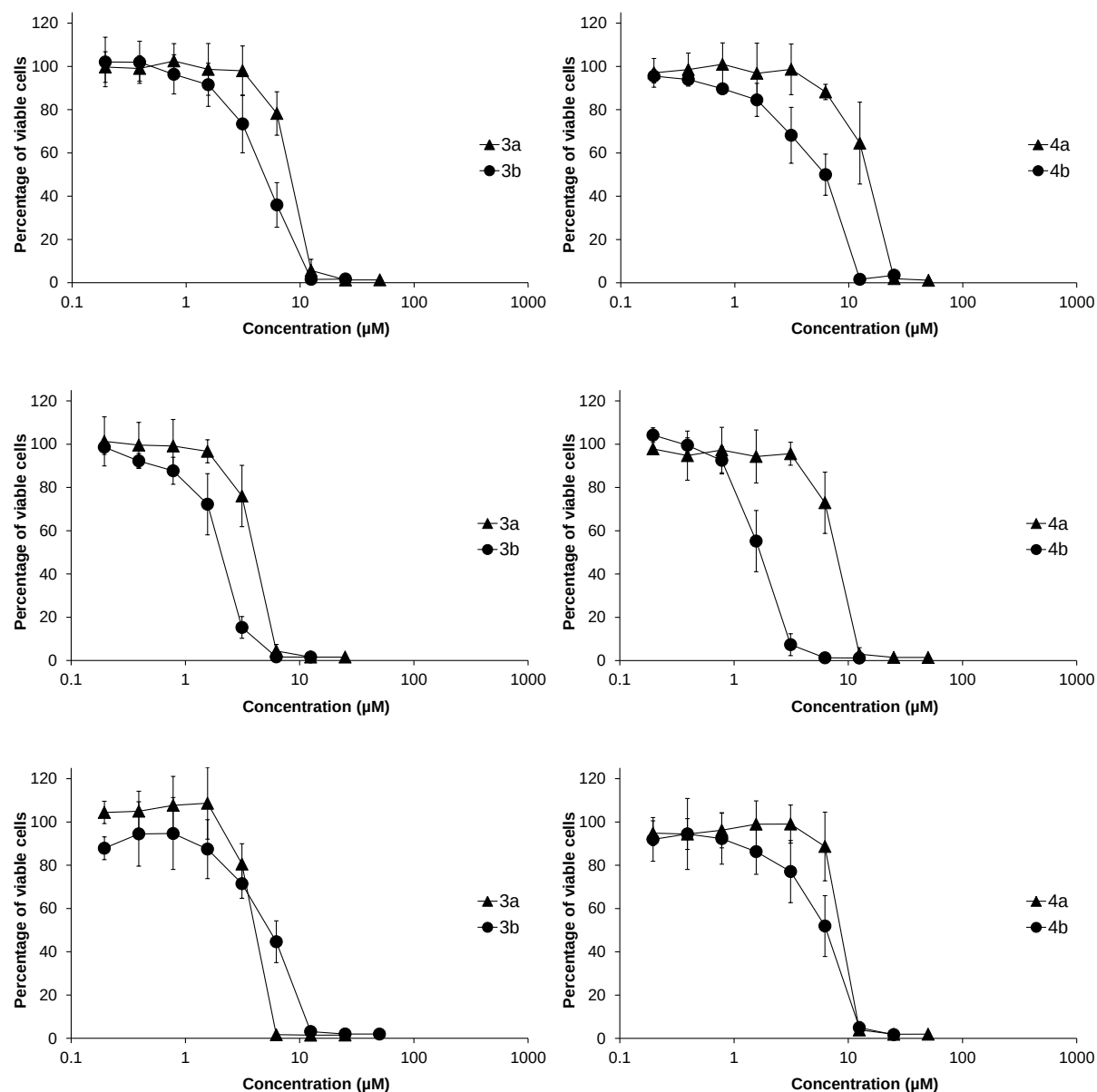


Figure S11: Concentration-effect curves of compounds 3a and 3b (left), 4a and 4b (right) in A549 (top), CH1/PA-1 (middle) and SW480 (bottom) cells, obtained by the MTT assay (exposure: 96 h). Values are means $\pm$ standard deviations from at least three independent experiments.

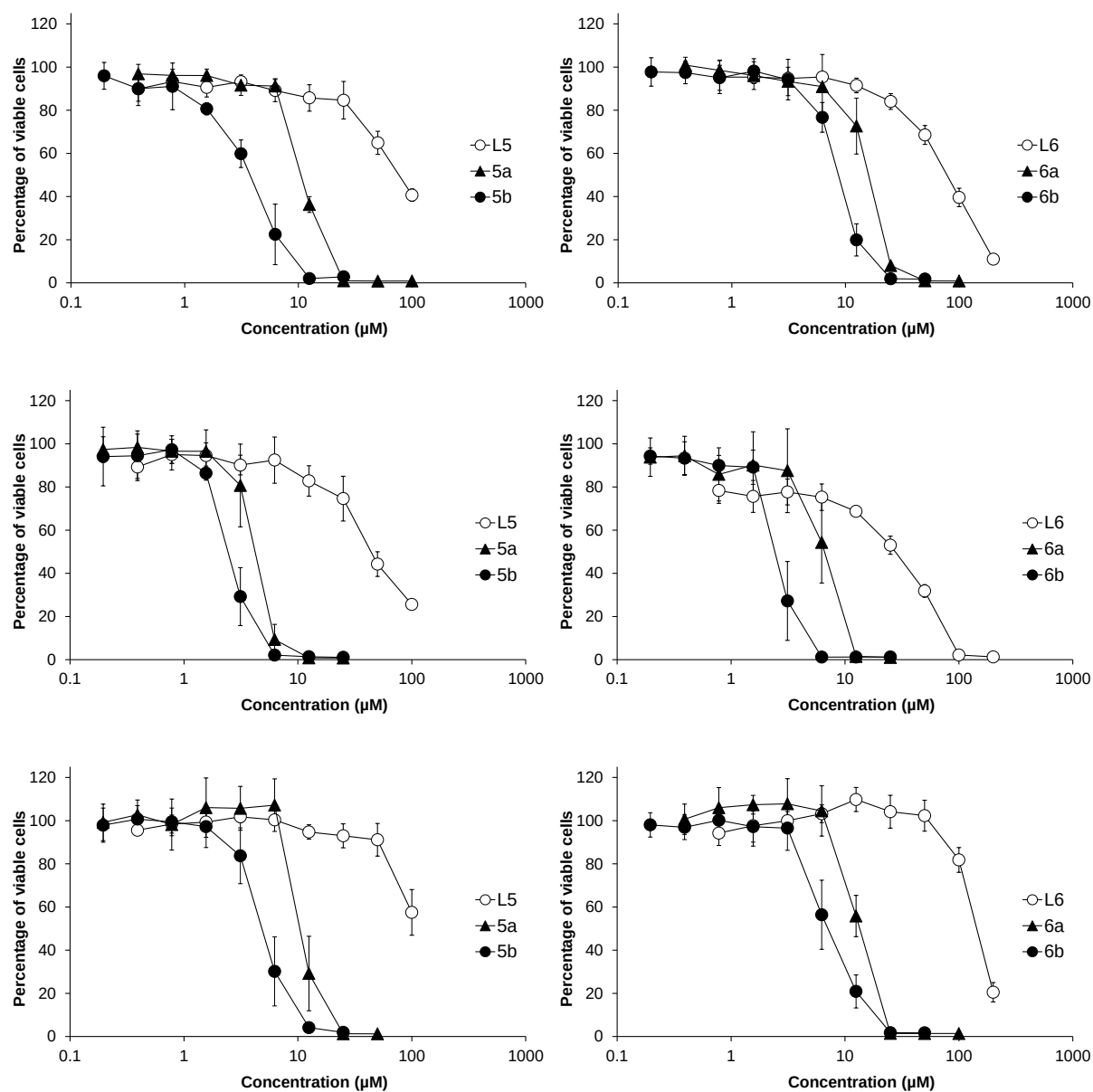


Figure S12: Concentration-effect curves of compounds L5, 5a and 5b (left), L6, 6a and 6b (right) in A549 (top), CH1/PA-1 (middle) and SW480 (bottom) cells, obtained by the MTT assay (exposure: 96 h). Values are means $\pm$ standard deviations from at least three independent experiments.

4.1 FACS Studies

Table S10. Apoptosis induction by 2a, 2b, 6a and 6b after 24 h in SW480 cells, analyzed by the flow-cytometric AV/PI assay differentiating viable (AV-/PI-), early apoptotic (AV+/PI-), late apoptotic (AV+/PI+) and necrotic (AV-/PI+) cell fractions. Percentages are means $\pm$ standard deviations from three independent experiments.

Compound	Conc. [μ M]	viable cells [%]	early apoptotic cells [%]	late apoptotic cells [%]	necrotic cells [%]
neg. control	0	95.4 $\pm$ 1.0	2.1 $\pm$ 0.8	2.2 $\pm$ 0.4	0.4 $\pm$ 0.2
pos. control	50	6.4 $\pm$ 5.5	1.8 $\pm$ 2.6	87.7 $\pm$ 5.9	4.1 $\pm$ 2.9
2a	5	91.6 $\pm$ 2.6	2.0 $\pm$ 1.6	5.7 $\pm$ 1.4	0.8 $\pm$ 0.3
	10	75.9 $\pm$ 2.2	13.7 $\pm$ 2.5	8.9 $\pm$ 0.7	1.6 $\pm$ 0.8
	20	69.3 $\pm$ 12.3	2.3 $\pm$ 0.9	22.4 $\pm$ 8.1	6.0 $\pm$ 5.1
	40	1.0 $\pm$ 0.2	3.0 $\pm$ 0.2	85.1 $\pm$ 2.3	10.9 $\pm$ 1.9
2b	5	95.4 $\pm$ 1.0	1.8 $\pm$ 0.7	2.5 $\pm$ 0.5	0.3 $\pm$ 0.2
	10	95.9 $\pm$ 1.0	1.0 $\pm$ 0.4	2.8 $\pm$ 0.6	0.3 $\pm$ 0.2
	20	95.3 $\pm$ 1.4	0.9 $\pm$ 0.4	3.4 $\pm$ 0.9	0.4 $\pm$ 0.3
	40	94.3 $\pm$ 1.3	1.0 $\pm$ 0.5	4.3 $\pm$ 1.0	0.4 $\pm$ 0.1
6a	5	95.6 $\pm$ 1.6	1.8 $\pm$ 1.2	2.1 $\pm$ 0.5	0.5 $\pm$ 0.1
	10	94.6 $\pm$ 1.0	2.3 $\pm$ 1.2	2.7 $\pm$ 0.3	0.6 $\pm$ 0.3
	20	71.0 $\pm$ 3.1	17.4 $\pm$ 3.4	10.6 $\pm$ 1.0	1.0 $\pm$ 0.5
	40	80.7 $\pm$ 3.3	7.1 $\pm$ 1.4	10.9 $\pm$ 2.0	1.4 $\pm$ 0.1
6b	5	95.3 $\pm$ 1.2	2.1 $\pm$ 1.2	2.2 $\pm$ 0.3	0.4 $\pm$ 0.3
	10	94.6 $\pm$ 1.1	2.2 $\pm$ 1.0	2.9 $\pm$ 0.3	0.4 $\pm$ 0.2
	20	93.9 $\pm$ 0.6	1.8 $\pm$ 0.7	3.8 $\pm$ 0.2	0.5 $\pm$ 0.2
	40	85.3 $\pm$ 1.0	3.2 $\pm$ 0.1	10.7 $\pm$ 1.0	0.8 $\pm$ 0.4

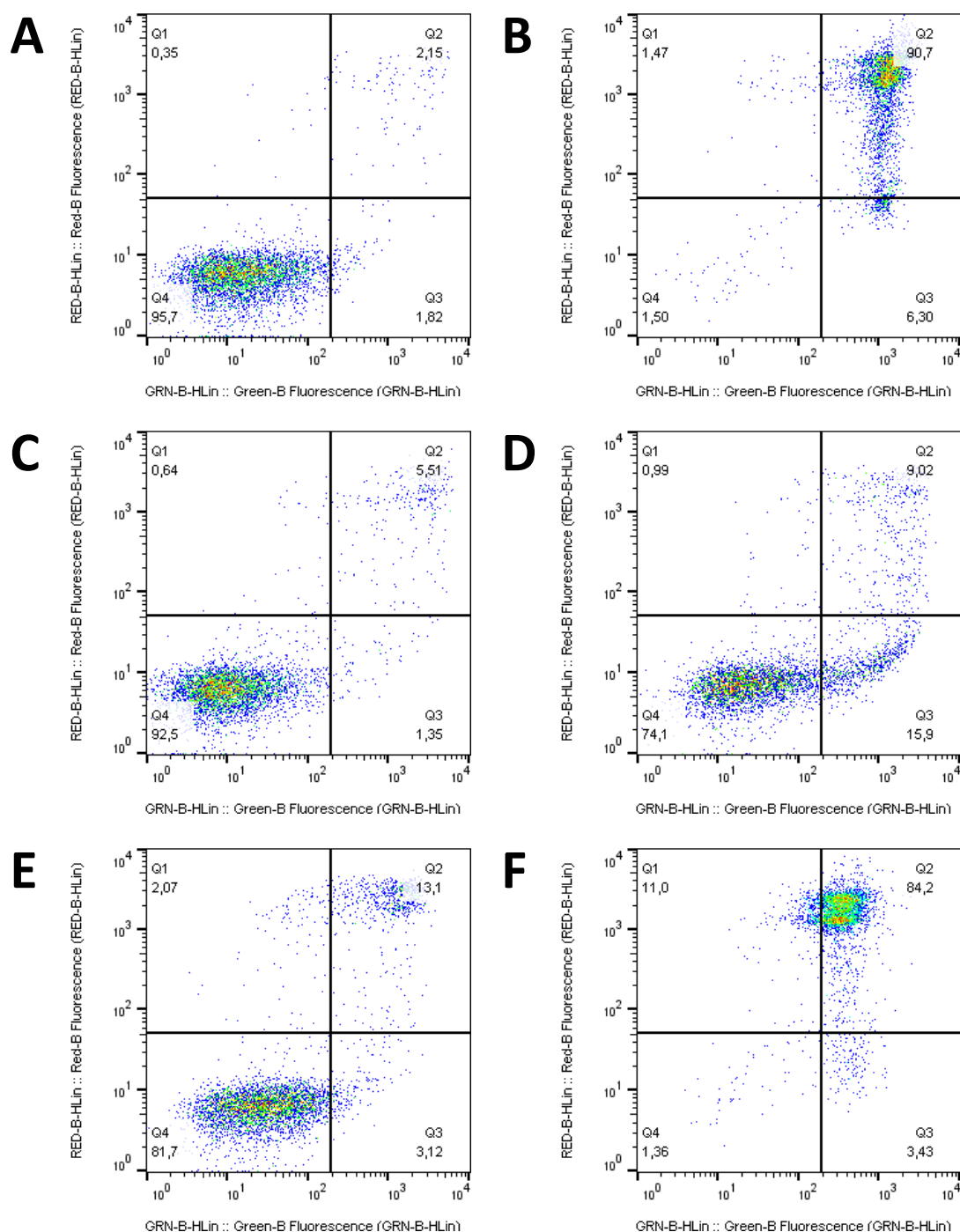


Figure S13. Apoptosis induction by 2a after 24 h in SW480 cells, analyzed by the flow-cytometric AV/PI assay. Dot plots represent necrotic (Q1: AV-/PI+), late apoptotic (Q2: AV+/PI+), early apoptotic (Q3: AV+/PI-) and viable (Q4: AV-/PI-) cell fractions (percentages indicated correspondingly) from a selected experiment: (A) untreated control; (B) positive control (50 μ M of Pt complex KP1998(Scaffidi-Domianello et al., 2012)); (C) 5 μ M, (D) 10 μ M, (E) 20 μ M and (F) 40 μ M of 2a.

4.2 Cellular Uptake Studies

Table S11. Instrumental parameters for ICP-MS analysis. (Klose et al., 2017)

	ICP-MS Agilent 7500ce
RF power [W]	1560
Cone material	Nickel
Carrier gas [L/min]	0.92–0.97
Make up gas [L/min]	0.22–0.27
Plasma gas [L/min]	15
Monitored isotopes	^{102}Ru , ^{115}In , ^{185}Re , ^{189}Os
Dwell time [s]	0.3
Number of replicates	10

For Ru, In served as internal standard, while Re was used as internal standard for Os.

5 References

- Klose, M.H.M., Hejl, M., Heffeter, P., Jakupec, M.A., Meier-Menches, S.M., Berger, W., et al. (2017). Post-digestion stabilization of osmium enables quantification by ICP-MS in cell culture and tissue. *Analyst* 142(13), 2327-2332. doi: 10.1039/c7an00350a.
- Scaffidi-Domianello, Y.Y., Legin, A.A., Jakupec, M.A., Roller, A., Kukushkin, V.Y., Galanski, M., et al. (2012). Novel Oximato-Bridged Platinum(II) Di- and Trimer(s): Synthetic, Structural, and in Vitro Anticancer Activity Studies. *Inorganic Chemistry* 51(13), 7153-7163. doi: 10.1021/ic300148e.



Article

Arene Variation of Highly Cytotoxic Tridentate Naphthoquinone-Based Ruthenium(II) Complexes and In-Depth In Vitro Studies

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Citation: Cseh, K.; Geisler, H.; Stanojkovska, K.; Westermayr, J.; Brunmayr, P.; Wenisch, D.; Gajic, N.; Hejl, M.; Schaier, M.; Koellensperger, G.; et al. Arene Variation of Highly Cytotoxic Tridentate Naphthoquinone-Based Ruthenium(II) Complexes and In-Depth In Vitro Studies.

Pharmaceutics **2022**, *14*, 2466.

<https://doi.org/10.3390/pharmaceutics14112466>

Academic Editors: Damiano Cirri and Federica De Castro

Received: 14 October 2022

Accepted: 5 November 2022

Published: 15 November 2022

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Abstract: The main purpose of this study was to synthesize a new set of naphthoquinone-based ruthenium(II) arene complexes and to develop an understanding of their mode of action. This study systematically reviews the steps of synthesis, aiming to provide a simplified approach using microwave irradiation. The chemical structures and the physicochemical properties of this novel group of compounds were examined by ¹H-NMR and ¹³C-NMR spectroscopy, X-ray diffractometry, HPLC-MS and supporting DFT calculations. Several aspects of the biological activity were investigated in vitro, including short- and long-term cytotoxicity tests, cellular accumulation studies, detection of reactive oxygen species generation, apoptosis induction and NAD(P)H:quinone oxidoreductase 1 (NQO1) activity as well as cell cycle analysis in A549, CH1/PA-1, and SW480 cancer cells. Furthermore, the DNA interaction ability was studied in a cell-free assay. A positive correlation was found between cytotoxicity, lipophilicity and cellular accumulation of the tested complexes, and the results offer some important insights into the effects of the arene. The most obvious finding to emerge from this study is that the usually very chemosensitive CH1/PA-1 teratocarcinoma cells showed resistance to these phthiocol-based organometallics in comparison to the usually less chemosensitive SW480 colon carcinoma cells, which pilot experiments suggest as being related to NQO1 activity.

Keywords: metal-based drugs; ruthenium; piano-stool complexes; anticancer

1. Introduction

Platinum-based compounds are an important pillar in the fight against cancer, particularly the worldwide-approved drugs cisplatin, carboplatin and oxaliplatin [1,2]. However, these drugs cause severe side effects such as nephrotoxicity, myelosuppression and neurotoxicity [3]. To overcome these drawbacks, scientists worldwide are searching for alternatives, such as small-molecule receptor tyrosine kinase inhibitors (e.g., sunitinib), monoclonal antibodies (e.g., cetuximab, bevacizumab, ipilimumab), CAR-T cells, vaccines,

oncolytic viruses and new cytostatic drugs (e.g., novel platinum-based compounds) [4–6]. Since the latter interfere with cell proliferation, not only cancer cells but also normal cells are affected, which leads to severe side effects.

One line of research is the use of ruthenium instead of platinum, due to the lower toxicity of active complexes [7]. There are two prominent ruthenium coordination compounds which have shown promising results in (pre)clinical trials, namely TLD-1433 and BOLD-100 (formerly KP1139, IT-139). The ruthenium(II) compound TLD-1433 (Figure 1A) is under investigation for photodynamic therapy and a clinical phase two trial in patients with non-muscle invasive bladder cancer is in progress (ClinicalTrials.gov, Identifier: NCT03945162) [8]. The ruthenium(III) drug BOLD-100 (Figure 1B) was granted an Orphan Drug Designation (ODD) in pancreatic cancer and a clinical trial of a combination therapy with FOLFOX (5-fluorouracil + folinic acid + oxaliplatin) in gastro-intestinal cancers is ongoing (ClinicalTrials.gov Identifier: NCT04421820) [9].

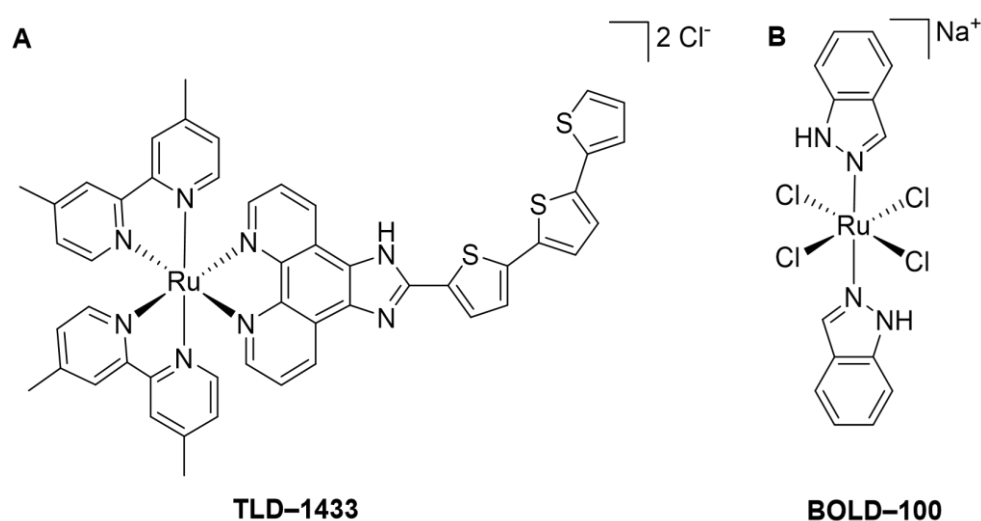


Figure 1. Chemical structures of clinically studied ruthenium compounds. Ru(II) coordination compound TLD-1433 (A) and Ru(III) drug BOLD-100 (B).

However, not only coordination compounds of ruthenium are in the focus of metal-based anticancer drug research, but also organometallic ruthenium(II) compounds. The best-studied compounds are RAPTA-C from Dysons' group, which mainly interacts with proteins, and RM175 from Sadlers' group, which mainly interferes with DNA [10]. This complex class exhibits a piano-stool structure, in which the metal center is surrounded by an arene and variable ligands with different denticities. The three coordination sites can be occupied by three monodentate, one bidentate and one monodentate, or one tridentate ligand. This pseudo-octahedral geometry provides a powerful tool for controlling pharmacokinetic and pharmacodynamic properties. Each building block of the complex can influence chemical and biological properties, such as stability, cytotoxicity, cellular uptake, electrochemical behavior, and hydrolysis [11–13]. Furthermore, bioactive ligands were often used in order to increase the cytotoxic effect or for targeting.

One interesting compound class is naphthoquinones, which demonstrate several potential modes of action, such as ROS (reactive oxygen species) generation, apoptosis induction through ER (endoplasmic reticulum) stress and inhibition of topoisomerase, as well as interference with p53 or NAD(P)H:quinone oxidoreductase 1 activity (NQO1) [14].

Due to this broad range of properties favoring anticancer activity, naphthoquinone-based organometallics were developed and investigated. Complexes with a labile chlorido-leaving group have shown promising results in vitro and in vivo [15,16]. However, low stability in an aqueous solution may explain the occurrence of severe side effects during in vivo experiments. Stability was improved by an in situ formation of a hemiaminal bond between an azole and a naphthoquinone moiety, yielding complexes with a tridentate

ligand, which increased the stability enormously [17]. Only a few examples of tridentate ligands are reported in the literature for anticancer drug research (Figure 2). The *N, N, N*-coordinated ruthenium complex from Babak et al. has shown high stability, which leads to inertness against biomolecules resulting in low activity *in vitro* [18]. However, our group synthesized novel tridentate naphthoquinone complexes, providing highly cytotoxic and more stable *N, O, O*-complexes compared to the parental bidentate chlorido compounds [17,19]. Furthermore, Martínez-De-León et al. published cationic *N, N, S*- and *N, N, Se*-iminophosphorane organometallics with good cytotoxicity in several cancer cell lines [20].

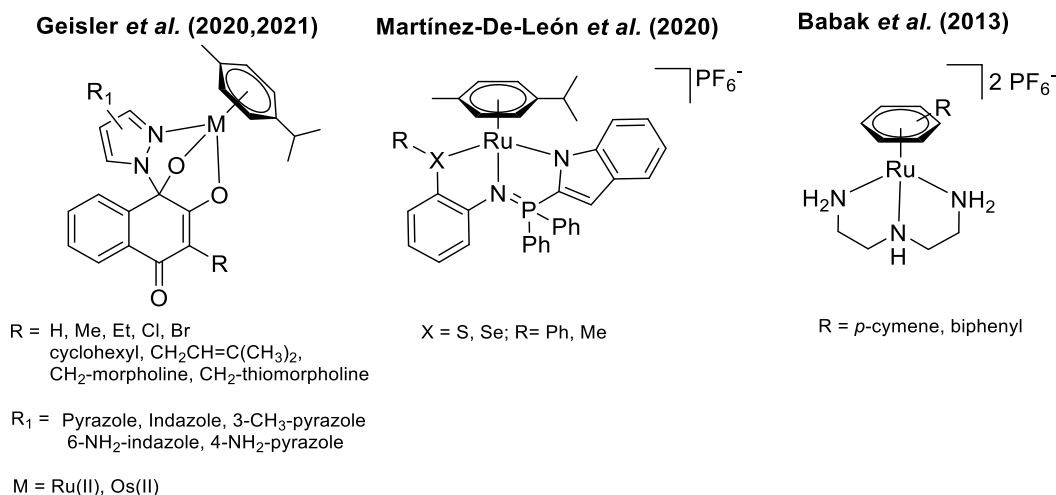


Figure 2. Tridentate ruthenium arene complexes with anticancer properties [17–20].

Within this work, we synthesized, characterized and investigated phthiocol-based organometallics with different arenes and their stabilities at physiological pH by HPLC-MS measurements. Density Functional Theory (DFT) calculations were performed to prove the stability and hydrolysis hypotheses. In addition, the most relevant aspects of their biological activity were investigated in several biological assays, such as determination of cytotoxicity, cellular accumulation, ROS generation ability, apoptosis induction, DNA interaction, as well as influence on the cell cycle and NQO1 activity.

2. Materials and Methods

The used dimeric metal precursors [Ru(*p*-cymene)Cl₂]₂, [RuCl₂(benzene)]₂, [RuCl₂(toluene)]₂, [RuCl₂(indane)]₂ and [RuCl₂(biphenyl)]₂ were synthesized according to the literature [21–23]. Phthiocol (L) [24,25] and complex 1 [17] were synthesized as reported. The following chemicals were used without further purification for the syntheses: RuCl₃ × H₂O (Johnson Matthey, London, UK), biphenyl, cyclohexa-1,3-diene, indane, sodium (Sigma-Aldrich, St. Louis, MO, USA), 1-methyl-1,4-cyclohexadiene, α-terpinene (Alfa Aesar, Haverhill, MA, USA), 1H-pyrazole, triethylamine, menadione (Acros-Fisher, Waltham, MA, USA), [RuCl₂(hexamethylbenzene)]₂ (TCI, Tokyo, Japan) and hydrogen peroxide (30%, VWR, Radnor, PA, USA).

Solvents were purchased from commercial suppliers and dried before use if needed. Microwave reactions were performed with a Biotage® Initiator+ system (Uppsala, Sweden). Purification via flash column chromatography was conducted with Biotage® Isolera system (Uppsala, Sweden) and silica gel (VWR, mesh 40–63 μm). Elemental analyses were performed by the Microanalytical Laboratory of the University of Vienna with a Eurovector EA 3000(2009), equipped with a high-temperature pyrolysis furnace (HT, Hekatech, Osnabrück Germany, 2009). Elemental analysis samples were weighed on a Sartorius SEC 2 ultra-micro balance with ±0.1 μg resolution. Sample weights from 1–3 mg were used. For calibration, two NIST-certified reference materials were used: sulfanilamide (C₆H₈N₂O₂S) and BBOT (2,

5-bis-(5-tert-butyl-2-benzoxazole-2-yl)-thiophenone, $C_{26}H_{26}N_2O_2S$). The limit of quantification (LOQ) was 0.05 w-% for C, H, N and 0.02 w-% for S. For samples without N and/or S, the content of these elements was determined and verified to be below LOQ. The presented values are the average of determinations in triplicate. The general numbering of carbons and hydrogens and the corresponding NMR spectra of complexes 2–6 were described in the SI (Figures S1–S10). 1H -, ^{13}C - and 2D-NMR spectra were recorded at 298 K on a Bruker AV III 600 or AV NEO 500 spectrometers at 600.25/500.10 MHz (1H) and 150.95 MHz (^{13}C) (Bruker, Billerica, MA, USA). Single crystals of four compounds (2–4, 6) were obtained by vapor diffusion or liquid–liquid diffusion method from dichloromethane/diethyl ether or dichloromethane/*n*-hexane. The X-ray intensity data were measured on a Bruker D8 Venture diffractometer equipped with a multilayer monochromator, Mo and Cu K/ α IN-COATEC microfocus sealed tube and Oxford cooling system (Bruker, Billerica, MA, USA).

2.1. Synthesis

General Procedure for Complex Syntheses (2–6) [17]

The corresponding dimer (0.10 mmol), 1*H*-pyrazole (0.19–0.21 mmol), NEt_3 (0.6 mmol) and the desired phthiocol (L) (0.19–0.21 mmol) were dissolved in 8–12 mL methanol and stirred at 50–60 °C for 20–30 minutes under microwave irradiation. The solvent was removed, and the residue was purified by flash chromatography (SiO_2) with a ternary eluent system (EtOAc/*n*-hexane/ NEt_3 or EtOAc/MeCN/ NEt_3). The fractions were combined, evaporated and dried in vacuo. Oily residues were dissolved in dichloromethane, precipitated with *n*-hexane, separated and washed with *n*-hexane, and dried in vacuo.

[3-Methyl-4-oxo-(1*H*- κN^2 -pyrazol-1-yl)-1,4-dihydronaphthalene-1,2-bis(olato)- κO^1 - κO^2](η^6 -benzene)ruthenium(II)](2)

The reaction was performed according to the general procedure using $[RuCl_2(benzene)]_2$ (150 mg, 0.30 mmol), 1*H*-pyrazole (39 mg, 0.57 mmol), phthiocol (107 mg, 0.57 mmol) and NEt_3 (251 μ L, 182 mg, 1.80 mmol). The mixture was stirred at 60 °C for 20 minutes under microwave irradiation. Flash chromatography with EtOAc/MeCN/ NEt_3 (88/10/2). Yield: 93 mg yellow/green solid (0.21 mmol, 38%). Elemental analysis found: C, 52.61; H, 4.03; N, 6.12; O, 14.23. Calc. for $C_{20}H_{16}N_2O_3Ru \cdot (H_2O)_{1.1}$ C, 53.00; H, 4.05; N, 6.18; O, 14.47%. 1H NMR (600.25 MHz, MeOD- d_4) δ 8.40 (dd, J = 2.1, 0.6 Hz, 1H, H3'), 8.12–8.08 (m, 1H, H5–8), 7.65–7.59 (m, 3H, H5–8), 6.70 (d, J = 2.0 Hz, 1H, H5'), 6.35 (dd, J = 2.4, 2.4 Hz, 1H, H4'), 5.93 (s, 5H, Harene) and 1.74 (s, 3H, H9). ^{13}C NMR (150.93 MHz, MeOD- d_4) δ 184.2 (C4), 182.3 (C2), 142.0 (C3'), 137.4 (C4a/8a), 134.0 (C4a/8a), 132.5 (C5–8), 131.4 (C5–8), 128.0 (C5'), 127.6 (C5–8), 127.2 (C5–8), 108.8 (C4'), 105.8 (C3), 94.9 (C1), 82.9 (Carene) and 8.12 (C9).

[3-Methyl-4-oxo-(1*H*- κN^2 -pyrazol-1-yl)-1,4-dihydronaphthalene-1,2-bis(olato)- κO^1 - κO^2](η^6 -toluene)ruthenium(II)](3)

The reaction was performed according to the general procedure using $[RuCl_2(toluene)]_2$ (150 mg, 0.28 mmol), 1*H*-pyrazole (36 mg, 0.53 mmol), phthiocol (100 mg, 0.53 mmol) and NEt_3 (234 μ L, 170 mg, 1.68 mmol). The mixture was stirred at 50 °C for 20 minutes under microwave irradiation. Flash chromatography with EtOAc/*n*-hexane/ NEt_3 (90/5/5). Yield: 117 mg yellow solid (0.26 mmol, 49%). Elemental analysis found: C, 54.62; H, 4.20; N, 6.27; O, 12.31. Calc. for $C_{21}H_{18}N_2O_3Ru \cdot (H_2O)_{0.7}$: C, 54.82; H, 4.25; N, 6.09; O, 12.87%. 1H NMR (600.25 MHz, MeOD- d_4) δ 8.34 (dd, J = 2.2, 0.6 Hz, 1H, H3'), 8.12–8.08 (m, 1H, H5–8), 7.65–7.58 (m, 3H, H5–8), 6.69 (dd, J = 2.6, 0.6 Hz, 1H, H5'), 6.34 (t, J = 2.4 Hz, 1H, H4'), 6.12 (dd, J = 5.7, 5.7 Hz, 1H, Hc), 5.98 (dd, J = 5.7, 5.7 Hz, 1H, Hc), 5.66 (d, J = 5.8 Hz, 1H, Hb), 5.64 (dd, J = 5.6, 5.6 Hz, 1H, Hd), 5.57 (d, J = 5.6 Hz, 1H, Hb), 2.37 (s, 3H, He) and 1.75 (s, 3H, H9). ^{13}C NMR (150.95 MHz, MeOD- d_4) δ : 184.0 (C4), 183.0 (C2), 141.8 (C3'), 137.6 (C4a/8a), 134.0 (C4a/8a), 132.4 (C5–8), 131.3 (C5–8), 128.0 (C5'), 127.7 (C5–8), 127.2 (C5–8), 108.7 (C4'), 105.7 (C3), 101.1 (Ca), 94.9 (C1), 86.2 (Cc), 85.8 (Cc), 79.9 (Cb), 79.5 (Cb), 77.0 (Cd), 18.6 (Ce) and 8.1 (C9).

[3-Methyl-4-oxo-(1*H*- κN^2 -pyrazol-1-yl)-1,4-dihydronaphthalene-1,2-bis(olato)- κO^1 - κO^2](η^6 -indane)ruthenium(II)](4)

The reaction was performed according to the general procedure using $[\text{RuCl}_2(\text{indane})]_2$ (150 mg, 0.26 mmol), 1*H*-pyrazole (40 mg, 0.59 mmol), phthiocol (111 mg, 0.59 mmol) and NEt_3 (259 μL , 1.86 mmol). The mixture was stirred at 50 °C for 20 minutes under microwave irradiation. Flash chromatography with $\text{EtOAc}/n\text{-hexane}/\text{NEt}_3$ (80/10/2). Yield: 144 mg yellow/green solid (0.30 mmol, 58%). Elemental analysis found: C, 57.58; H, 4.29; N, 6.08; O, 10.50. Calc. for $\text{C}_{23}\text{H}_{20}\text{N}_2\text{O}_3\text{Ru}\cdot(\text{H}_2\text{O})_{0.25}$: C, 57.79; H, 4.32; N, 5.86; O, 10.88%. ^1H NMR (600.25 MHz, MeOD-d_4) δ 8.32 (d, J = 2.3 Hz, 1H, H3'), 8.12–8.08 (m, 1H, Harom.), 7.63–7.57 (m, 3H, Harom.), 6.68 (d, J = 2.6 Hz, 1H, H5'), 6.34 (dd, J = 2.4, 2.4 Hz, 1H, H4'), 5.97 (d, J = 5.7 Hz, 1H, Hb/d), 5.93 (d, J = 5.8 Hz, 1H, Hb/d), 5.87 (dd, J = 5.6, 5.6 Hz, 1H, Ha/c), 5.65 (dd, J = 5.5, 5.5 Hz, 1H, Ha/c), 2.99–2.92 (m, 1H, Hf), 2.90–2.84 (m, 1H, Hf), 2.83–2.75 (m, 2H, Hf), 2.17–2.04 (m, 2H, Hg) and 1.75 (s, 3H, H9). ^{13}C NMR (150.93 MHz, MeOD-d_4) δ 183.9 (C4), 183.4 (C2), 141.5 (C3'), 137.8 (C4a/8a), 134.1 (C4a/8a), 132.3 (C5–8), 131.3 (C5–8), 128.0 (C5'), 127.8 (C5–8), 127.1 (C5–8), 108.7 (C4'), 105.7 (C3), 102.4 (Ce), 100.1 (Ce), 94.8 (C1), 82.2 (Ca/c), 80.4 (Ca/c), 79.4 (Cb/d), 78.9 (Cb/d), 30.9 (2xCf), 24.1 (Cg) and 8.1 (C9).

[3-Methyl-4-oxo-(1*H*- κN^2 -pyrazol-1-yl)-1,4-dihydronaphthalene-1,2-bis(olato)- κO^1 - κO^2)(η^6 -biphenyl)ruthenium(II)](5)

The reaction was performed according to the general procedure using $[\text{RuCl}_2(\text{biphenyl})]_2$ (100 mg, 0.15 mmol), 1*H*-pyrazole (21 mg, 0.31 mmol), phthiocol (55 mg, 0.29 mmol) and NEt_3 (128 μL , 93 mg, 0.92 mmol). The mixture was stirred at 50 °C for 20 minutes under microwave irradiation. Flash chromatography with $\text{EtOAc}/\text{MeCN}/\text{NEt}_3$ (88/10/2). Yield: 144 mg yellow/green solid (0.19 mmol, 66%). Elemental analysis found: C, 60.76; H, 4.12; N, 5.53; O, 9.86. Calc. for $\text{C}_{26}\text{H}_{20}\text{N}_2\text{O}_3\text{Ru}\cdot(\text{H}_2\text{O})_{0.2}$: C, 60.86; H, 4.01; N, 5.46; O, 9.98%. ^1H NMR (600.25 MHz, MeOD-d_4) δ 8.10–8.07 (m, 1H, H5–8), 7.99 (d, J = 2.2 Hz, 1H, H3'), 7.87–7.82 (m, 2H, Hf–h), 7.61–7.57 (m, 3H, H5–8), 7.53–7.49 (m, 3H, Hf–h), 6.66 (d, J = 2.5 Hz, 1H, H5'), 6.26 (dd, J = 2.4, 2.4 Hz, 1H, H4'), 6.24 (d, J = 6.2 Hz, 2H, Hb), 6.20 (dd, J = 5.9, 5.9 Hz, 1H, Hc), 6.09 (dd, J = 5.9, 5.9 Hz, 1H, Hc), 5.92 (dd, J = 5.6, 5.6 Hz, 1H, Hd), 1.67 (s, 3H, H9). ^{13}C NMR (150.95 MHz, MeOD-d_4) δ 184.0 (C4), 182.6 (C2), 141.3 (C3'), 137.4 (C4a–8a), 136.2 (Ce), 134.0 (C4a–8a), 132.4 (Cf–h), 131.3 (C5–8), 130.9 (C5–8), 130.0 (Cf–h), 129.9 (Cf–h), 128.0 (C5'), 127.7 (C5–8), 127.2 (C5–8), 108.6 (C4'), 105.7 (C3), 99.3 (Ca), 95.0 (C1), 84.1 (Cc), 83.8 (Cc), 81.1 (Cb), 80.9 (Cb), 80.7 (Cd) and 8.1 (C9).

[3-Methyl-4-oxo-(1*H*- κN^2 -pyrazol-1-yl)-1,4-dihydronaphthalene-1,2-bis(olato)- κO^1 - κO^2)(η^6 -hexamethylbenzene)ruthenium(II)](6)

The reaction was performed according to the general procedure using $[\text{RuCl}_2(\text{hexamethylbenzene})]_2$ (150 mg, 0.23 mmol), 1*H*-pyrazole (37 mg, 0.54 mmol), phthiocol (102 mg, 0.54 mmol) and NEt_3 (226 μL , 164 mg, 1.62 mmol). The mixture was stirred at 60 °C for 30 minutes under microwave irradiation. Flash chromatography with $\text{EtOAc}/n\text{-hexane}/\text{NEt}_3$ (80/15/5). The fractions were combined, evaporated and the orange residue was dissolved in dichloromethane. Crystallization with *n*-hexane provides orange crystals, which were separated and dried in vacuo at 60 °C. Yield: 80 mg orange crystals (0.15 mmol, 27%). Elemental analysis found: C, 59.04; H, 5.39; N, 5.46; O, 9.21. Calc. for $\text{C}_{26}\text{H}_{28}\text{N}_2\text{O}_3\text{Ru}\cdot(\text{CH}_2\text{Cl}_2)_{0.15}$: C, 59.22; H, 5.38; N, 5.28; O, 9.05%. ^1H NMR (700.40 MHz, MeOD-d_4) δ 8.11 (s, 1H, H3'), 8.10–8.07 (m, 1H, H5–8), 7.63–7.53 (m, 3H, H5–8), 6.63 (d, J = 2.5 Hz, 1H, H5'), 6.34 (dd, J = 2.4, 2.4 Hz, 1H, H3'), 2.30 (s, 18H, Hb), 1.76 (s, 3H, H9). ^{13}C NMR (176.13 MHz, MeOD-d_4) δ 185.4 (C4), 183.5 (C2), 139.1 (C3'), 138.4 (C4a/8a), 134.3 (C4a/8a), 132.0 (C5–8), 131.0 (C5–8), 128.2 (C5'), 128.1 (C5–8), 126.9 (C5–8), 108.6 (C4'), 105.4 (C3), 94.5 (C1) 91.8 (Ca), 16.0 (Cb) and 8.0 (C9).

2.2. HPLC-MS Stability Measurements

The 10 mM stock solutions were prepared by dissolving compounds 2–6 in DMSO. 4 μL of stock solution was mixed with 990 μL of PBS buffer (pH 7.4) and 6 μL DMSO to obtain a final concentration of 40 μM . After injection, the chromatogram at 225 nm and the corresponding mass spectra (positive mode) were recorded hourly (4 h) and after 24 h at 20 °C. Peak areas were determined by integrating complex signals of the

respective chromatogram. The measurements were performed with an HPLC system (Agilent Technologies, 126 Infinity) equipped with a C18 column (Waters, Atlantis T3 3 μ M, 1.0×150 mm²) coupled to an MS (Bruker, amaZon SL, ESI, positive mode) with a flow rate of 0.2 mL/min at 20 °C and a gradient (5–95%) with Milli-Q water/ACN.

2.3. Theoretical Simulations

Optimizations of energetic minima and maxima (transition states) were carried out with the ORCA program suite [26] and the PBEh-3c method [27]. The PBEh-3c method is based on DFT that uses the PBE0 functional, reparametrized with 42% Hartree–Fock exchange, and the def2-mSVP double-zeta basis set. It further accounts for dispersion correction, the basis set superposition error, and includes the ZORA effective core potential for the Ru atom. This method has been shown to be more reliable than most frequently applied DFT protocols, such as B3LYP/6-31G*, and turned out to be most suitable for the purpose of this study [26,27]. A tight convergence was set for the self-consistent field and the DFT grid was set to 4. The conductor-like polarizable continuum model [28] was used to model the solvent with a dielectric constant of 80.4 and a refractive index of 1.33. Due to numerical instabilities and to obtain smoother potentials, Gaussian smearing [29] was applied to the point charge. After every converged optimization, a frequency calculation was carried out to confirm a minimum or transition state on the potential energy landscape.

The structure of the aqua complex was estimated by elongation of the bond between the metal and the weaker bound oxygen (next to the C=C double bond in the dihydronaphthalene unit) and by placing a water molecule close to the Ru atom. The initial structures were pre-optimized at the semi-empirical HF-3c level of theory. The relaxed structure was used as an input for subsequent optimization with PBE3h-3c.

In order to estimate the energy barrier for the aquation process, we carried out a transition state search. The optimization was carried out in ORCA with the settings described above. A frequency calculation with a single imaginary frequency confirmed the found transition state.

2.4. Cell Culture

CH1/PA-1 ovarian teratocarcinoma cells (provided by L. R. Kelland, CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK; confirmed by STR profiling as PA-1 ovarian teratocarcinoma cells at Multiplexion, Heidelberg, Germany), SW480 colon carcinoma and A549 non-small cell lung cancer cells (both obtained from the American Type Culture Collection, Manassas, VA, USA) were grown as adherent cultures in 75 cm² culture flasks (CytoOne, Starlab, Hamburg, Germany) by using minimal essential medium (MEM) supplemented with 1 mM sodium pyruvate, 4 mM L-glutamine, 1% (*v/v*) nonessential amino acids from 100-fold stock (all purchased from Sigma-Aldrich) and 10% heat-inactivated fetal bovine serum (BioWest, Nuaille, France). Cells were maintained under standard culture conditions with 5% CO₂ at 37 °C in a humidified atmosphere.

2.5. MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Acros Organics, Geel, Belgium) assay was used to detect the cytotoxicity of the compounds after 24 h and 96 h incubation. In brief, cells were harvested from culture flasks and the following cell numbers (each in 100 μ L) were seeded into 96-well culture plates (CytoOne, tissue culture treated, Starlab, Hamburg, Germany) for 24 h incubation: 1×10^4 (CH1/PA-1), 1×10^4 (SW480) and 1.5×10^4 (A549) cells per well; and for 96 h incubation: 1×10^3 (CH1/PA-1), 2×10^3 (SW480) and 3×10^3 (A549) cells per well. After 24 h, cells resuming their exponential growth rate were treated with test compounds at nine concentrations obtained by serial two-fold dilution. Stock solutions were prepared in DMSO (20 mM or 40 mM) and diluted in supplemented MEM. An amount of 100 μ L of each concentration was added to the plates in triplicates and cells were exposed for 24 or 96 h. Afterward, the medium was replaced with 100 μ L of MTT solution (5 mg/mL) in phosphate-buffered

saline (PBS, Sigma-Aldrich), diluted 1:7 in RPMI 1640 medium (supplemented with 4 mM L-glutamine and 10% heat-inactivated FBS). Upon cell-viability-dependent conversion of the yellow, water-soluble tetrazolium salt into purple formazan products for 4 h, the crystals were dissolved in 150 μ L DMSO. The absorbance of the resulting solutions, which is directly proportional to the number of viable cells, was measured at 550 nm (and at 690 nm as a reference) with a microplate reader (ELx808, BioTek, Winoosky, VT, USA) and Gen5TM 3.08 software (BioTek). Blank-corrected optical densities were subjected to graphical evaluation and interpolation of IC₅₀ values. At least three biologically and technically independent experiments were performed.

2.6. Colony Formation Assay

For the colony formation assay, SW480 cells were seeded at a density of 350 cells/2 mL into each well of 6-well plates (CytoOne, tissue culture treated) and left to adhere for 24 h. To determine the long-term effects of the test compounds, the cells were exposed to them for 11 days at 37 °C and 5% CO₂. After incubation, the wells were gently washed with 1 mL cold PBS and the colonies were fixed with 1 mL cold (4 °C) 100% methanol (Thermo Fisher, Waltham, MA, USA) for 30 min at 4 °C and washed with 1 mL PBS again. The fixed colonies were stained with 1 mL of 0.5% *w/v* crystal violet (Sigma-Aldrich) in methanol for 2 min and the plates were washed under tap water. After drying the plates, images of the wells were captured, and colonies were counted with the colony counting function of Fusion Fx7 software (Vilber Lourmat, Collégien, France). Results were normalized to untreated controls. Data are averaged from at least three independent experiments.

2.7. Cellular Ruthenium Accumulation

SW480 cells were seeded at densities of 1.8×10^5 cells/well in 12-well plates (CytoOne, tissue culture treated) in 1 mL of supplemented MEM per well. (As CH1/PA-1 cells tend to detach during the required washing steps, cellular accumulation studies were performed only in SW480 cells). After 24 h, compounds dissolved in DMSO and diluted in MEM were added to the cells to final concentrations of 2 μ M and 5 μ M, and cells were incubated at 37 °C for 2 h. During incubation, the cell number was determined from three control wells of a plate seeded in parallel to the treatment plates. After the 2 h incubation, the medium was removed and all wells were washed with 3×1 mL PBS, and cells were lysed by adding 400 μ L HNO₃ per well for 1 h at room temperature. An amount of 300 μ L of each lysate was added to 7.7 mL MilliQ water to get a total volume of 8 mL, and determination of the ruthenium content was carried out by ICP-MS. The measured amount of accumulated metal was normalized to the cell number and results were given in fg [Ru]/cell from at least three independent experiments. An Agilent 7800 ICP-MS instrument (Agilent Technologies, Tokyo, Japan) equipped with an Agilent SPS 4 autosampler (Agilent Technologies, Tokyo, Japan) was used to determine the ruthenium content. The ICP-MS parameters were tuned on a daily base to achieve high sensitivity. The ICP-MS was equipped with a MicroMist nebulizer with a sample uptake rate of ~ 0.2 mL/min and standard nickel cones. The Agilent MassHunter software package (Workstation Software, Version C.01.04, 2018) was used for data evaluation. The instrumental parameters can be found in the supplementary information (Table S9).

2.8. Cell Cycle Studies

For the flow-cytometric determination of cell cycle distribution, CH1/PA-1 (8×10^4 cells/well) and SW480 (1.2×10^5 cells/well) cells were seeded into 12-well plates (CytoOne, tissue culture treated) in 1 mL supplemented MEM. In the first 24 h, the cells were allowed to settle and resume exponential growth. Afterward, the cells were treated with three different concentrations of the test compounds ($\sim$ half of IC₅₀, $\sim$ IC₅₀ and $\sim$ two times IC₅₀) and continuously exposed for 24 h at 37 °C and at 5% CO₂. Gemcitabine (Sigma-Aldrich, 0.01 and 0.05 μ M) and etoposide (Sigma-Aldrich, 0.25, 0.5 and 1 μ M) were used as positive controls. After removal of the treatment medium, cells were washed with

1 mL PBS and trypsinized. Cells were collected in Eppendorf tubes and centrifuged (300 g, 3 min), and the supernatant was discarded. In the following step, cells were washed with 1 mL PBS and resuspended in 500 μ L PI/HFS buffer (0.1% *v/v* Triton X-100, 0.1% *w/v* sodium citrate, in PBS) containing 25 μ g/mL propidium iodide (PI, 1.0 mg/mL, Sigma-Aldrich). The staining was carried out overnight at 4 °C in the dark, and 200 μ L of the cell suspension was loaded into 96-well round bottom plates (Falcon). Samples were measured with a Millipore Guava easyCyte 8HT flow cytometer with GuavaSoft™ software in the InCyte-modus, and 10,000 events/sample were detected. Data were analyzed with FlowJo software v 10.8.1 (Tree Star, Ashland, OR, USA) using Watson algorithm. Percentages of cells in the three cell cycle phases (G1/G0, S and G2/M) were calculated by integrating the resulting histograms of fluorescence signals (Figures S29 and S30). Experiments were repeated at least three times. For statistical evaluation, an unpaired *t*-test with Welch's correction was performed by using GraphPad PRISM software.

2.9. Apoptosis Assay (Annexin V/PI)

Cell death induction was quantitatively analyzed via flow cytometry using double staining with FITC-conjugated annexin V (eBioscience, San Diego, CA, USA) and propidium iodide (PI, 1.0 mg/mL, Sigma-Aldrich). SW480 cells were seeded into 24-well plates (7×10^4 cells/well) in 600 μ L MEM per well and allowed to settle for 24 h. Afterward, cells were exposed to two or three different concentrations (0.1–100 μ M) of the compounds (based on their cytotoxic activity) for 24 h. After treatment, the medium was collected, and cells were washed once with 37 °C warm PBS and harvested. Following trypsinization, the cell suspension was added to the pre-collected medium and cells were centrifuged (300 g, 3 min). The supernatant was removed, and the cell pellet was resuspended with 1.5 μ L FITC-conjugated annexin V in 150 μ L binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl and 2.5 mM CaCl_2) and incubated at 37 °C for 15 min. An amount of 1 μ L of propidium iodide (PI, Thermo Fisher) was added shortly before flow-cytometric analysis using a Guava easyCyte 8HT instrument (Merck Millipore, Burlington, MA, USA) with InCyte software. Results were quantified by using the FlowJo software (TreeStar). At least three independent experiments were conducted.

2.10. DCFH/DA Assay

To monitor the cellular levels of reactive oxygen species (ROS), SW480 cells were seeded into 96-well plates (2.5×10^4 cells/well) in volumes of 100 μ L per well. After a 24 h pre-incubation, the cells were washed with 200 μ L Hanks' balanced salt solution (HBSS, Sigma-Aldrich, containing 1% FCS) and incubated with 100 μ L of 25 μ M DCFH-DA (2',7'-dichlorofluorescein diacetate, Sigma-Aldrich) in HBSS (containing 1% FCS) for 45 min at 37 °C. Cells were washed with 200 μ L HBSS and exposed to the compounds in a range of concentrations (0.2–200 μ M) in 200 μ L phenol-red-free Opti-MEM (Gibco, with 1% FCS) in triplicates. All experiments included blanks (phenol-red-free medium with 1% FCS), negative controls (non-drug-treated cells) and positive controls (cells treated with 200 μ M and 400 μ M tert-butyl hydroperoxide (Sigma Aldrich)). ROS production was measured at 10 min intervals with the Biotek Synergy HT reader (excitation: 485/20 nm, emission: 516/20 nm) over a 2 h period. For representation, the values were given as the ratios of the means of treated samples and the means of the negative control $\pm$ standard deviations from two independent experiments (due to a lack of effect, no more repeats were performed).

2.11. DNA Interaction Assay

For detecting changes in DNA secondary structure upon drug treatment, 400 ng of the pUC19 plasmid (2686 bp, New England Biolabs, Ipswich, MA, USA) was incubated with the test compounds at a final concentration of 50 μ M (dissolved and diluted in MilliQ water). The incubation was performed at 37 °C for different time intervals (15 min to 6 h) with continuous shaking. Additionally, 4 μ L aliquots of a 6 $\times$ DNA loading dye (Thermo Fisher) were added to the 20 μ L reaction solution, and the DNA samples were separated

in a 1% agarose gel in $1 \times$ Tris-Borate-EDTA (TBE) buffer. Electrophoresis was initiated at 60 V for 5 min and continued at 120 V for 90 min in $1 \times$ TBE buffer. For visualization of the DNA modification degree, the agarose gel was stained with ethidium bromide (EtBr) in $1 \times$ TBE (0.75 $\mu\text{g/mL}$) for 20 min. Images were captured with the GelDoc-It Imaging System Fusion Fx7 (Vilber Lourmat, Eberhardzell, Germany), and data were quantified with ImageJ/Fiji 1.46 software.

2.12. NQO1 Activity Assay

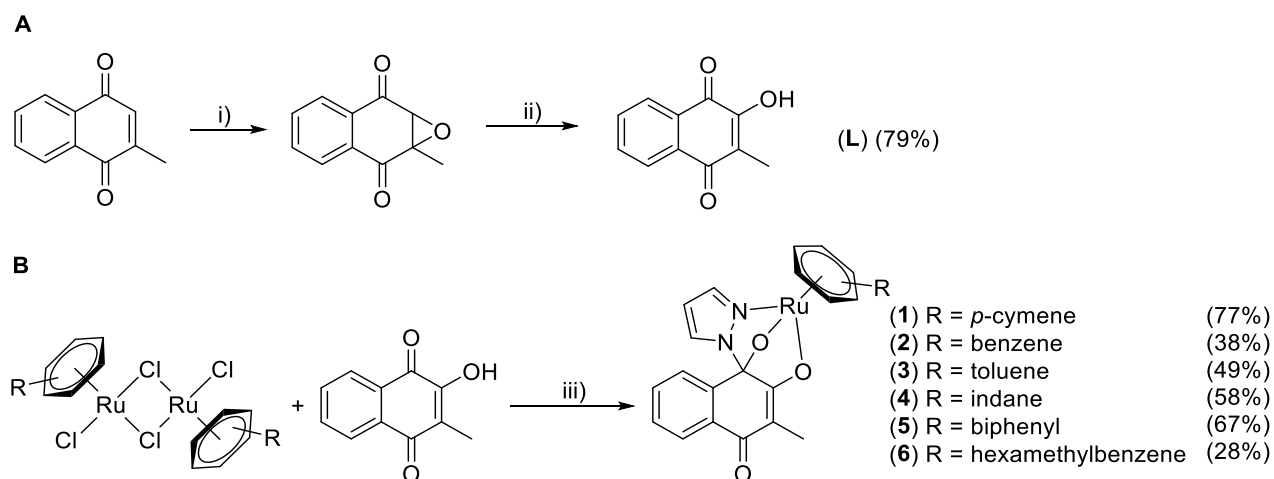
To determine NQO1 activity, SW480 and CH1/PA-1 cells were seeded into 6-well plates in densities of 2×10^5 cells/well in 2 mL supplemented MEM. After the recovery time of 24 h, cells were treated with two or three different concentrations of test compounds (0.1–100 μM , based on the corresponding IC_{50} values). The test substances were dissolved in DMSO/medium. To exclude that assay validity was compromised by the use of DMSO, cells treated with DMSO alone (at concentrations corresponding to those in substance solutions) were measured in parallel. After 24 h drug exposure, the medium was aspirated, and the cells were gently washed twice with PBS. The further steps were performed following the manual instructions of the NQO1 activity assay kit (ab184867, abcam, Cambridge, UK). Protease inhibitor phenylmethylsulfonyl fluoride (PMSF, Merck, Darmstadt, Germany) and phosphatase inhibitor PhosSTOPTM (Roche, Basel, Switzerland) were applied. For determining the protein concentration, the Micro BCATM protein assay (prod # 23235, Thermo Scientific) kit was used. For NQO1 activity determination, 100 $\mu\text{g/mL}$ of total protein was loaded. The development of yellow color was monitored for 5 min in 20-sec intervals with a microplate reader (BioTek ELx808) at 450 nm. The measured data were evaluated according to the instructions of the NQO1 activity assay kit: “NQO1 activity was calculated by subtracting the OD value with Inhibitor from the one without Inhibitor. The activity is expressed as the change in absorbance per minute per amount of sample loaded into the well” [30]. Data are expressed as rates of increase of mean absorbance $\pm$ SDs from three independent experiments.

3. Results and Discussion

3.1. Syntheses and Characterization

The dimeric ruthenium arene precursors were synthesized according to the literature's procedures or purchased from commercial suppliers ($[\text{Ru}(\eta^6\text{-hexamethylbenzene})\text{Cl}_2]_2$) [21–23]. The desired dienes were obtained via Birch reduction (2,3,4,7-tetrahydro-1*H*-indene and 1,4-dihydro-1,1'-biphenyl) or were commercially available (α -terpinene, 1,4-dihydrocyclohexen and 1-methylcyclohexa-1,4-diene) [31,32]. Treatment of the respective diene with $\text{RuCl}_3 \times \text{H}_2\text{O}$ yielded the desired dimeric precursors in good to excellent yields (71–98%). 2-Hydroxy-3-methyl-1,4-naphthoquinone (phthiocol, L) was synthesized according to the literature, where menadione was formed via epoxidation and subsequent SiO_2 -mediated ring opening (yield: 79%) [24,25]. The tridentate complexes were obtained by treating the respective ruthenium dimer, 1*H*-pyrazole and an excess of triethylamine in methanol under microwave irradiation, resulting in moderate to good yields (28–77%) (Scheme 1) [17,19]. The enhanced complex stabilities allowed a straight-forward purification by column chromatography with a ternary eluent system. Furthermore, this complex class exhibits two stereo centers, one at the C1 atom, which connects the pyrazole moiety with the phthiocol ligand, and one at the Ru(II) itself. Theoretically, four stereoisomers should occur, however, only two isomers can be formed due to geometrical reasons (R_{C1} , R_{Ru} and S_{C1} , S_{Ru}) [17].

The low yield of **6** may result from the favored formation of the parental chlorido complex, which could be isolated via column chromatography during the work up. However, the bidentate chlorido compound was not investigated in this project, due to the focus on tridentate complexes.



Scheme 1. Synthetic pathway of phthiocol (**L**) (**A**) and complex syntheses (**1–6**) (**B**). (i) = H_2O_2 , Na_2CO_3 , MeOH; 0 °C to room temperature, 1.5 h; (ii) = SiO_2 , H_2SO_4 , THF, 70 °C; (iii) = MeOH, microwave, 50–60 °C, 20–30 min.

All complexes were characterized with standard analytical methods and the purity was proved by using elemental analysis. In the ^1H -NMR spectra of **1–6**, the signal corresponding to the pyrazolate proton in position three (denoted by an asterisk in Figure 3) was shifted the most upon complex formation due to the spatial proximity to the arene. The electron-rich arenes shift the signal to higher fields. The other pyrazolate protons and those of the naphthoquinone showed only negligible shift changes, due to the increased distance to the arene. The occurrence of a quaternary carbon signal around 90–100 ppm confirmed the formation of a hemiaminal bond (Figures S1–S10).

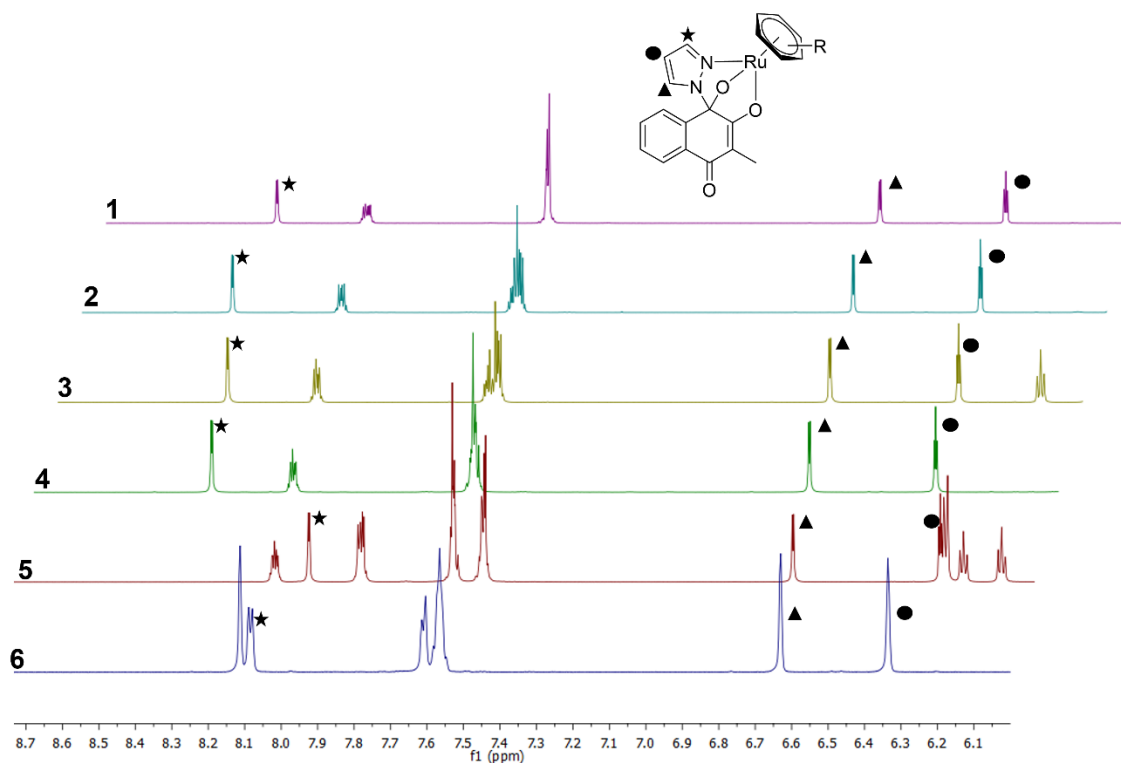


Figure 3. Arene influence towards the chemical shifts of pyrazolate in MeOD-d_4 . R = *p*-cymene = **1**, R = benzene = **2**, R = toluene = **3**, R = indane = **4**, R = biphenyl = **5**, R = hexamethylbenzene = **6**.

3.2. X-ray Crystallographic Studies

Single crystals of four compounds (**2–4**, **6**) were obtained by vapor diffusion or the liquid–liquid diffusion method from dichloromethane/diethyl ether or dichloromethane/*n*-hexane. The respective ORTEP plots and sample data can be found in the supplementary information (Figures S11–S14 and Tables S1–S5). The toluene derivative (**3**) crystallized in the orthorhombic space group *Ibca*, compound **4** in the triclinic group *P*-1 and the other complexes in monoclinic space groups (*C2/c* for **1**, *P2₁/n* for **2** and **6**). For all complexes, the following order of bond lengths was observed: Ru-O2 > Ru-N2 > Ru-O1. It confirmed the hydrolysis mechanism reported in our previous work, where the Ru-O2 bond cleaved firstly to obtain the aqua complex [19]. A comparison of the bond lengths and the stability data showed no correlation (Table 1, Figure 4).

Table 1. Selected bond lengths of complexes **1–6** obtained X-ray single crystal diffraction [Å].

	Ru-O1	Ru-O2	Ru-N2	C1-N1	M-Arene
1 ^(a)	2.059(2)	2.121(2)	2.096(3)	1.528(4)	1.651
2	2.077(2)	2.086(2)	2.088(2)	1.514(3)	1.650
3	2.057(2)	2.104(2)	2.111(3)	1.507(4)	1.655
4	2.049(1)	2.116(1)	2.086(2)	1.516(2)	1.649
6	2.062(1)	2.117(1)	2.094(2)	1.523(2)	1.659

^(a) Bond lengths data from reference [17].

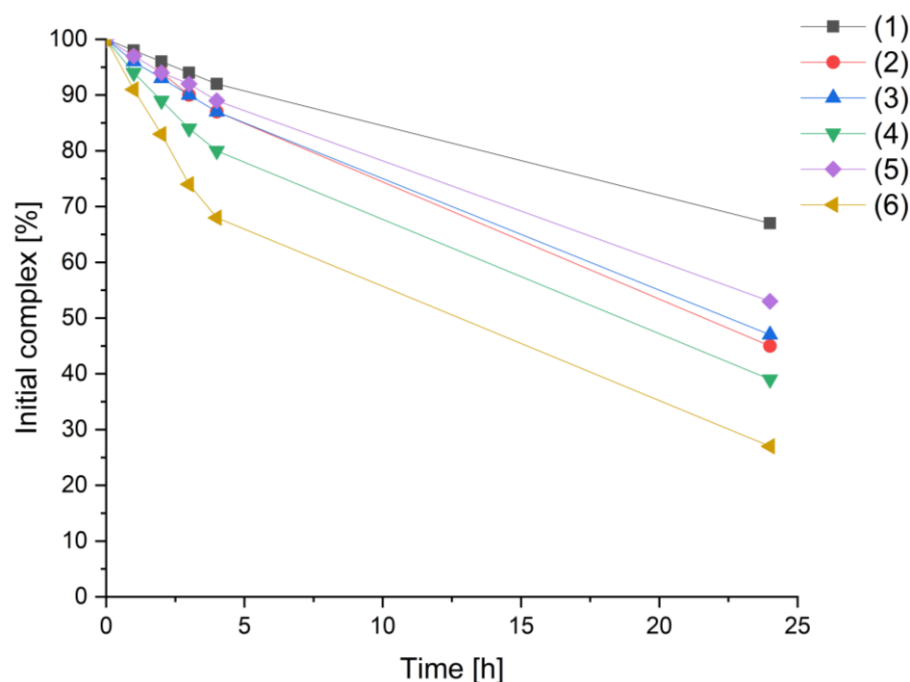


Figure 4. The percentage amount of initial complex vs. time in PBS (pH = 7.4) at 20 °C.

3.3. Stability Studies

Complex stability in an aqueous solution is an important parameter for drug candidates since the intact compound should reach the tumor tissue in sufficient amounts to take effect [33]. Transition metal complexes tend to hydrolyze in an aqueous solution, yielding highly reactive aqua species which can rapidly interact with biomolecules. Therefore, the aqueous behavior of all complexes (**1–6**) under physiologically relevant pH (7.4) at 20 °C was investigated via HPLC-MS. The percentage amount of the initial complex was determined by the integration of the complex signal hourly (for 4 h) and after 24 h (Figure 4).

The complex signals were detected between 12.1–15.9 min with the corresponding masses $[M+H]^+$ and $[M-pz]^+$ (Figures S15–S20). Depending on the arene, hydroxido- and pyrazolato-bridged dimers were observed in a time-dependent manner.

The *p*-cymene complex (**1**) showed the highest stability, with more than 70% of the original complex still existing after 24 h. Similar aqueous behavior was observed for compounds **2–5**, with 40–60% of the initial complexes persisting. The hexamethylbenzene derivative (**6**) exhibited the lowest stability, with less than 70% of the complex remaining intact after 4 h. The reduced stability, poor yield and formation of the parental bidentate chlorido complex suggest that tridentate pyrazolate naphthoquinone complex **6** exhibits an unfavorable structure.

3.4. DFT Calculations

In order to obtain further insight into the complexes' stabilities, DFT calculations of all complexes (**1–6**), their corresponding hydrolyzed aqua species, and the respective transition states were carried out (Table 2). The energies of complexes (**1–6**) are set to zero for better comparison of barrier heights (Figure 5). According to the results, the experimentally found stability (Figure 4) can only be explained by a complex interplay of follow-up reactions, kinetic effects and thermodynamics.

Table 2. Computed energies in [a.u.] of Ru-naphthoquinone-complexes in their initial- and transition-state, as well as their corresponding aqua complexes.

Arene	Reactant [a.u.]	Transition State [a.u.]	Aqua-Complex [a.u.]
<i>p</i> -Cymene (1)	−1356.045552	−1432.265348	−1432.285092
Benzene (2)	−1199.219445	−1275.437208	−1275.459690
Toluene (3)	−1238.431142	−1314.648960	−1314.670467
Indane (4)	−1315.656248	−1391.875753	−1391.894567
Biphenyl (5)	−1429.689526	−1505.909387	−1505.919352
HMB (6)	−1434.464410	−1510.686025	−1510.704607

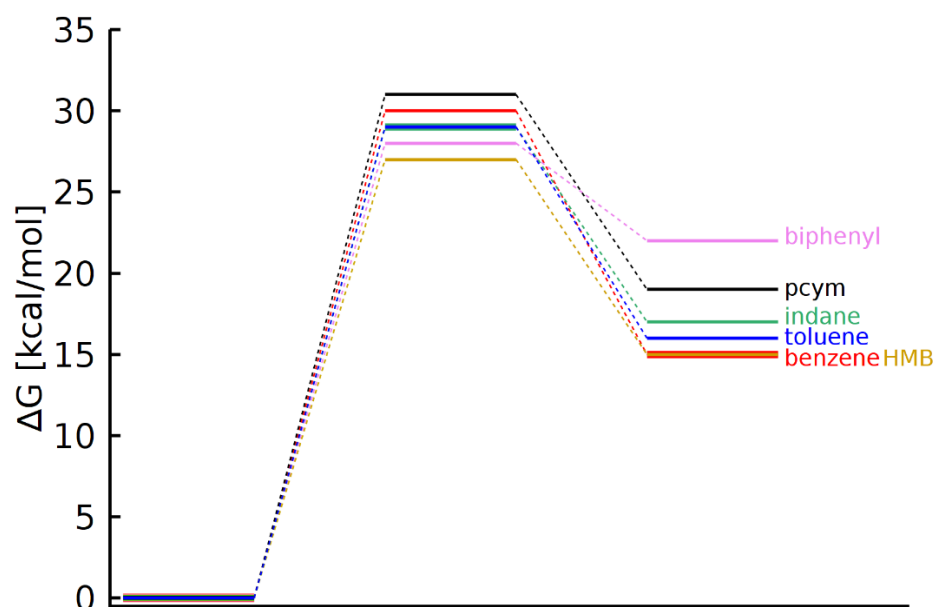


Figure 5. DFT calculations regarding the hydrolysis of complexes (**1–6**). Relative free energies of complexes (**1–6**) are set to zero (left) in order to compare the energetic order of the corresponding transition states (middle) and aqua complexes (right).

The first striking point is that the complexes (**1–6**) should be more stable than their hydrolyzed counterparts. The experimentally found decomposition is likely due to follow-

up reactions, where, e.g., the aqua complexes dimerize and, in this way, shift the equilibrium of the hydrolyzation reaction towards the aqua products. Furthermore, the relative stability of the complexes (1–6) can neither be explained alone by the energetic order of the product energies (aqua complexes) nor alone by the energetic order of the respective transition states. Instead, an interplay of transition barrier heights and product energy (i.e., a combination of kinetic and thermodynamic effects) leads to the experimentally found stabilities. This complex interplay obstructs the rational design of more stable complexes because the hydrolyzation stability cannot be predicted with a simple rule of thumb.

3.5. Biological Studies

3.5.1. Effects on Cell Proliferation and Cytotoxicity

The cytotoxicity of compounds 1–6 was determined in three different cancer cell lines after 24 h and 96 h incubation (Figures S21 and S22). These compounds exhibit a similar cytotoxic profile to those reported previously, where low activity was observed in CH1/PA-1 and high activity in A549 and SW480 cancer cells [17,19]. The IC₅₀ values in CH1/PA-1 cells of the complexes (1–6) were only slightly lower or in the same range as that of phthiocol (L) (Table 3). In contrast, the data indicate a pronounced selectivity for SW480 cells. In the latter, the lowest IC₅₀ values were observed for compounds 1 and 5 (bearing an electron-rich arene) after 24 h as well as after 96 h incubation.

Table 3. Cytotoxicity of compounds (L, 1–6), 50% inhibitory concentrations in human carcinoma cell lines A549, SW480 and CH1/PA-1. Values are means ± SDs obtained by the MTT assay (exposure time: 24 h and 96 h), n.d. = not determined.

Compound	IC ₅₀ Values [μM]					
	A549		SW480		CH1/PA-1	
	24 h	96 h	24 h	96 h	24 h	96 h
1	3.8 ± 2.6	1.2 ± 0.2 ^(a)	0.46 ± 0.14	0.094 ± 0.031 ^(a)	59 ± 18	>50 ^(a)
2	>200	141 ± 38	31 ± 3	9.9 ± 2.5	170 ± 23	157 ± 8
3	>200	72 ± 12	11 ± 2	2.8 ± 0.7	121 ± 18	138 ± 10
4	119 ± 21	9.1 ± 1.5	3.5 ± 1.6	0.52 ± 0.09	146 ± 18	88 ± 10
5	4.3 ± 1.6	1.2 ± 0.2	0.77 ± 0.24	0.072 ± 0.019	42 ± 4	30 ± 3
6	38 ± 21	3.4 ± 0.6	2.8 ± 1.6	0.27 ± 0.06	57 ± 16	35 ± 4
L	>400	210 ± 32 ^(b)	>400	116 ± 37 ^(b)	>400	129 ± 29 ^(b)
[Ru(<i>p</i> -cym)Cl ₂] ₂ ^(c)	n.d.	>100	n.d.	>100	n.d.	>100
[Ru(benzene)Cl ₂] ₂ ^(c)	n.d.	>100	n.d.	>100	n.d.	>100
[Ru(toluene)Cl ₂] ₂ ^(c)	n.d.	>100	n.d.	>100	n.d.	>100
[Ru(indane)Cl ₂] ₂ ^(c)	n.d.	257 ± 66	n.d.	166 ± 35	n.d.	51 ± 13
Cisplatin	n.d.	3.8 ± 1.0	n.d.	2.3 ± 0.2	n.d.	0.073 ± 0.001
Carboplatin ^(d)	n.d.	38 ± 3	n.d.	42 ± 10	n.d.	0.79 ± 0.11
Oxaliplatin ^(e)	n.d.	0.98 ± 0.21	n.d.	0.29 ± 0.05	n.d.	0.18 ± 0.01

^(a) IC₅₀ values from reference [15]. ^(b) IC₅₀ values from reference [17]. ^(c) IC₅₀ values from reference [34]. ^(d) IC₅₀ values from reference [35]. ^(e) IC₅₀ values from reference [36].

These observations lead to the assumption that lipophilicity plays a major role in the activity profile, with more lipophilic compounds showing higher cytotoxicity. Plotting the IC₅₀ values (Table 3) against the calculated miLogP values of the different arenes (www.molinspiration.com, Table S6, (accessed on 13 October 2022)) showed a good agreement between cytotoxicity and calculated lipophilicity (Figure S23), demonstrating that the more lipophilic compounds 1, 5 and 6 had the lowest IC₅₀ values. The benzene and toluene derivatives (2, 3), on the other hand, exhibited the lowest cytotoxicity in all treated cancer cell lines. Complex 4, with indane as the arene, is an exception since it has similar miLogP and stability values to 2 and 3 but an enhanced cytotoxicity in all three cancer cells.

To determine the long-term effect of drugs on proliferating tumor cells, a colony formation assay (CFA) was performed for 11 days [37]. The ability of single cells to grow

into a colony was tested at three different concentrations based on IC_{50} values of 96 h MTT results in SW480 cells (Figures S24–S27 and 6). After 11 days of exposure, only a minor fraction of seeded cells retained the capacity for growth at a concentration of about half of the 96-h IC_{50} values. For the two most cytotoxic compounds, 1 and 5, $18 \pm 5\%$ and $19 \pm 7\%$ colonies, respectively, were counted (relative to the untreated control) (Table S7). The determination of clonogenic growth at a concentration of about the 96-h IC_{50} value yielded the expected results: $3.1 \pm 0.8\%$ (compound 1) and $4.0 \pm 2.0\%$ (compound 5), which correlates well with the results of the MTT assay (24 and 96 h). Treatment with a concentration of approximately the double 96-h IC_{50} value significantly decreased the colony formation upon treatment with all substances, as almost no colonies were observed. Remarkably, the phthiocol ligand (L) showed strong inhibition of colony growth (at the 96-h IC_{50} no colonies could be detected), even though it showed modest cytotoxicity ($116 \pm 37 \mu\text{M}$) after 96 h treatment in the MTT assay.

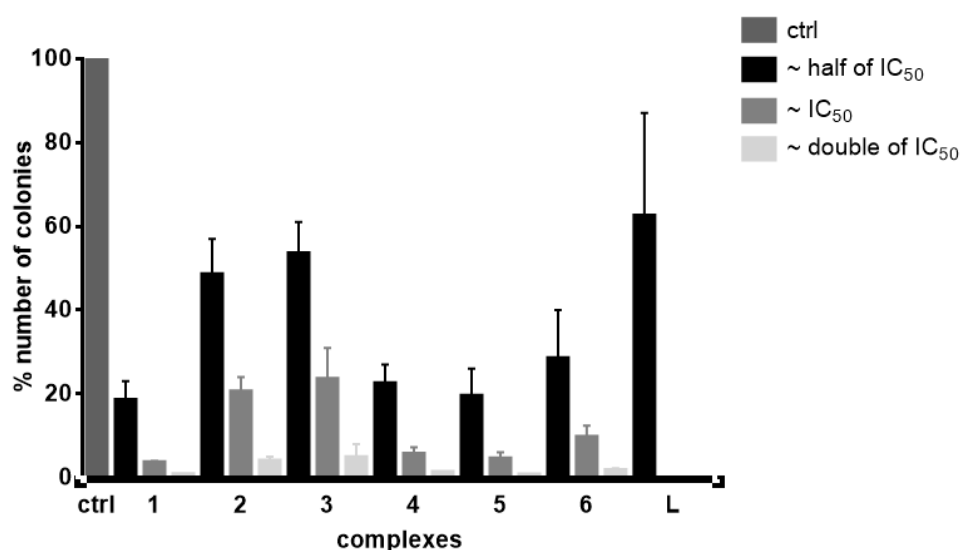


Figure 6. Inhibition of colony formation in SW480 cells exposed to L and 1–6 at two or three different concentrations for 11 days. The results are given in percentage (%) relative to the untreated control. Columns represent the mean $\pm$ standard deviation of three independent experiments.

3.5.2. Cellular Accumulation

In order to understand if there is a correlation between cytotoxicity, cellular accumulation and lipophilicity, the total cellular ruthenium content was quantified by ICP-MS. After 2 h of incubation with the compounds 1–6, SW480 cells were lysed and analytically diluted for metal quantification. The diversity in structure among the (arene) ligands is reflected in a wide range of cellular accumulation of ruthenium from 4.5 fg to 107 fg per cell (treated with $2 \mu\text{M}$ of the test compound) and from 11 fg to 336 fg per cell (treated with $5 \mu\text{M}$ of the test compound, Table S8). According to the quantified ruthenium content, the ligands can be ranked with regard to cellular accumulation of the corresponding complex as follows: benzene (2) < toluene (3) < indane (4) < hexamethylbenzene (6) < *p*-cymene (1) < biphenyl (5) (Figure S28). The highest cellular ruthenium levels were observed for the most cytotoxic compounds (1, 5 and 6). Complex 4, with milder cytotoxicity, yielded a lower level of cellular accumulation, as expected. The ruthenium accumulation was relatively limited in complexes 2 and 3, as only minor amounts of the metal were detected, suggesting that the coordinated arene may be unfavorable for membrane permeation. Overall, drug accumulation correlates fairly well with the cytotoxicity and lipophilicity of the complexes (Figure 7).

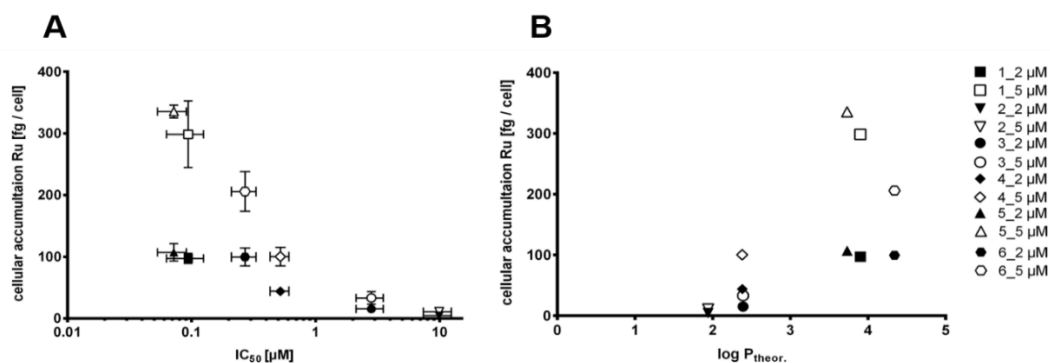


Figure 7. Semi-logarithmic plot of cellular accumulation after 2 h exposure to compounds 1–6 vs. their IC_{50} values (96 h exposure) in SW480 cells (A). Correlation between drug accumulation and a theoretical lipophilicity coefficient (96 h exposure) (B). The symbols indicate the compound concentrations applied in cellular accumulation experiments, with filled symbols for 2 μM and open symbols for 5 μM.

3.5.3. Analysis of Cell Cycle Distribution

To deepen the understanding of how the novel naphthoquinone complexes exert their biological activity, cell cycle distribution was studied in CH1/PA-1 and SW480 cells upon treatment with these compounds (Figures S29 and S30). In several publications, a significant impact on the cell cycle was reported for naphthoquinone derivatives, which can target the checkpoints of the cell cycle and thereby affect cell proliferation [14,38,39]. In this approach, percentages of cells in G1/G0 (cells preparing for DNA replication/quiescent state), S (cells performing DNA synthesis) and G2/M (cells preparing for/undergoing mitosis) phases can be detected by flow cytometry. In both cell lines, three different concentrations (based on IC_{50} values) were applied to cover a wider range for the detection upon 24 h treatment.

In the CH1/PA-1 cell line, a characteristic pattern could be observed for all tested complexes (Figure S31 and Table S10). The G1/G0 phase fraction is reduced by complexes 1–6 at all concentrations tested, whereas the S phase fraction remains unchanged, or is slightly increased by complex 1 (up to 11% relative to untreated control) in a concentration-dependent manner. An increase in the cell fraction in the G2/M phase relative to the control was measured in all cases. The effect of the ligand (L) on cell cycle distribution deviates from the trend observed for complexes 1–6 in CH1/PA1 cells. The ligand caused a gradual increase in the G1/G0 cell fraction and a decrease in the G2/M fraction, while the S phase was unaffected.

In the SW480 cell line (which is more sensitive to complexes 1–6), the cell cycle distribution was affected more strongly than in the CH1/PA-1 cell line (Figure S32 and Table S11). All compounds induced a significant reduction of cell number in the G1/G0 phase and correspondingly increased the cell fraction in S and G2/M phases. In general, a G2/M phase arrest (up to 28% increase relative to untreated control) or S phase arrest (up to 22% increase relative to the untreated control) could be observed. The ligand (L) did not show any cell cycle perturbation in SW480 cells at tested concentrations. In conclusion, the investigated complexes have effects on DNA replication and cell division. The most pronounced effect on the cell cycle was measured for complexes 1 and 5 at concentrations around the respective IC_{50} values in both cell lines (Figure 8).

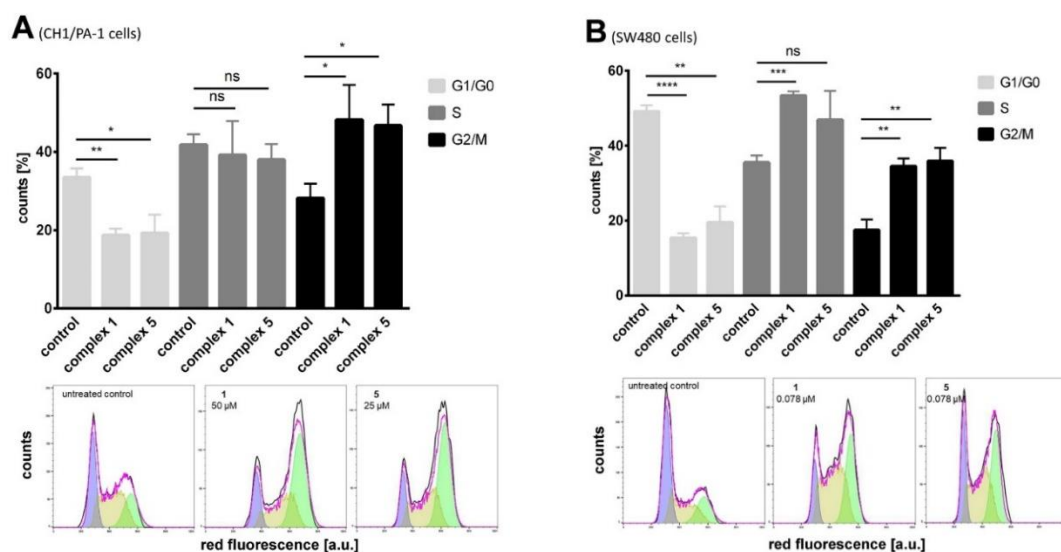


Figure 8. Impact of the most cytotoxic complexes **1** and **5** on cell cycle distribution in CH1/PA-1 (A) and SW480 cells (B) upon exposure for 24 h. Complex **1** was applied at 50 μM concentration in CH1/PA-1 cells and 0.078 μM in SW480 cells. Complex **5** was applied at 25 μM in CH1/PA-1 cells and 0.078 μM in SW480 cells. Histograms represent the effects of the IC_{50} (according to MTT assays) relative to untreated control in a representative experiment. The different colors indicate the cell fractions in G1/G0 phase (purple), S phase (yellow) and G2/M phase (green), calculated by integrating the histograms of fluorescence signals by using Watson algorithm (indicated by magenta lines). In the column diagrams, the data are given as means $\pm$ SDs from at least three independent experiments. Unpaired *t*-test with Welch's correction was performed for statistical evaluation (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns: not significant).

3.5.4. Apoptosis Induction

Regarding the types of cell death that are induced by anticancer compounds, two main categories can be distinguished: necrosis and programmed cell death, with the latter comprising apoptosis and non-apoptotic mechanisms (such as autophagy, necroptosis, and apoptosis-like programmed cell death) [40,41]. In this study, the ability of apoptosis and necrosis induction was examined by the flow-cytometric annexin V/propidium iodide (AV/PI) assay. The double staining applied in this assay enables the differentiation of viable cells (AV-/PI-), early apoptosis (AV+/PI-), late apoptosis (AV+/PI+) and necrosis (AV-/PI+). If cancer cells undergo early or late apoptosis, phosphatidylserines (PS) are located from the inner leaflet to the outer side of the lipid bilayer of the plasma membrane and (FITC-coupled) annexin V can selectively bind to PS. At the same time, PI can be used for the identification of cells with damaged cell membranes such as necrotic or late apoptotic cells.

The SW480 cells were treated with complexes **1–6** and free ligand **L** at concentrations that roughly cover the wide IC_{50} range (24 h incubation) (Table S12, Figure S33). Only complexes **1** and **5** induced apoptosis to a high extent, which increased with concentration (Figure 9). With the highest applied concentration (10 μM) they had a strong influence on the percentages of viable (AV-/PI-), early apoptotic (AV+/PI-) and late apoptotic (AV+/PI+) cells. The increase of late apoptotic cells to $64 \pm 6\%$ (complex **1**) and $40 \pm 6\%$ (complex **5**) is very prominent. In accordance with the data above, apoptotic events were expected of complex **6** carrying a hexamethylbenzene ligand, but neither apoptotic nor necrotic cells exceeded the percentages in untreated controls. The other tested complexes **2–4** as well as **L** did not induce any of these processes either.

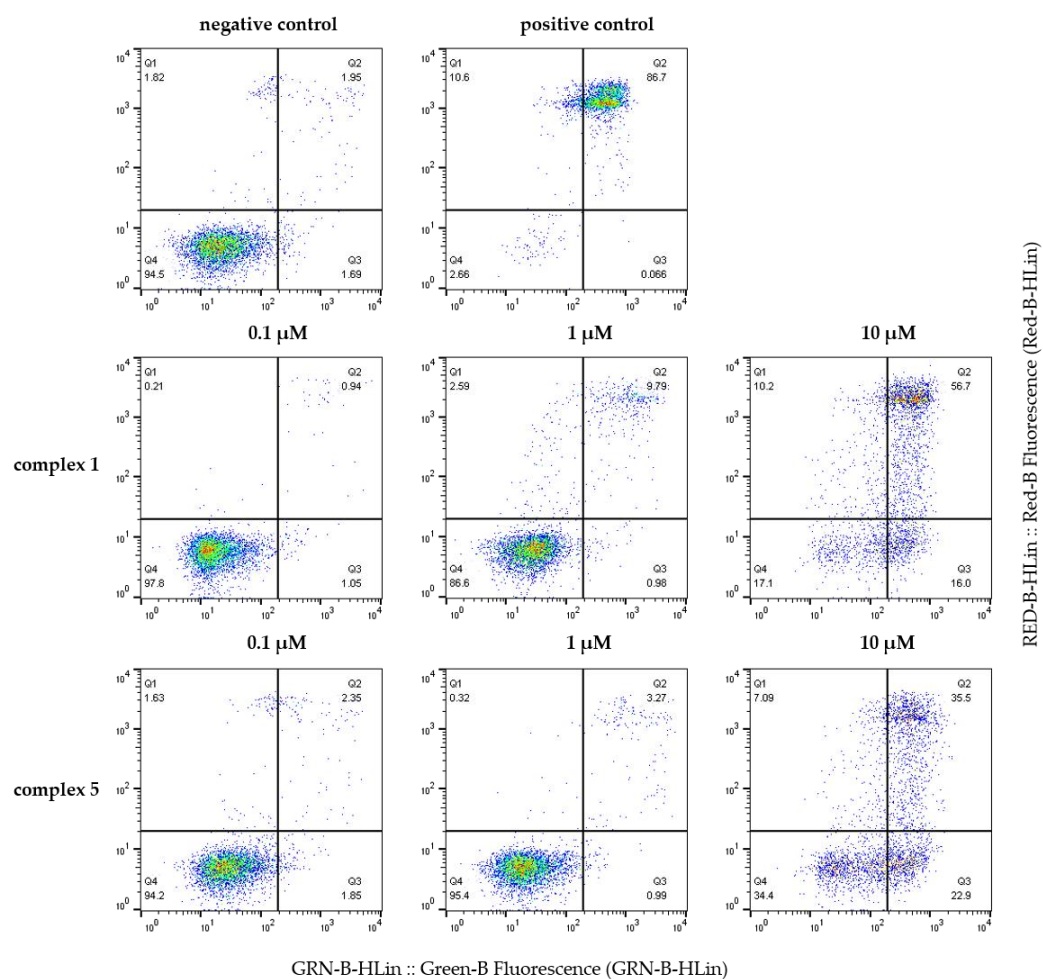


Figure 9. To distinguish the viable (Q4), early apoptotic (Q3), late apoptotic (Q2) and necrotic (Q1) cell counts, SW480 cells treated with complexes 1 and 5 at three different concentrations were subject to annexin V-FITC (x-axis) and PI (y-axis) staining, detected by flow cytometry. As a positive control, 1-P was used [42]. The dot plots represent a representative experiment out of at least three.

3.5.5. ROS Assay

Naphthoquinones are obvious candidates for testing the capacities of reactive oxygen species (ROS) production due to their ability to easily accept electrons, resulting in the formation of semi-quinones/hydroquinones on the one hand, and radical formation of an electron-donor substrate on the other hand. Those radical species undergo reactions rapidly, thereby causing damage such as DNA mutations, which cells might not be able to repair properly. Alternatively, ROS are able to inactivate biologically important enzymes, e.g., ubiquitin-specific proteases 1 and 2 (USP 1 and USP 2), which are associated with the repair of DNA damage and resistance to apoptosis induction [14].

To check their ability to induce ROS in cancer cells, the DCFH-DA-assay has been applied to the new naphthoquinone-comprising metallodrugs (complexes 1–6) and their parental ligand (L). The 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) precursor is oxidized by reactive oxygen species, and the product DCF emits fluorescence signals, which may increase or decrease in a time-dependent manner. As compounds are most cytotoxic in SW480 and less active in CH1/PA-1 cells, both cell lines were treated with the compounds. Results indicate no noteworthy increase in ROS levels for all tested complexes or the parental naphthoquinone ligand (Figures S34 and S35). At higher applied concentrations (200 μ M for CH1/PA-1 and 20 μ M for SW480) slightly reduced levels of ROS could be detected compared to the negative control, while complex 1 even caused overall lowered ROS levels (approximately half the amount of the negative control: T/C $\approx$ 0.5) with a

further decrease at the highest concentration in CH1/PA-1 cells. Intriguingly, complex **5** seems to be the most constant compound, with T/C-values around 0.9–1 throughout the concentration range. Therefore, increased amounts of reactive oxygen species do not seem to be responsible for the cytotoxic potency of the complexes.

3.5.6. DNA Interaction Studies

The compounds' capacities to alter DNA secondary structure were investigated in a cell-free environment with a dsDNA assay to assess whether DNA could be a possible target of their mode of action. For the assay, intact pUC19 plasmid (2.7 kbp) was incubated with complexes (**2–6**) for up to 6 h, and the possible changes in plasmid secondary structure were detected by agarose gel electrophoresis. The plasmid dsDNA mostly holds a negatively supercoiled (sc) form, and may, upon drug treatment, gradually converge to the open circular (oc) form. For the compounds studied here, the only signs of interaction detected were single-strand breaks induced by a few of them (Figure S36). However, other forms of DNA interaction such as cross-linking, intercalation, or double-strand breakage could not be observed, in contrast to effects reported for the ruthenium(III) complexes KP1019 and KP418, which may cause untwisting and bending of DNA [43]. Compounds **2–4** moderately induced single-strand breaks; after 6 h of incubation the oc form reached $11.8 \pm 0.9\%$, $11.7 \pm 1.6\%$ and $12.1 \pm 1.9\%$, respectively (Figure S37). The interaction showed a clear time dependency, with the detected amount of oc form increasing over time in comparison to the baseline value of $3.6 \pm 0.7\%$ (control after 6 h). These findings are nevertheless remarkable because the other compounds (**1**, **5**, **6** and **L**) demonstrated only a minor influence on pUC19 [19]. Apparently, DNA interaction may not contribute to the cytotoxic behavior of the tested complexes, as **2–4** only exhibited moderate cytotoxicity in comparison to the other test substances. Overall, this supports the hypothesis that this series of complexes do not have a platinum-like mode of action (*cf.* cisplatin and related platinum drugs) [19,44].

3.5.7. NQO1 Activity

In the literature, it has been reported that naturally occurring naphthoquinones, such as β -lapachone, act as effective cytotoxic agents and selectively kill cancer cells with high expression levels of the NQO1 enzyme (located primarily in the cytosol) [45]. Furthermore, NQO1 activity has been reviewed by Pereyra et al. as a potential mechanism of how naphthoquinones eliminate cancer cells [14]. NAD(P)H:quinone oxidoreductase 1 (NQO1, E.C.1.6.99.2) is a quinone-detoxifying enzyme in normal cells, which utilizes NADH or NADPH as electron donors for the two-electron reduction of quinones to unstable, reactive hydroquinones. The enzyme protects the structure and functioning of normal cells against quinones (exogenous and/or endogenous) and prevents tumor progression. Inhibiting its activity in cancer cells may support therapeutic efficacy by suppressing cancer cell growth or by potentiating the activity of chemotherapeutic drugs [46,47]. One of the most studied and widely used inhibitors of the NQO1 enzyme is dicoumarol, which suppresses the enzymatic activity by competing with NAD(P)H [48].

Compounds **L** and **1–6** are assumed to be substrates of the NQO1 enzyme. They may induce structural changes in NQO1, resulting in the inhibition of the enzyme's activity *in vitro*. In SW480 colon carcinoma cells, complexes **1** and **5** lowered NQO1 activity tremendously at 10 μ M concentration, which is 21- and 13-fold higher than the corresponding IC₅₀ values (cytotoxicity) after 24 h of incubation (Figure 10). At lower concentrations (0.1 μ M and 1 μ M), negligible effects were detected compared to the untreated control. Compound **4** exhibited a moderate suppression of enzyme activity, also at 10 μ M (2.8-fold higher than IC₅₀), which may explain its enhanced cytotoxicity compared to **2** and **3** with similar miLogP values and stability. Phthiocol (**L**) at 10 μ M moderately inhibited the activity of NQO1 to a similar extent as compound **4** (Figure S38). Experiments were also performed in CH1/PA-1 cells to better understand the unexpected chemoresistance of the usually very sensitive ovarian teratocarcinoma cancer cell line. Surprisingly, CH1/PA-1 cells showed a very low baseline level of NQO1 activity (Figures 10 and S39). We assume that their lack of

NQO1 activity might be a key to understanding their pronounced resistance to the tested compounds compared to SW480 cells.

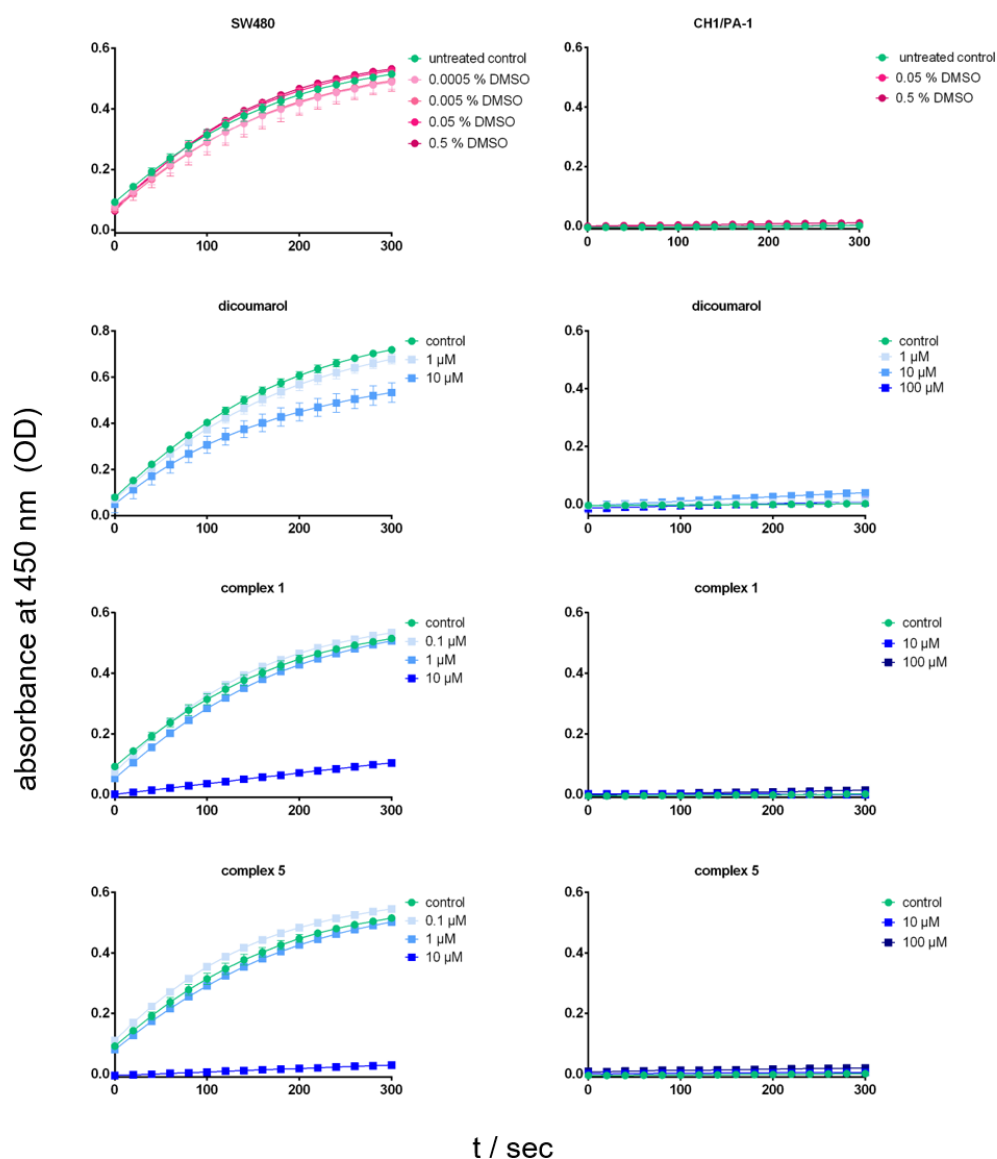


Figure 10. Time-dependent impact on NQO1 activity in SW480 (left panel) and CH1/PA-1 (right panel) cell extracts after treatment with the most active complexes, 1 and 5, or dicoumarol (positive control) compared to negative controls with and without DMSO (concentrations corresponding to the content in solutions of the test compounds). Data are expressed as OD (450 nm) per 100 $\mu\text{g}/\text{mL}$ of total cellular protein.

4. Conclusions

Phthiocol (L) and complexes 1–6 with six different arenes, *p*-cymene (1), benzene (2), toluene (3), indane (4), biphenyl (5) and hexamethylbenzene (6), were successfully synthesized via microwave irradiation. This new set of tridentate phthiocol-based ruthenium arene complexes was characterized by using ^1H -NMR and ^{13}C -NMR spectroscopy, the crystal structures were examined by X-ray single-crystal spectroscopy. The aqueous stability of all complexes 1–6 was studied by HPLC-MS chromatography. DFT calculations were performed, and the theoretical results were in good agreement with the experimental stability data.

Cytotoxicity assays yielded remarkable results, as the broadly chemosensitive CH1/PA-1 teratocarcinoma cells were considerably less affected upon treatment with L and com-

plexes 1–6 than SW480 colon carcinoma cells, which usually show moderate response to anticancer agents compared to CH1/PA-1 cells. The long-term cytotoxic effect was tested by a colony formation assay for 11 days, the results of which correspond well to those of the 24-h and 96-h MTT assays. Based on these data, the most promising compounds seem to be complexes 1 and 5 with a *p*-cymene and biphenyl arene, respectively. The cellular accumulation was determined by ICP-MS, where the most cytotoxic compounds, 1 and 5 (with the highest aqueous stability), yielded the highest total ruthenium content in the cell lysates. Moreover, complex 6 showed enhanced cellular accumulation with a similar magnitude as compounds 1 and 5, despite the lowest stability in the aqueous milieu. A correlation could be found between cellular accumulation and lipophilicity, with complexes having higher calculated miLogP values for the arene showing significantly enhanced cellular ruthenium levels.

Since Pereyra et al. reviewed various aspects of the mechanisms of action of naphthoquinone derivatives, we focused on some of these, such as the influence on cell cycle, apoptosis induction, ROS generation, DNA damage and inhibition of NQO1 activity. As for cytotoxicity, the experimental evidence from cell cycle studies suggests that the compounds have a stronger impact on SW480 cells than on CH1/PA-1 cells. Data from the apoptosis assay revealed a pronounced increase of early and late apoptotic cells by complexes 1 and 5 in SW480 cells. Neither ROS generation nor a meaningful extent of interaction with DNA could be observed. Remarkably, NQO1 enzyme activity and its inhibition by some of the compounds at high concentrations could be detected only in SW480 but not CH1/PA-1 cells, which suggests that the unexpected resistance of CH1/PA-1 cells to the new agents might be due to a negligible baseline activity of this enzyme in the latter cell line.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pharmaceutics14112466/s1>. Materials, NMR spectra of complexes (Figures S1–S10), experimental parameters for X-ray analysis, crystal structures (Figures S11–S14), sample and crystal data, data collection, and structure refinement (Tables S1–S5), HPLC and HPLC-MS chromatograms (Figures S15–S20), calculated miLogP values of used arenes (Table S6), concentration–effect curves (Figures S21 and S22), correlation between IC₅₀ values and miLogP values (Figure S23), colony formation assay (Table S7, Figures S24–S27), cellular accumulation results (Table S8, Figure S28); ICP-MS parameters (Table S9), cell cycle distribution and histograms (Tables S10 and S11, Figures S29–S32), apoptosis induction (Table S12, Figure S33), ROS assay results (Figures S34 and S35), dsDNA plasmid assay results (Figures S36 and S37) and NQO1 activity assay results (Figures S38 and S39). (References [49–55] are cited in the supplementary materials).

Author Contributions: Conceptualization, K.C., H.G. and W.K.; methodology, K.C., H.G. and W.K.; software, P.M.; validation, G.K., M.A.J., J.W., P.M. and W.K.; formal analysis, K.C., H.G. and D.W.; investigation, K.C., H.G., K.S., P.B., D.W. and M.H.; resources, G.K., M.A.J., P.M. and W.K.; data curation, K.C., H.G., K.S., N.G., J.W., P.B., M.S. and M.H.; writing—original draft preparation, K.C., H.G., D.W. and W.K.; writing—review and editing, K.C., H.G., M.A.J., J.W., P.M. and W.K.; visualization, K.C., H.G., N.G., K.S. and D.W.; supervision, G.K., M.A.J., P.M. and W.K.; project administration, M.A.J., P.M. and W.K.; funding acquisition, G.K., M.A.J., P.M. and W.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding. Open Access Funding by the University of Vienna.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: CCDC 2210525–2210528 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44-1223-336033.

Acknowledgments: We express our gratitude to Anton Legin, who provided his expertise for cell cycle analysis and helped with the data evaluation.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Kelland, L. The resurgence of platinum-based cancer chemotherapy. *Nat. Rev. Cancer* **2007**, *7*, 573–584. [CrossRef] [PubMed]
- Cheff, D.M.; Hall, M.D. A drug of such damned nature. 1 challenges and opportunities in translational platinum drug research. *J. Med. Chem.* **2017**, *60*, 4517–4532. [CrossRef] [PubMed]
- Oun, R.; Moussa, Y.E.; Wheate, N.J. The side effects of platinum-based chemotherapy drugs: A review for chemists. *Dalton Trans.* **2018**, *47*, 6645–6653. [CrossRef] [PubMed]
- Schirmmayer, V. From chemotherapy to biological therapy: A review of novel concepts to reduce the side effects of systemic cancer treatment. *Int. J. Oncol.* **2019**, *54*, 407–419. [CrossRef] [PubMed]
- Köpf, H.; Köpf-Maier, P. Titanocene Dichloride—The first metallocene with cancerostatic activity. *Angew. Chem. Int. Ed. Engl.* **1979**, *18*, 477–478. [CrossRef] [PubMed]
- Guo, M.; Guo, Z.; Sadler, P. Titanium(IV) targets phosphoesters on nucleotides: Implications for the mechanism of action of the anticancer drug titanocene dichloride. *J. Biol. Inorg. Chem.* **2001**, *6*, 698–707. [CrossRef]
- Vatansever, H.S.; Kabadayı, H.; Korkmaz, M.; Özdal-Kurt, F.; Kavukcu, S.B.; Türkmen, H. Apoptotic properties of ruthenium complexes on different type of cancer cell lines. *Proceedings* **2018**, *2*, 1593. [CrossRef]
- Intravesical Photodynamic Therapy (PDT) in BCG Refractory/Intolerant Non-Muscle Invasive Bladder Cancer (NMIBC) Patients. Available online: <https://clinicaltrials.gov/ct2/show/NCT03945162> (accessed on 13 October 2022).
- BOLD-100 in Combination With FOLFOX for the Treatment of Advanced Solid Tumours. Available online: <https://clinicaltrials.gov/ct2/show/NCT04421820> (accessed on 13 October 2022).
- Adhireksan, Z.; Davey, G.E.; Campomanes, P.; Groessl, M.; Clavel, C.M.; Yu, H.; Nazarov, A.A.; Yeo, C.H.F.; Ang, W.H.; Dröge, P.; et al. Ligand substitutions between ruthenium-cymene compounds can control protein versus DNA targeting and anticancer activity. *Nat. Commun.* **2014**, *5*, 3462. [CrossRef]
- Pizarro, A.M.; Habtemariam, A.; Sadler, P.J. Activation mechanisms for organometallic anticancer complexes. In *Medicinal Organometallic Chemistry*; Jaouen, G., Metzler-Nolte, N., Eds.; Springer: Berlin/Heidelberg, Germany, 2010; pp. 21–56.
- Peacock, A.F.A.; Habtemariam, A.; Fernández, R.; Walland, V.; Fabbiani, F.P.A.; Parsons, S.; Aird, R.E.; Jodrell, D.I.; Sadler, P.J. Tuning the reactivity of Osmium(II) and Ruthenium(II) arene complexes under physiological conditions. *J. Am. Chem. Soc.* **2006**, *128*, 1739–1748. [CrossRef]
- Ang, W.H.; Daldini, E.; Scolaro, C.; Scopelliti, R.; Juillerat-Jeannerat, L.; Dyson, P.J. Development of organometallic ruthenium–arene anticancer drugs that resist hydrolysis. *Inorg. Chem.* **2006**, *45*, 9006–9013. [CrossRef]
- Pereyra, C.E.; Dantas, R.F.; Ferreira, S.B.; Gomes, L.P.; Silva-Jr, F.P. The diverse mechanisms and anticancer potential of naphthoquinones. *Cancer Cell Int.* **2019**, *19*, 207. [CrossRef] [PubMed]
- Hackl, C.M.; Schoenhacker-Alte, B.; Klose, M.H.M.; Henke, H.; Legina, M.S.; Jakupec, M.A.; Berger, W.; Keppler, B.K.; Brüggemann, O.; Teasdale, I.; et al. Synthesis and in vivo anticancer evaluation of poly(organo)phosphazene-based metallodrug conjugates. *Dalton Trans.* **2017**, *46*, 12114–12124. [CrossRef] [PubMed]
- Kandioller, W.; Balsano, E.; Meier, S.M.; Jungwirth, U.; Göschl, S.; Roller, A.; Jakupec, M.A.; Berger, W.; Keppler, B.K.; Hartinger, C.G. Organometallic anticancer complexes of lapachol: Metal centre-dependent formation of reactive oxygen species and correlation with cytotoxicity. *Chem. Commun.* **2013**, *49*, 3348–3350. [CrossRef] [PubMed]
- Geisler, H.; Wernitznig, D.; Hejl, M.; Gajic, N.; Jakupec, M.A.; Kandioller, W.; Keppler, B.K. Novel phthiocol-based organometallics with tridentate coordination motif and their unexpected cytotoxic behaviour. *Dalton Trans.* **2020**, *49*, 1393–1397. [CrossRef]
- Babak, M.V.; Meier, S.M.; Legin, A.A.; Adib Razavi, M.S.; Roller, A.; Jakupec, M.A.; Keppler, B.K.; Hartinger, C.G. Am(m)ines make the difference: Organoruthenium Am(m)ine complexes and their chemistry in anticancer drug development. *Chem. A Eur. J.* **2013**, *19*, 4308–4318. [CrossRef]
- Geisler, H.; Westermayr, J.; Cseh, K.; Wenisch, D.; Fuchs, V.; Harringer, S.; Plutzar, S.; Gajic, N.; Hejl, M.; Jakupec, M.A.; et al. Tridentate 3-substituted naphthoquinone ruthenium arene complexes: Synthesis, characterization, aqueous behavior, and theoretical and biological studies. *Inorg. Chem.* **2021**, *60*, 9805–9819. [CrossRef]
- Martínez-De-León, C.G.; del Carmen Flores Vallejo, R.; Rodríguez-Álvarez, A.; Villareal, M.L.; Grévy, J.-M. Synthesis, characterization and cytotoxic activity of cationic half-sandwich Ru(II) complexes stabilized by iminophosphorane N,N,S and N,N,Se tridentate ligands. *New J. Chem.* **2020**, *44*, 20676–20687. [CrossRef]
- Bennett, M.A.; Huang, T.N.; Matheson, T.W.; Smith, A.K.; Ittel, S.; Nickerson, W. 16. (η⁶-Hexamethylbenzene)Ruthenium Complexes. In *Inorganic Syntheses*; John and Wiley and Sons: Hoboken, NJ, USA, 1982; pp. 74–78.
- Ma, L.; Ma, R.; Wang, Z.; Yiu, S.-M.; Zhu, G. Heterodinuclear Pt(IV)–Ru(II) anticancer prodrugs to combat both drug resistance and tumor metastasis. *Chem. Commun.* **2016**, *52*, 10735–10738. [CrossRef]
- Queyriaux, N.; Giannoudis, E.; Lefebvre, J.F.; Artero, V.; Chavarot-Kerlidou, M. Synthesis of Ruthenium Tris-Diimine photosensitizers substituted by four methylphosphonate anchoring groups for dye-sensitized photoelectrochemical cell applications. *Eur. J. Inorg. Chem.* **2019**, *2019*, 2154–2161. [CrossRef]
- Kathawate, L.; Gejji, S.P.; Yeole, S.D.; Verma, P.L.; Puranik, V.G.; Salunke-Gawali, S. The first naphthoquinone complex of K⁺ with vitamin K3 analog: Experiment and density functional theory. *J. Mol. Struct.* **2015**, *1088*, 56–63. [CrossRef]

25. Zhu, R.; Xing, L.; Wang, X.; Cheng, C.; Liu, B.; Hu, Y. Practical Preparation of Diosphenols by Ring Opening of α,β -Epoxyketones Catalyzed by Silica Gel Supported Acids. *Synlett* **2007**, 2007, 2267–2271. [CrossRef]
26. Neese, F. The ORCA program system. *WIREs Comput. Mol. Sci.* **2012**, 2, 73–78. [CrossRef]
27. Grimme, S.; Brandenburg, J.G.; Bannwarth, C.; Hansen, A. Consistent structures and interactions by density functional theory with small atomic orbital basis sets. *J. Chem. Phys.* **2015**, 143, 054107. [CrossRef] [PubMed]
28. Barone, V.; Cossi, M. Quantum calculation of molecular energies and energy gradients in solution by a conductor solvent model. *J. Phys. Chem. A* **1998**, 102, 1995–2001. [CrossRef]
29. York, D.M.; Karplus, M. A smooth solvation potential based on the conductor-like screening model. *J. Phys. Chem. A* **1999**, 103, 11060–11079. [CrossRef]
30. Available online: <https://www.abcam.com/nqo1-activity-assay-kit-ab184867.html#> (accessed on 13 October 2022).
31. Condon, S.E.; Buron, C.; Tippmann, E.M.; Tinner, C.; Platz, M.S. Generation and characterization of phenylsulfanylcabene. *Org. Lett.* **2004**, 6, 815–818. [CrossRef] [PubMed]
32. O'Mahony, M.J.; More O'Ferrall, R.A.; Boyd, D.R.; Lam, C.M.; O'Donoghue, A.C. Substituent effects on the dehydration of arene hydrates in aqueous solution. *J. Phys. Org. Chem.* **2013**, 26, 989–996. [CrossRef]
33. Di, L.; Kerns, E.H. Stability challenges in drug discovery. *Chem. Biodivers.* **2009**, 6, 1875–1886. [CrossRef]
34. Geisler, H.; Harringer, S.; Wenisch, D.; Urban, R.; Jakupec, M.A.; Kandiolle, W.; Keppler, B.K. Systematic study on the cytotoxic potency of commonly used dimeric metal precursors in human cancer cell lines. *ChemistryOpen* **2022**, 11, e202200019. [CrossRef]
35. Harringer, S.; Hejl, M.; Enyedi, É.A.; Jakupec, M.A.; Galanski, M.S.; Keppler, B.K.; Dyson, P.J.; Varbanov, H.P. Multifunctional Pt(IV) prodrug candidates featuring the carboplatin core and deferoxamine. *Dalton Trans.* **2021**, 50, 8167–8178. [CrossRef]
36. Varbanov, H.P.; Goschl, S.; Heffeter, P.; Theiner, S.; Roller, A.; Jensen, F.; Jakupec, M.A.; Berger, W.; Galanski, M.S.; Keppler, B.K. A novel class of bis- and tris-chelate diam(m)inebis(dicarboxylato)platinum(IV) complexes as potential anticancer prodrugs. *J. Med. Chem.* **2014**, 57, 6751–6764. [CrossRef] [PubMed]
37. Franken, N.A.; Rodermond, H.M.; Stap, J.; Haveman, J.; van Bree, C. Clonogenic assay of cells in vitro. *Nat. Protoc.* **2006**, 1, 2315–2319. [CrossRef] [PubMed]
38. Li, K.; Wang, B.; Zheng, L.; Yang, K.; Li, Y.; Hu, M.; He, D. Target ROS to induce apoptosis and cell cycle arrest by 5,7-dimethoxy-1,4-naphthoquinone derivative. *Bioorg. Med. Chem. Lett.* **2018**, 28, 273–277. [CrossRef] [PubMed]
39. Wang, H.; Luo, Y.H.; Shen, G.N.; Piao, X.J.; Xu, W.T.; Zhang, Y.; Wang, J.R.; Feng, Y.C.; Li, J.Q.; Zhang, Y.; et al. Two novel 1,4-naphthoquinone derivatives induce human gastric cancer cell apoptosis and cell cycle arrest by regulating reactive oxygen species-mediated MAPK/Akt/STAT3 signaling pathways. *Mol. Med. Rep.* **2019**, 20, 2571–2582. [CrossRef]
40. Jan, R.; Chaudhry, G.E. Understanding apoptosis and apoptotic pathways targeted cancer therapeutics. *Adv. Pharm. Bull.* **2019**, 9, 205–218. [CrossRef]
41. Elmore, S. Apoptosis: A review of programmed cell death. *Toxicol. Pathol.* **2007**, 35, 495–516. [CrossRef]
42. Ma, T.; Yang, P.; Dammann, I.; Lin, Z.; Mougharbel, A.S.; Li, M.X.; Adascalitei, F.; Mitea, R.; Silvestru, C.; Thorstenson, C.; et al. Tetra-(p-tolyl)antimony(III)-Containing Heteropolytungstates, $[(p\text{-tolyl})\text{Sb(III)}]_4(\text{A-}\alpha\text{-XW}_9\text{O}_{34})_2(\text{n-})$ (X = P, As, or Ge): Synthesis, structure, and study of antibacterial and antitumor activity. *Inorg. Chem.* **2020**, 59, 2978–2987. [CrossRef]
43. Hartinger, C.G.; Zorbas-Seifried, S.; Jakupec, M.A.; Kynast, B.; Zorbas, H.; Keppler, B.K. From bench to bedside—Preclinical and early clinical development of the anticancer agent indazolium trans-[tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019 or FFC14A). *J. Inorg. Biochem.* **2006**, 100, 891–904. [CrossRef]
44. Goschl, S.; Schreiber-Brynzak, E.; Pichler, V.; Cseh, K.; Heffeter, P.; Jungwirth, U.; Jakupec, M.A.; Berger, W.; Keppler, B.K. Comparative studies of oxaliplatin-based platinum(IV) complexes in different in vitro and in vivo tumor models. *Met. Integr. Biometal Sci.* **2017**, 9, 309–322. [CrossRef]
45. Bey, E.A.; Bentle, M.S.; Reinicke, K.E.; Dong, Y.; Yang, C.R.; Girard, L.; Minna, J.D.; Bornmann, W.G.; Gao, J.; Boothman, D.A. An NQO1- and PARP-1-mediated cell death pathway induced in non-small-cell lung cancer cells by beta-lapachone. *Proc. Natl. Acad. Sci. USA* **2007**, 104, 11832–11837. [CrossRef]
46. Srijiwangsa, P.; Na Bangchang, K. Roles of NAD (P) H-Quinone Oxidoreductase 1 (NQO1) on cancer progression and chemoresistance. *J. Clin. Exp. Oncol.* **2017**, 6, 1–6. [CrossRef]
47. Pidugu, L.S.; Mbimba, J.C.; Ahmad, M.; Pozharski, E.; Sausville, E.A.; Emadi, A.; Toth, E.A. A direct interaction between NQO1 and a chemotherapeutic dimeric naphthoquinone. *BMC Struct. Biol.* **2016**, 16, 1. [CrossRef] [PubMed]
48. Asher, G.; Dym, O.; Tsvetkov, P.; Adler, J.; Shaul, Y. The crystal structure of NAD(P)H quinone oxidoreductase 1 in complex with its potent inhibitor dicoumarol. *Biochemistry* **2006**, 45, 6372–6378. [CrossRef] [PubMed]
49. Bruker AXS. *Bruker SAINT v.838B Copyright(C)*; Bruker AXS: Billerica, MA, USA, 2005–2019.
50. Sheldrick, G.M. *Sadabs*; University of Göttingen: Göttingen, Germany, 1996.
51. Dolomanov, O.V.; Bourhis, L.J.; Gildea, R.J.; Howard, J.A.K.; Puschmann, H. OLEX2: A complete structure solution, refinement and analysis program. *J. Appl. Crystallogr.* **2009**, 42, 339–341. [CrossRef]
52. Hubschle, C.B.; Sheldrick, G.M.; Dittrich, B. ShelXle: A Qt graphical user interface for SHELXL. *J. Appl. Crystallogr.* **2011**, 44, 1281–1284. [CrossRef]
53. Sheldrick, G. SHELXS v 2016/4. 2015.
54. Sheldrick, G. Crystal structure refinement with SHELXL. *Acta Crystallogr. Sect. C* **2015**, 71, 3–8. [CrossRef]
55. Spek, A. Structure validation in chemical crystallography. *Acta Crystallogr. Sect. D* **2009**, 65, 148–155. [CrossRef]

Supporting information

Arene variation of highly cytotoxic tridentate naphthoquinone-based ruthenium(II) complexes and in-depth *in vitro* studies

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NMR spectra

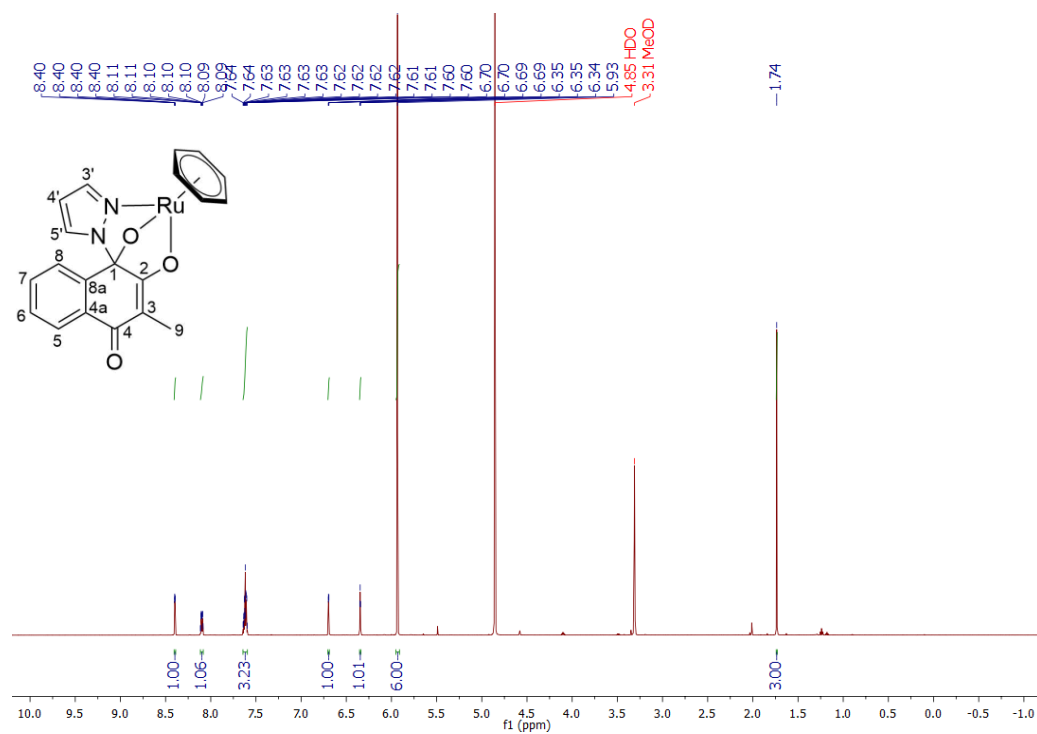


Figure S1: ¹H-NMR (MeOD-*d*₄, 600.25 MHz) spectrum of (2)

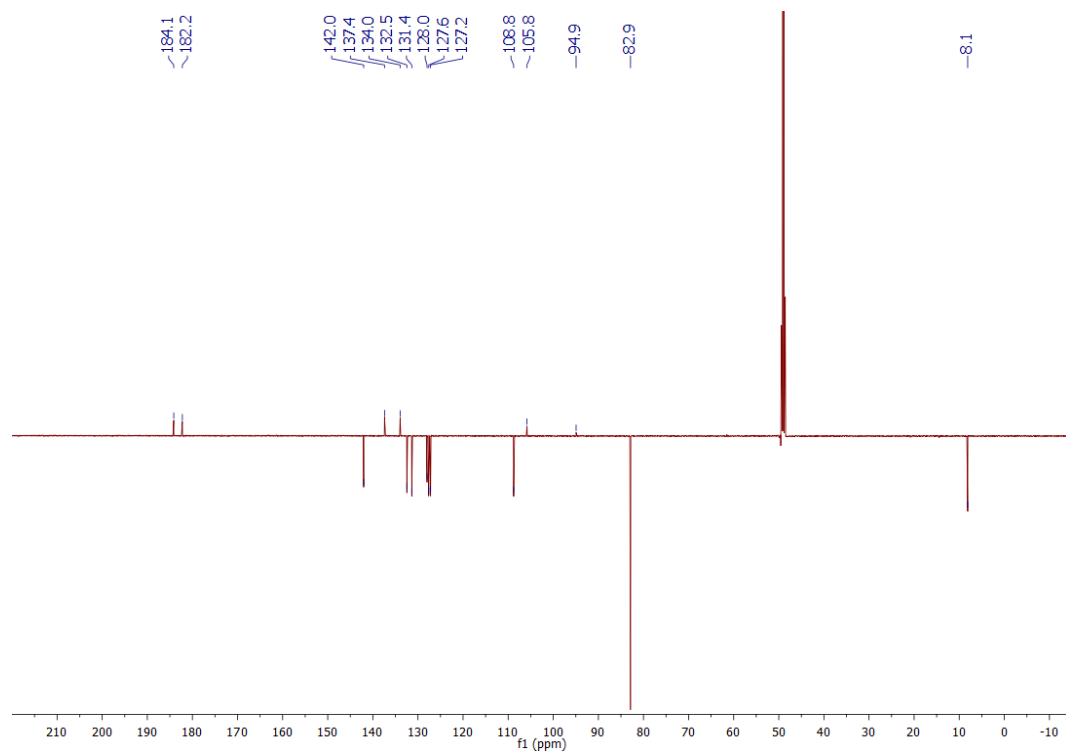
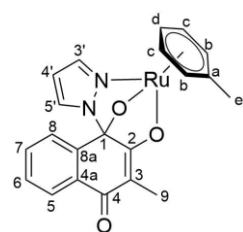


Figure S2: ¹³C-NMR (MeOD-*d*₄, 150.93 MHz) spectrum of (2)



¹³C NMR spectrum (CDCl₃) of compound 10b. The spectrum shows peaks at the following chemical shifts (ppm): 183.9, 183.3, 141.5, 137.7, 134.1, 132.3, 131.3, 128.0, 127.7, 127.1, 108.7, 105.6, 102.4, 100.1, 94.8, 82.2, 80.3, 79.4, 78.9, 50.0 (CDCl₃), 30.9, 24.1, and 8.1.

S2

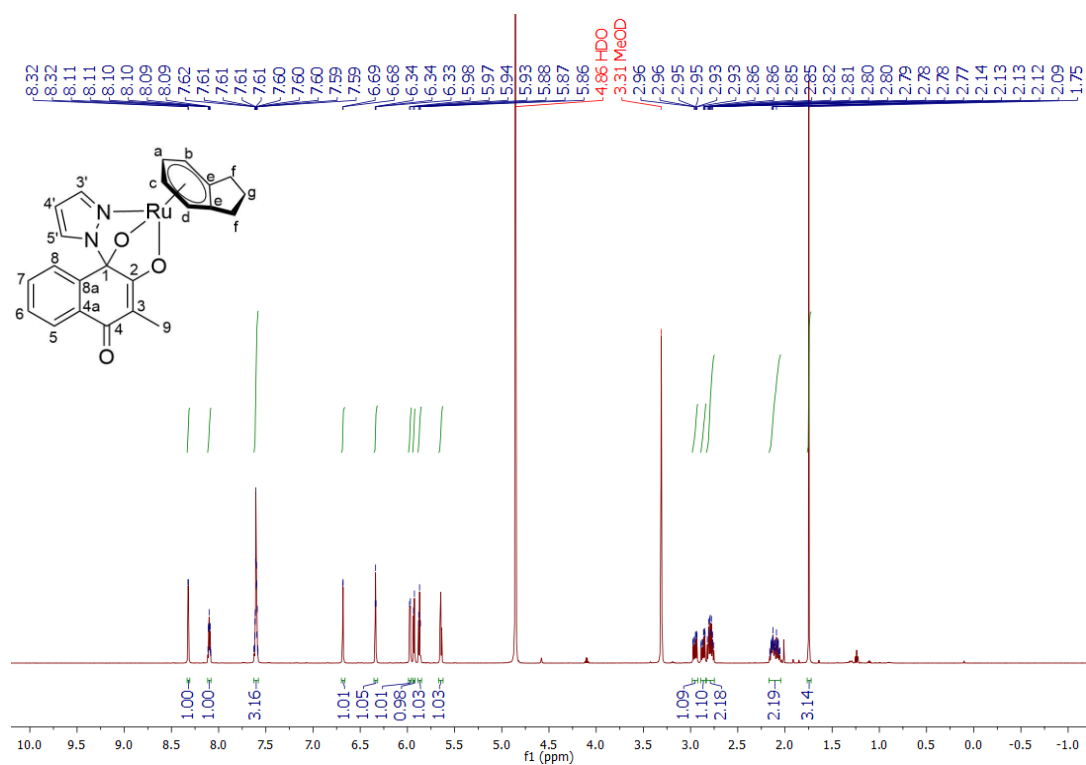


Figure S5: ¹H-NMR (MeOD-*d*₄, 600.25 MHz) spectrum of (**4**)

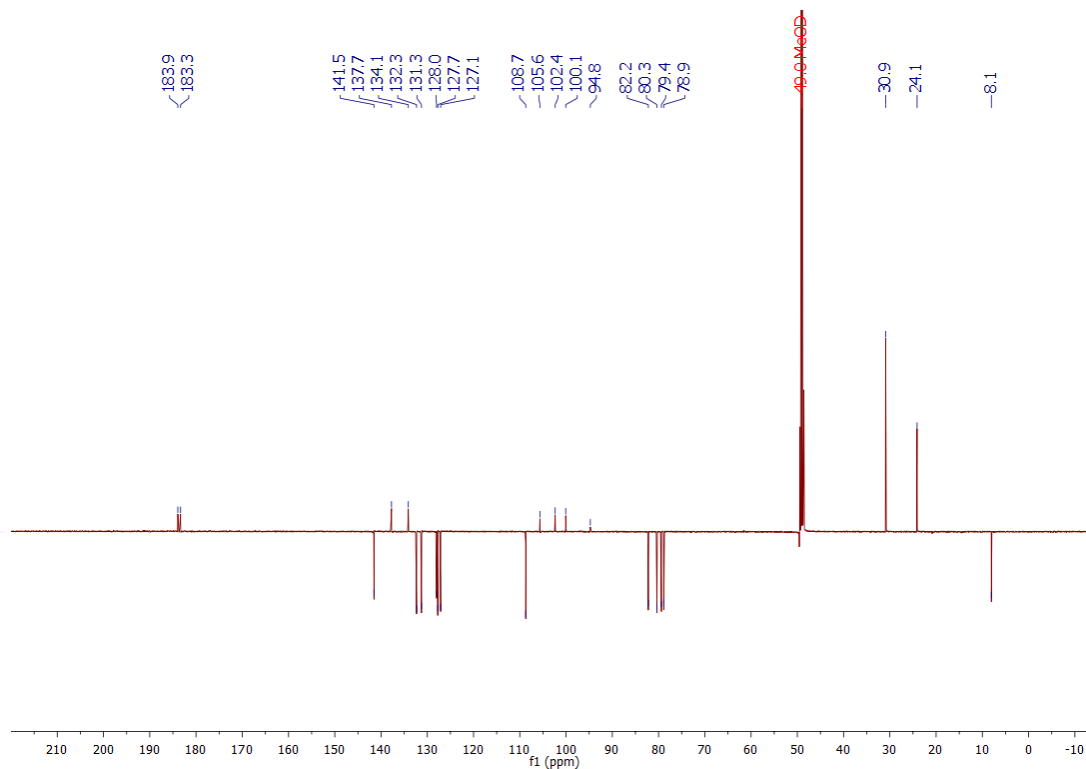


Figure S6: ¹³C-NMR (MeOD-*d*₄, 150.93 MHz) spectrum of (**4**)

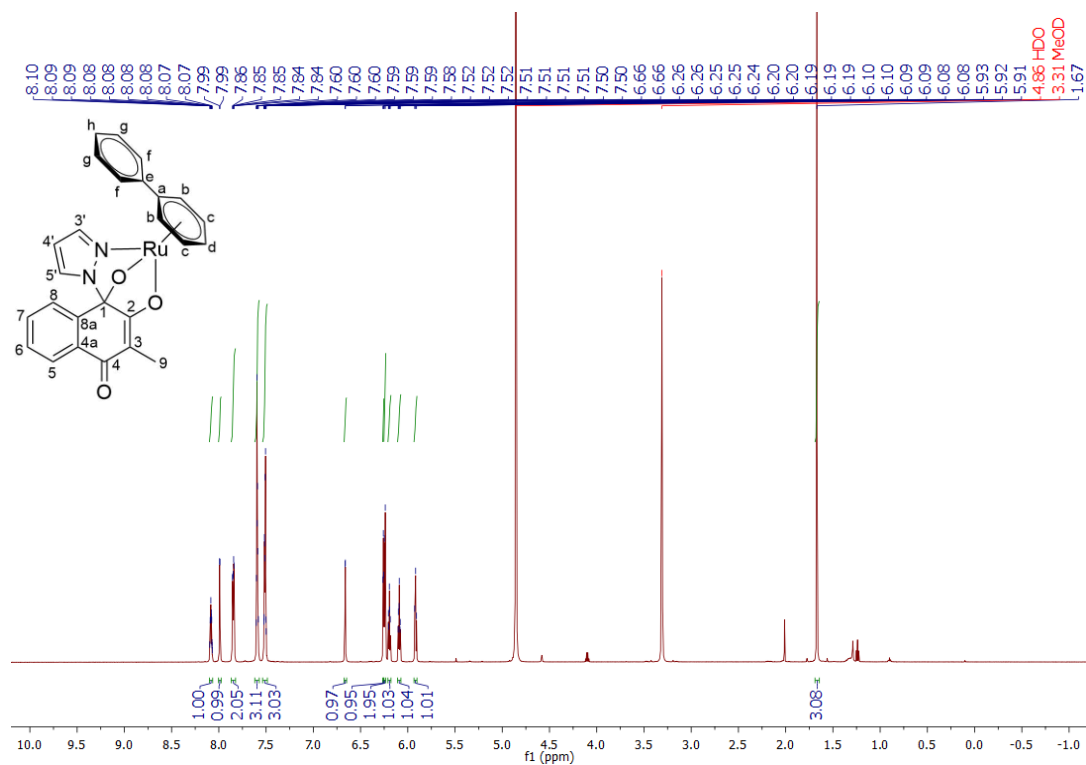


Figure S7: ¹H-NMR (MeOD-*d*₄, 600.25 MHz) spectrum of (5)

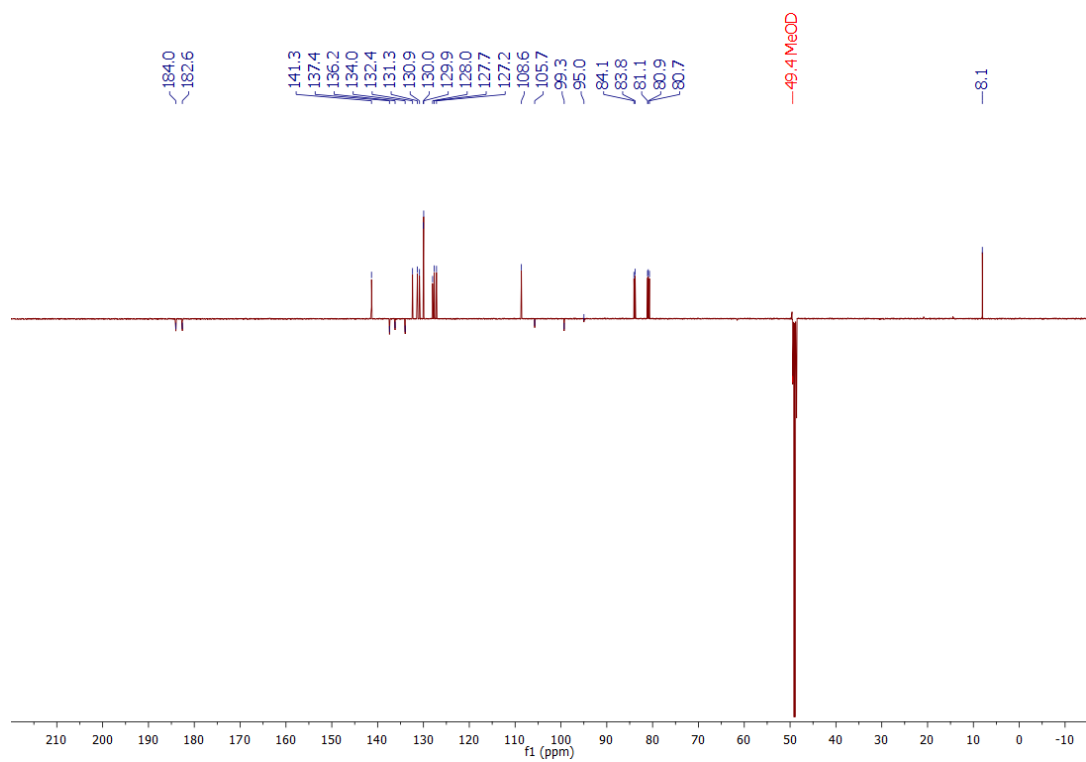


Figure S8: ¹³C-NMR (MeOD-*d*₄, 150.93 MHz) spectrum of (5)

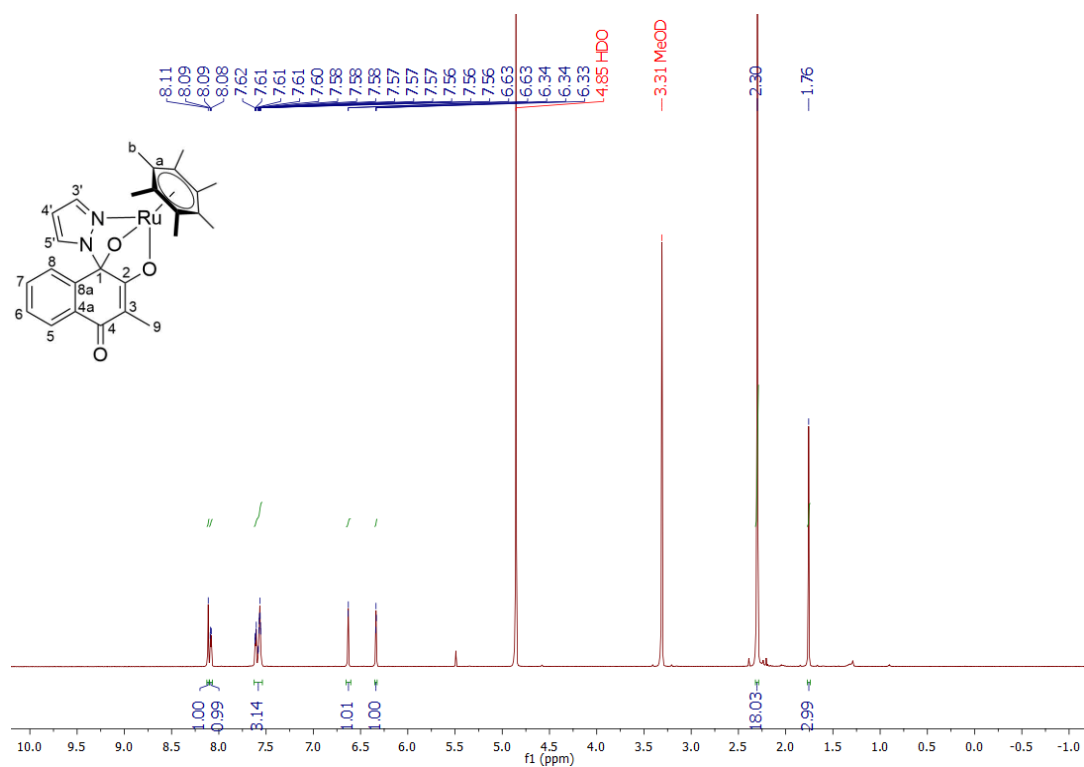


Figure S9: ¹H-NMR (MeOD-*d*₄, 700.40 MHz) spectrum of (6)

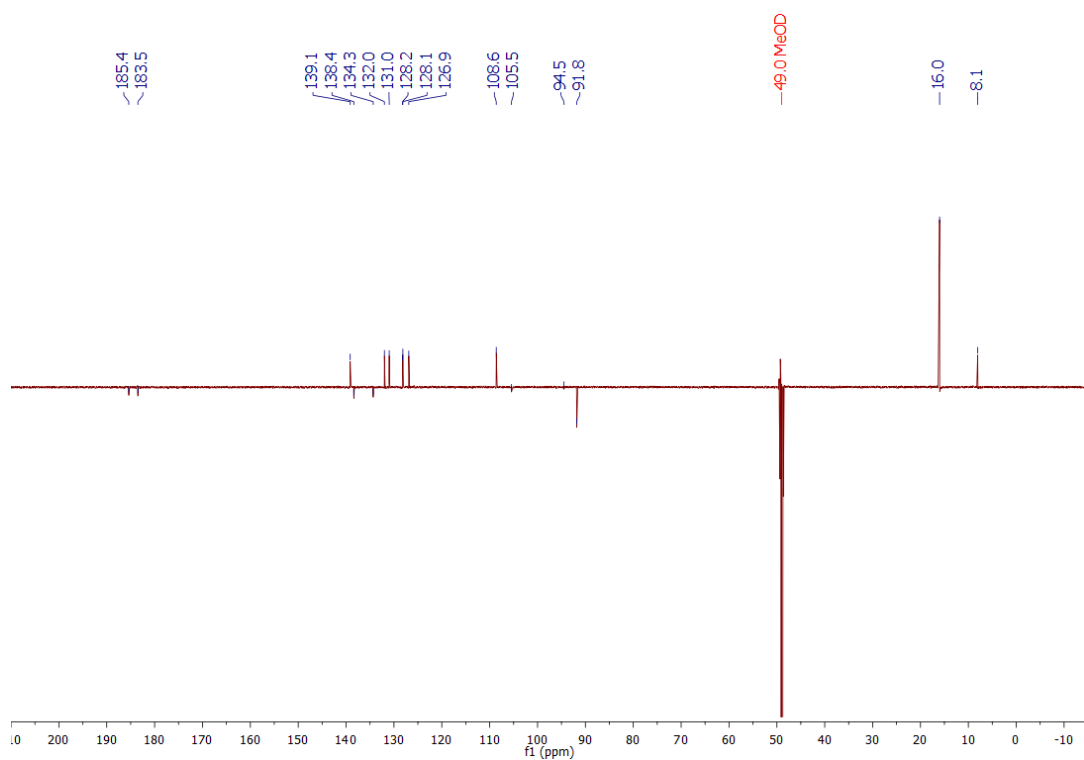


Figure S10: ¹³C-NMR (MeOD-*d*₄, 176.13 MHz) spectrum of (6)

X-Ray Diffraction Analysis

The X-ray intensity data were measured on Bruker D8 Venture diffractometer equipped with multilayer monochromator, Mo K/ α INCOATEC sealed tubes and Oxford cooling system. The structures were solved by *Direct and Iterative Methods*. Non-hydrogen atoms were refined with *anisotropic displacement parameters*. Hydrogen atoms were inserted at calculated positions and refined with riding model. The following software was used: *Bruker SAINT software package* [1] using a narrow-frame algorithm for frame integration, *SADABS* [2] for absorption correction, *OLEX2* [3] for structure solution, refinement, molecular diagrams and graphical user-interface, *Shelxle* [4] for refinement and graphical user-interface, *SHELXS-2015* [5] for structure solution, *SHELXL-2015* [6] for refinement, *Platon* [7] for symmetry check. Experimental data and CCDC-Codes Experimental data (Available online: <http://www.ccdc.cam.ac.uk/conts/retrieving.html>) can be found in Table S1. Crystal data, data collection parameters, and structure refinement details are given in Tables S2–S5. Asymmetric Units visualized in Figure S11–S14.

Table S1: Experimental parameter and CCDC-Code.

Sample	Machine	Source	Temp.	Detector Distance	Time/ Frame	#Frames	Frame width	CCDC
			[K]	[mm]	[s]		[°]	
2	Bruker D8	Mo	100	40	10	360	0.5	2210525
3	Bruker D8	Mo	100	40	15	209	0.5	2210526
4	Bruker D8	Mo	100	40	10	2403	0.5	2210527
6	Bruker D8	Mo	100	36	1	1896	0.36	2210528

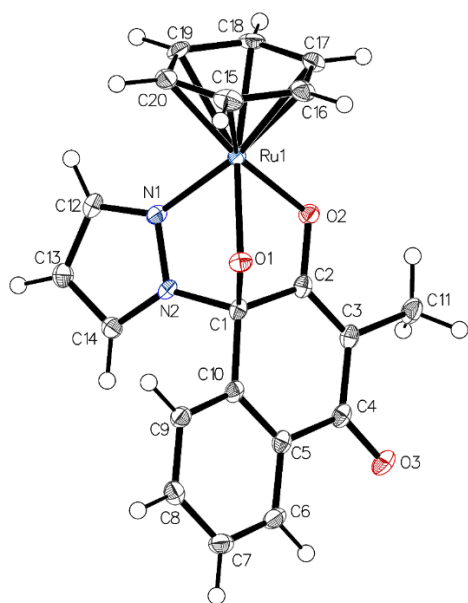


Figure S11: Asymmetric Unit drawn with 50% displacement ellipsoid. Solvent omitted for clarity. The bond precision for C-C single bonds is 0.0037 Å.

Table S2: Sample and crystal data, data collection and structure refinement

Identification code	mo_hege_PhBr004_P21n	b [Å]	9.7557(8)
Empirical formula	C ₂₀ H ₁₈ N ₂ O ₃ Ru	c [Å]	14.7947(11)
Formula weight [g/mol]	451.43	α [°]	90
Temperature [K]	100.0	β [°]	94.244(5)
Crystal system	monoclinic	γ [°]	90
Space group	P21/n	Volume[Å ³]	1691.2(3)
a [Å]	11.7496(12)	Z	4
ρ _{calc} [g/cm ³]	1.773	Reflections collected	9577
μ [mm ⁻¹]	0.958	Independent reflections	3074 [R _{int} = 0.0377, R _{sigma} = 0.0406]
F(000)	912.0	Data/restraints/parameters	3074/0/248
Crystal size[mm ³]	0.1 × 0.09 × 0.04	Goodness-of-fit on F ²	1.066
Radiation	MoKα (λ = 0.71073)	Final R indexes [I > 2σ (I)]	R1 = 0.0246, wR2 = 0.0619
2θ range for data collection [°]	4.596 to 50.686	Final R indexes [all data]	R1 = 0.0274, wR2 = 0.0640
Index ranges	-14 ≤ h ≤ 14, -11 ≤ k ≤ 11, -17 ≤ l ≤ 17	Largest diff. peak/hole [e ⁻ Å ⁻³]	0.49/-0.42

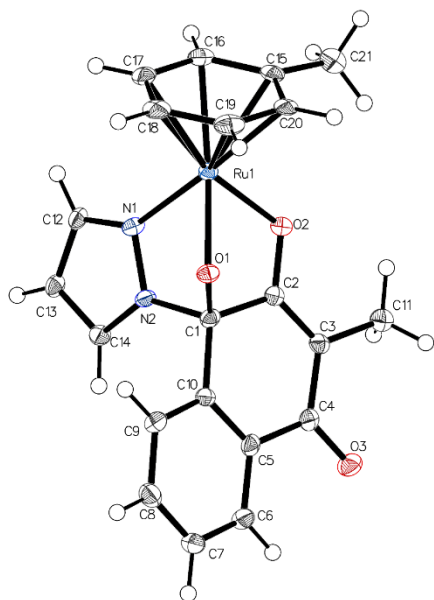


Figure S12: Asymmetric Unit drawn with 50% displacement ellipsoid. Solvent omitted for clarity. The bond precision for C-C single bonds is 0.0046 Å.

Table S3: Sample and crystal data, data collection and structure refinement

Identification code	mo_hege_PhBr005_lbca	b [Å]	18.5085(11)
Empirical formula	C ₂₁ H ₁₉ N ₂ O ₃ Ru	c [Å]	24.2359(14)
Formula weight [g/mol]	456.45	α [°]	90
Temperature [K]	100.0	β [°]	90
Crystal system	orthorhombic	γ [°]	90
Space group	lbca	Volume[Å ³]	7146.1(7)
a [Å]	15.9307(8)	Z	16
ρ _{calc} [g/cm ³]	1.697	Reflections collected	12602
μ [mm ⁻¹]	0.906	Independent reflections	3276 [R _{int} = 0.0638, R _{sigma} = 0.0508]
F(000)	3696.0	Data/restraints/parameters	3276/0/254
Crystal size[mm ³]	0.06 × 0.04 × 0.02	Goodness-of-fit on F ²	1.019
Radiation	MoKα (λ = 0.71073)	Final R indexes [I > 2σ (I)]	R ₁ = 0.0330, wR ₂ = 0.0706
2θ range for data collection [°]	4.762 to 50.692	Final R indexes [all data]	R ₁ = 0.0451, wR ₂ = 0.0783
Index ranges	-19 ≤ h ≤ 17, -21 ≤ k ≤ 22, -26 ≤ l ≤ 29	Largest diff. peak/hole [e ⁻ Å ⁻³]	0.38/-0.56

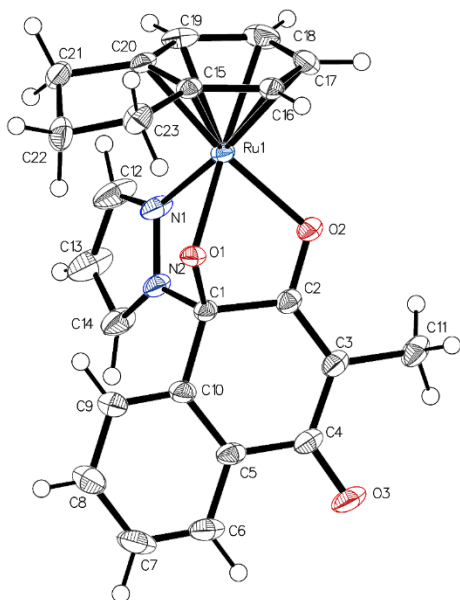


Figure S13: Asymmetric Unit drawn with 50% displacement ellipsoid. The bond precision for C-C single bonds is 0.0027 Å.

Table S4: Sample and crystal data, data collection and structure refinement

Identification code	mo_hege_PhBr015_P-1	b [Å]	9.9658(4)
Empirical formula	C ₂₃ H ₂₀ N ₂ O ₃ Ru	c [Å]	14.1016(5)
Formula weight [g/mol]	473.48	α [°]	96.1229(14)
Temperature [K]	100.0	β [°]	104.6352(14)
Crystal system	triclinic	γ [°]	101.1567(14)
Space group	P-1	Volume[Å ³]	1047.14(7)
a [Å]	7.9569(3)	Z	2
ρ _{calc} [g/cm ³]	1.502	Reflections collected	51436
μ [mm ⁻¹]	0.774	Independent reflections	6115 [R _{int} = 0.0371, R _{sigma} = 0.0208]
F(000)	480.0	Data/restraints/parameters	6115/0/263
Crystal size[mm ³]	0.09 × 0.07 × 0.05	Goodness-of-fit on F ²	1.053
Radiation	MoKα (λ = 0.71073)	Final R indexes [I>=2σ (I)]	R ₁ = 0.0248, wR ₂ = 0.0579
2θ range for data collection [°]	4.776 to 60.114	Final R indexes [all data]	R ₁ = 0.0278, wR ₂ = 0.0596
Index ranges	-11 ≤ h ≤ 11, -14 ≤ k ≤ 14, -19 ≤ l ≤ 19	Largest diff. peak/hole [e ⁻ Å ⁻³]	0.87/-0.66

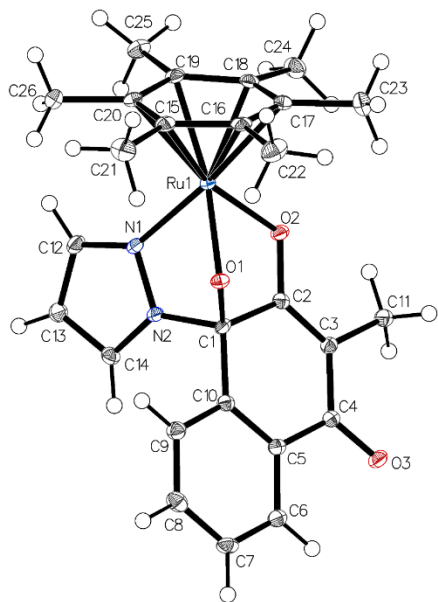


Figure S14: Asymmetric Unit drawn with 50% displacement ellipsoid. Solvent omitted for clarity. The bond precision for C-C single bonds is 0.0023 Å. A solvent mask was calculated, and 40 electrons were found in a volume of 130 Å³ in 1 void per unit cell. This is consistent with the presence of 2[H₂O] per Unit Cell which account for 40 electrons per unit cell.

Table S5: Sample and crystal data, data collection and structure refinement

Identification code	HeGe764_P21n	b [Å]	20.8855(3)
Empirical formula	C ₂₇ H ₃₀ Cl ₂ N ₂ O ₃ Ru	c [Å]	14.4325(2)
Formula weight [g/mol]	602.50	α [°]	90
Temperature [K]	100.0	β [°]	105.1468(6)
Crystal system	monoclinic	γ [°]	90
Space group	P2 ₁ /n	Volume[Å ³]	2470.99(6)
a [Å]	8.49260(10)	z	4
ρ _{calc} [g/cm ³]	1.620	Reflections collected	73651
μ [mm ⁻¹]	0.884	Independent reflections	7494 [R _{int} = 0.0659, R _{sigma} = 0.0383]
F(000)	1232.0	Data/restraints/parameters	7494/6/342
Crystal size[mm ³]	0.176 × 0.16 × 0.087	Goodness-of-fit on F ²	1.035
Radiation	MoKα (λ = 0.71073)	Final R indexes [I > 2σ (I)]	R ₁ = 0.0287, wR ₂ = 0.0680
2θ range for data collection [°]	4.874 to 61.09	Final R indexes [all data]	R ₁ = 0.0451, wR ₂ = 0.0726
Index ranges	-12 ≤ h ≤ 12, -29 ≤ k ≤ 29, -17 ≤ l ≤ 20	Largest diff. peak/hole [e ⁻ Å ⁻³]	0.64/-0.78

HPLC-MS data

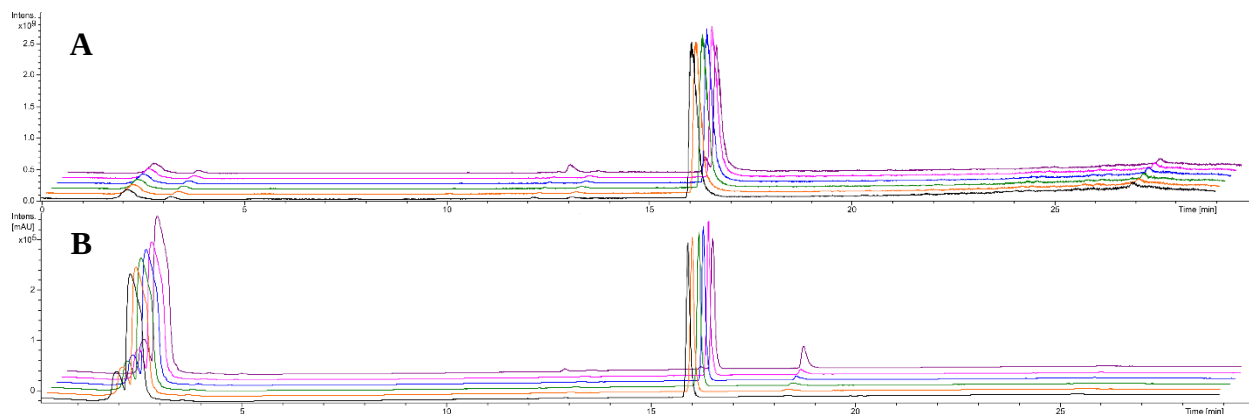


Figure S15: HPLC-MS chromatogram (A) and HPLC chromatogram (B) of compound 1 after 0, 1, 2, 3, 4, 24 h (from front to back; conditions see experimental section).

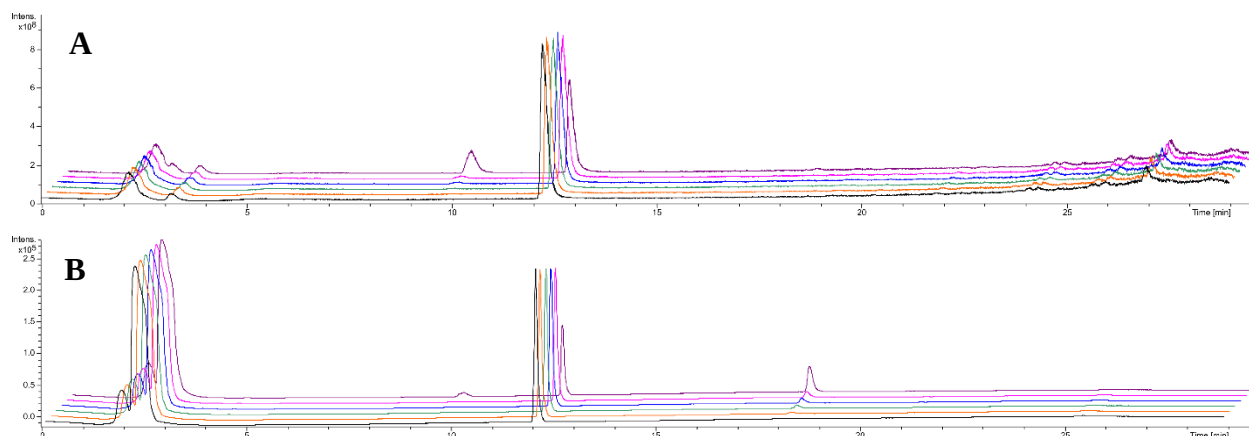


Figure S16: HPLC-MS chromatogram (A) and HPLC chromatogram (B) of compound 2 after 0, 1, 2, 3, 4, 24 h (from front to back; conditions see experimental section).

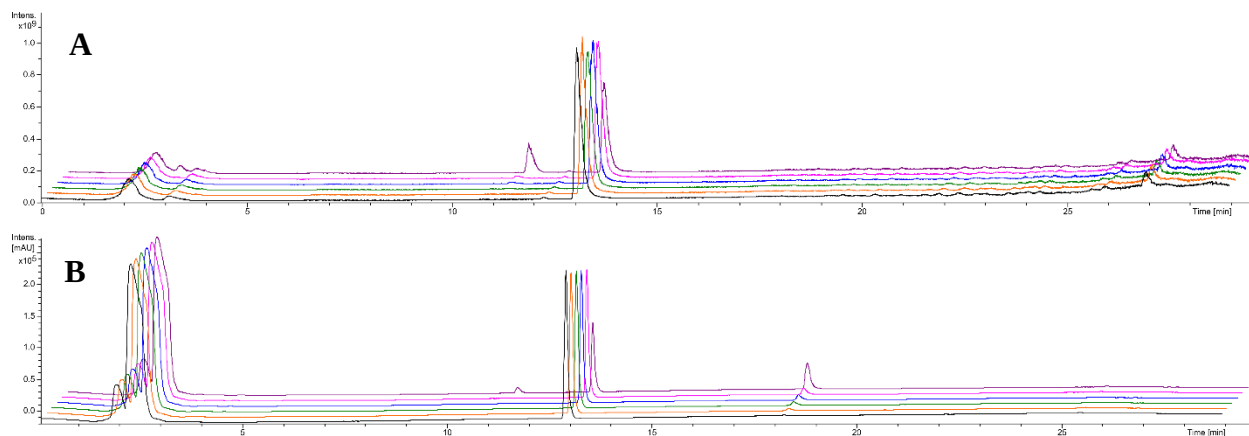


Figure S17: HPLC-MS chromatogram (A) and HPLC chromatogram (B) of compound 3 after 0, 1, 2, 3, 4, 24 h (from front to back; conditions see experimental section).

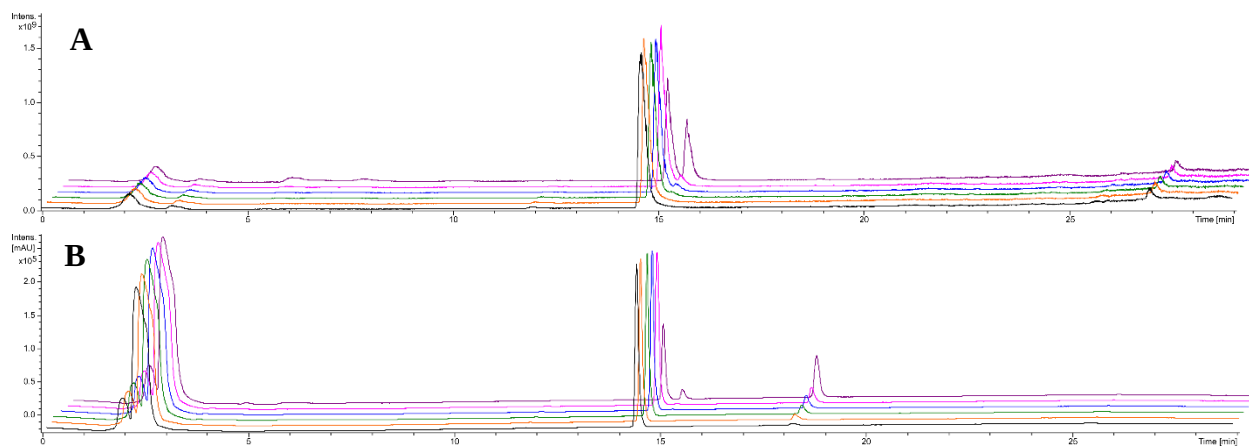


Figure S18: HPLC chromatogram-MS (A) and HPLC chromatogram (B) of compound **4** after 0, 1, 2, 3, 4, 24 h (from front to back; conditions see experimental section).

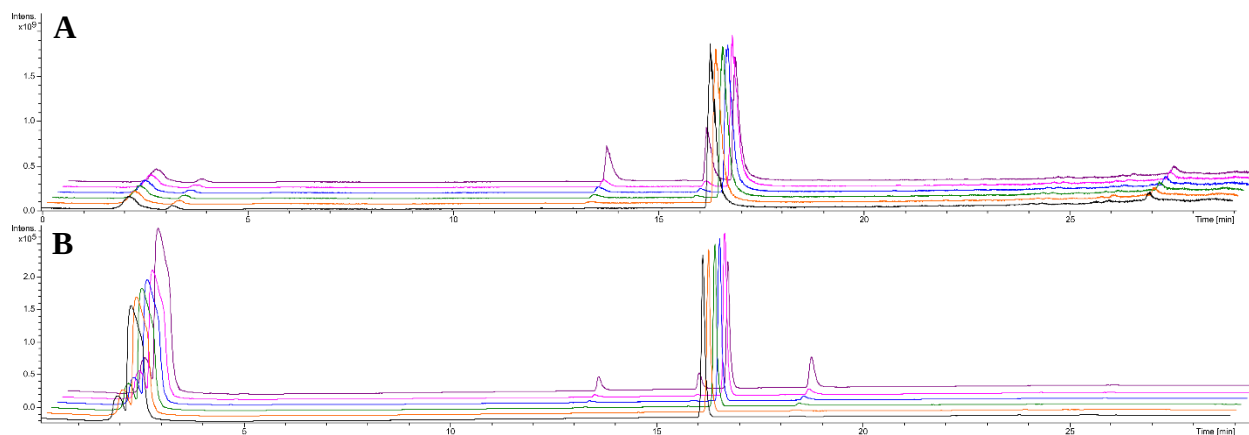


Figure S19: HPLC chromatogram-MS (A) and HPLC chromatogram (B) of compound **5** after 0, 1, 2, 3, 4, 24 h (from front to back; conditions see experimental section).

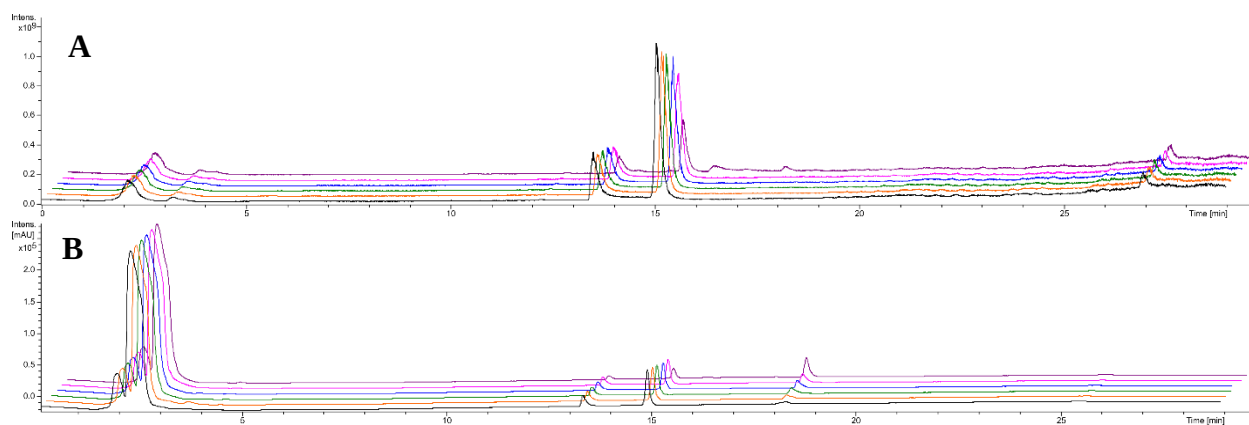
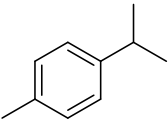
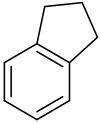
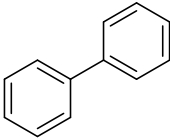
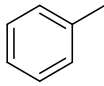
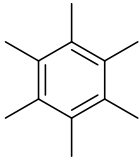


Figure S20: HPLC chromatogram-MS (A) and HPLC chromatogram (B) of compound **6** after 0, 1, 2, 3, 4, 24 h (from front to back; conditions see experimental section).

Calculated miLogP values

Table S6: Calculated miLogP values of used arenes. Calculations were performed at www.molinspiration.com

Arene	miLogP	Arene	miLogP
 <i>p</i> -Cymene	3.90	 Indane	2.38
 Benzene	1.94	 Biphenyl	3.73
 Toluene	2.39	 Hexamethylbenzene (HMB)	4.34

Biological studies

MTT assay

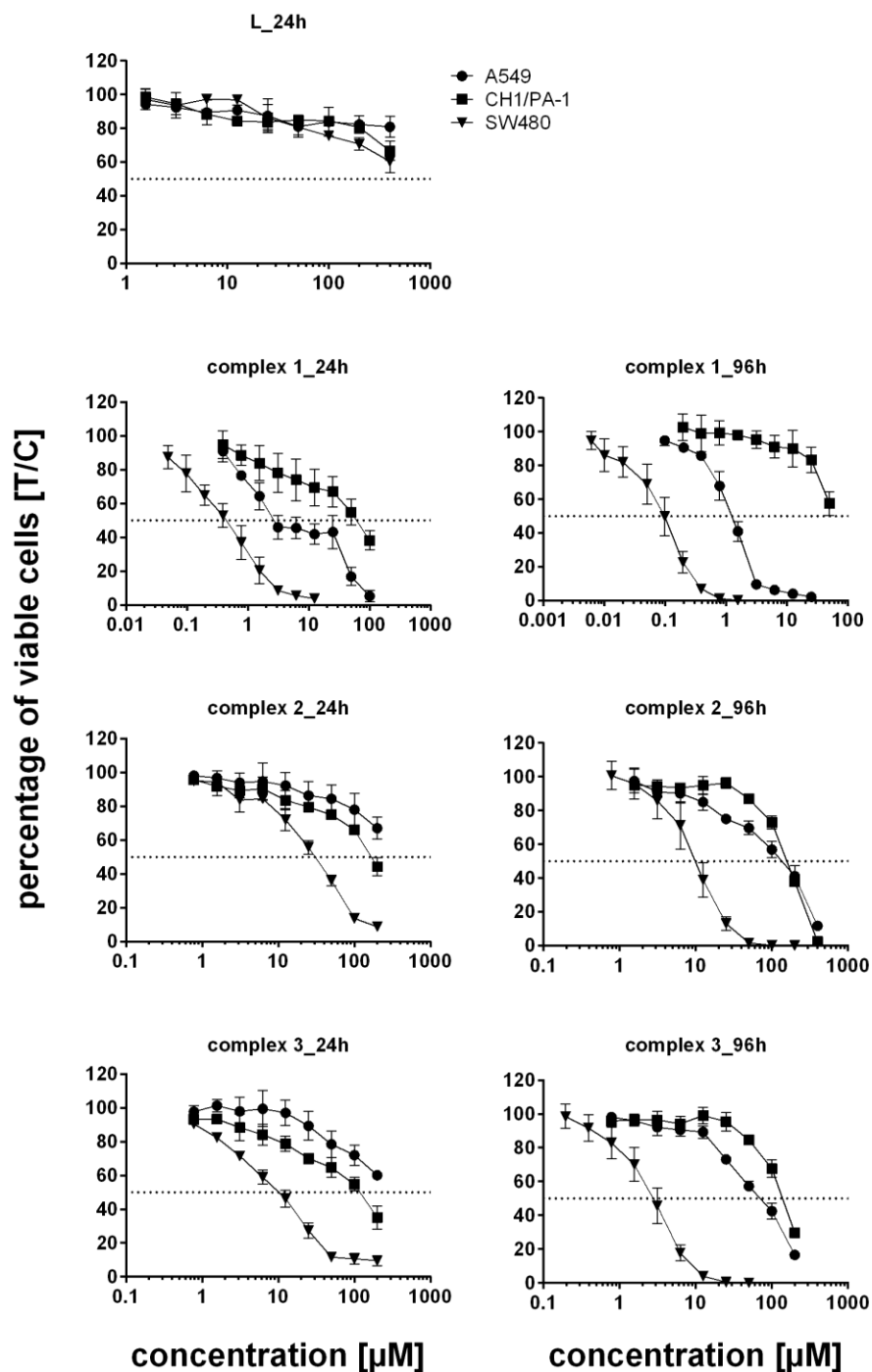


Figure S21: Concentration-effect curves of compounds L and 1–3 in three cell lines A549, SW480 and CH1/PA-1 after 24 h and 96 h exposure in the MTT assay.

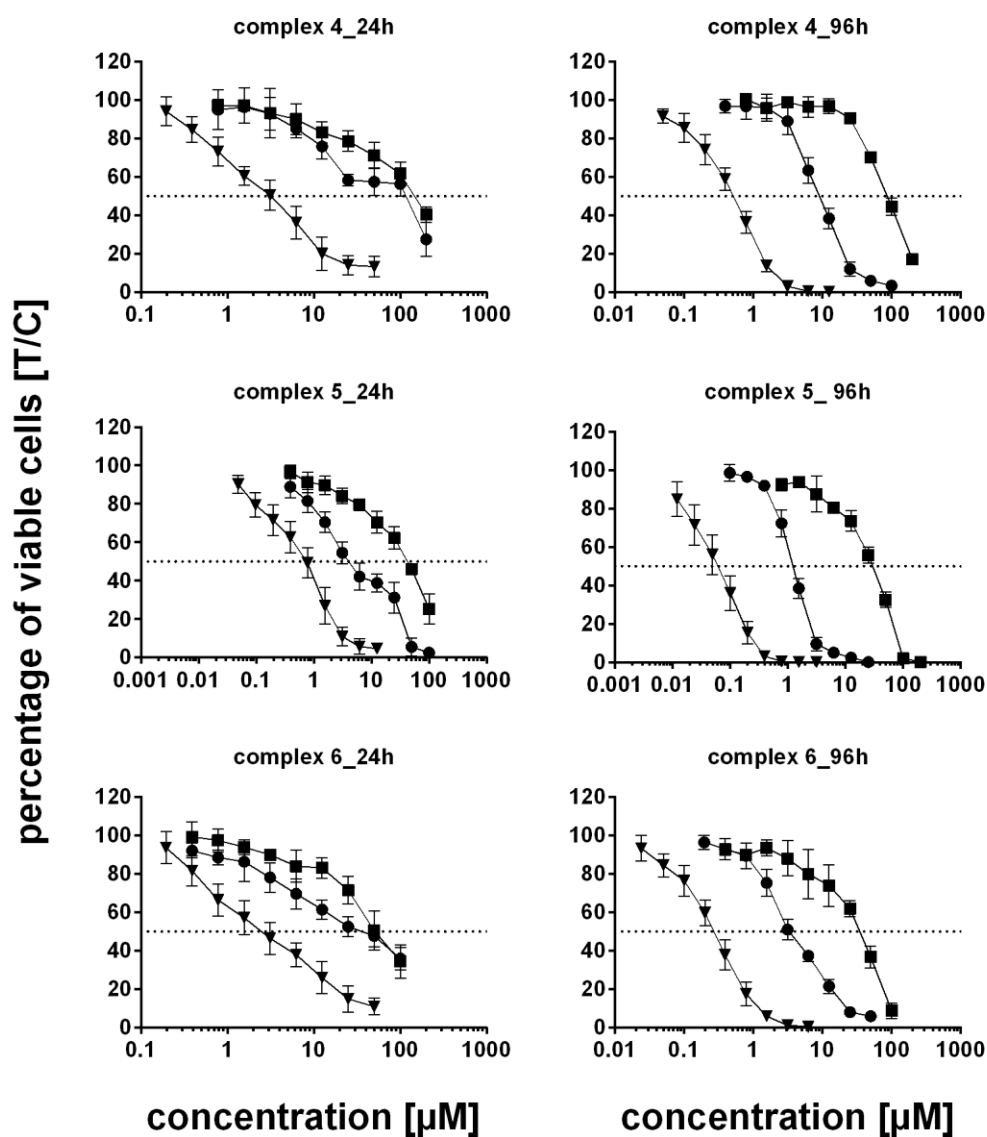


Figure S22: Concentration-effect curves of compounds 4–6 in three cell lines A549, SW480 and CH1/PA-1 after 24 h and 96 h exposure in the MTT assay.

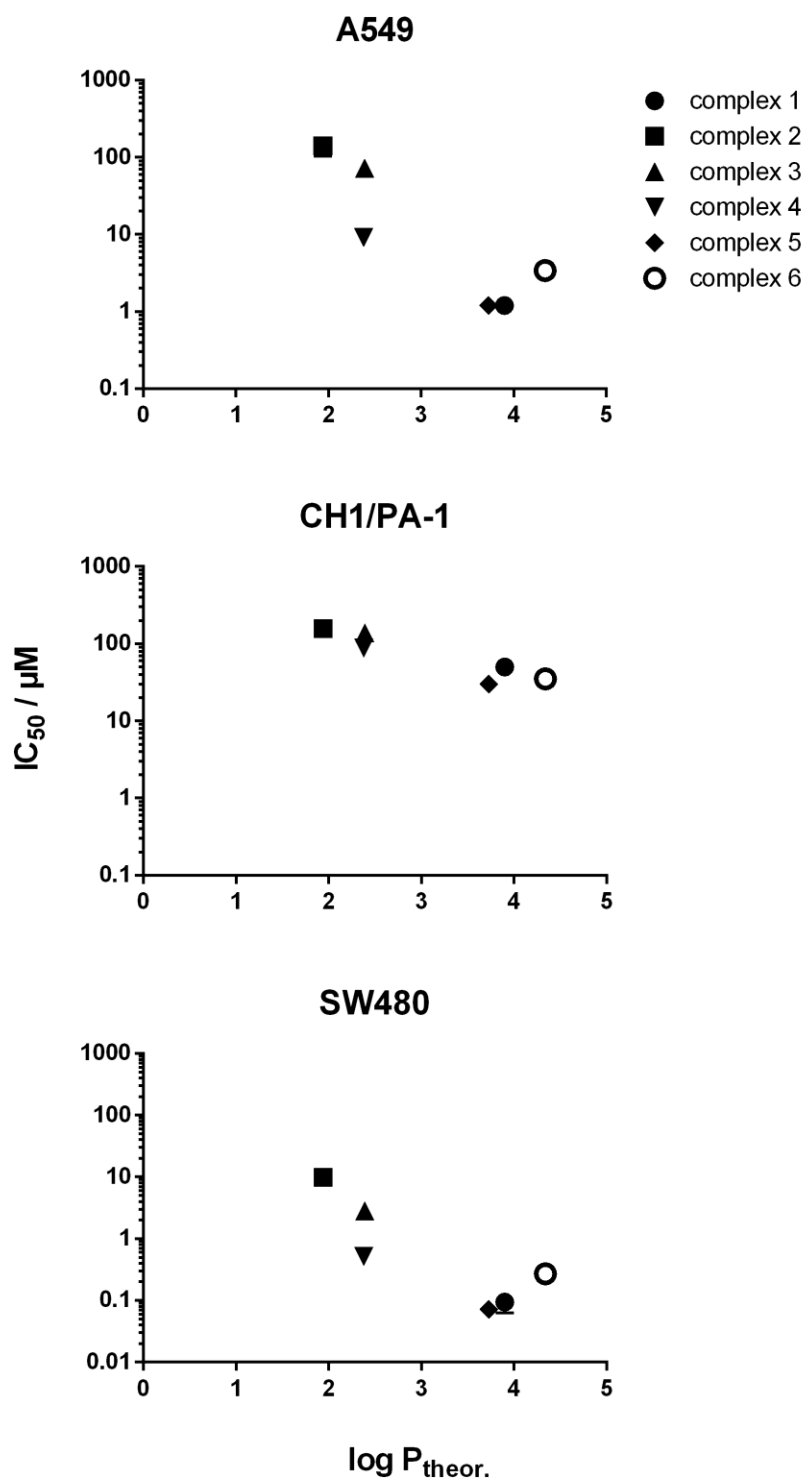


Figure S23: Correlation between IC_{50} values (96 h exposure) and theoretical lipophilicity coefficient in A549, CH1/PA-1 and SW480 cells.

Colony formation assay

Table S7: Impact of compounds **1–6** and **L** on colony formation in SW480 cells. Data are means $\pm$ standard deviations of three independent experiments and normalized to the negative control. (In negative controls 168 ± 18 colonies were counted.)

Compound	Concentration [μM]	Number of colonies [%]
control	0	100
1	0.039	18 ± 5
	0.078	3.1 ± 0.8
	0.16	0.26 ± 0.43
2	5	48 ± 9
	10	20 ± 4
	20	3.5 ± 1.5
3	1.25	53 ± 8
	2.5	23 ± 8
	5	4.3 ± 3.7
4	0.31	22 ± 5
	0.63	5.1 ± 2.1
	1.25	0.82 ± 0.85
5	0.039	19 ± 7
	0.078	4.0 ± 2.0
	0.16	0.22 ± 0.48
6	0.16	28 ± 12
	0.31	9.2 ± 3.2
	0.63	1.2 ± 1.1
L	50	62 ± 25
	100	0 ± 0
	150	0 ± 0

Ligand IC_{50} (SW480) $116 \pm 37 \mu M$

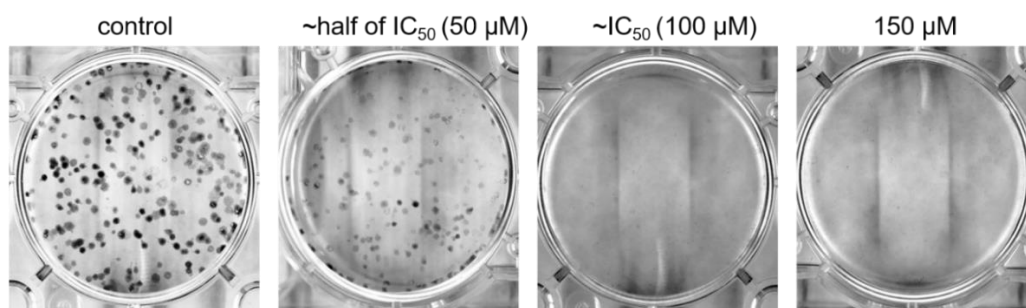
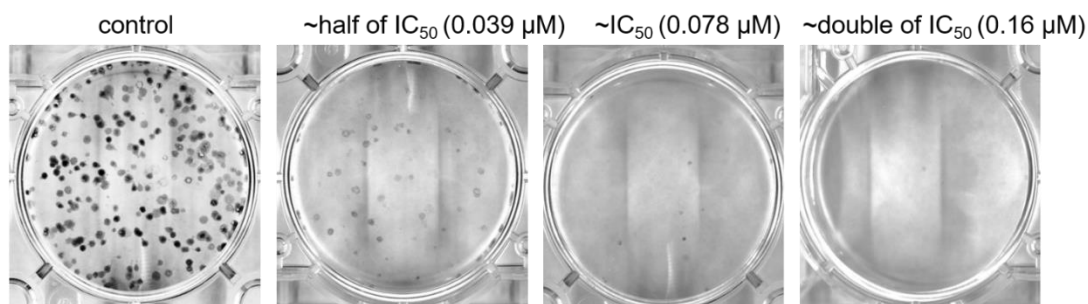


Figure S24: Representative images of the colony formation assay with compound L, which reduced the long-term proliferation capacity of SW480 cells *in vitro*. Chosen concentrations are based on 96-h IC_{50} values in the MTT assay.

Complex 1 IC_{50} (SW480) $0.094 \pm 0.031 \mu M$



Complex 2 IC_{50} (SW480) $9.88 \pm 2.49 \mu M$

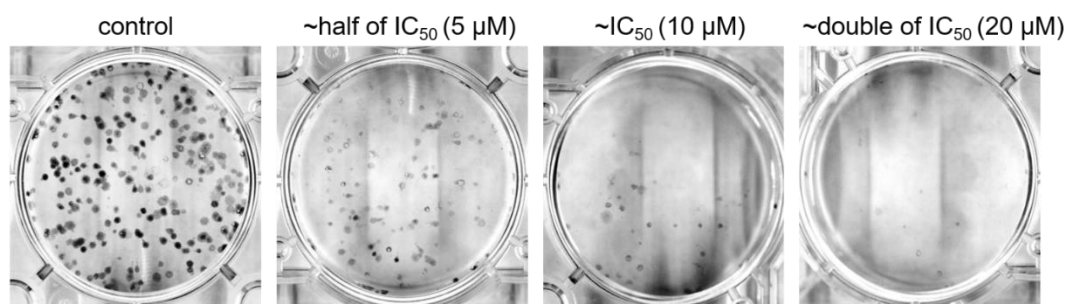
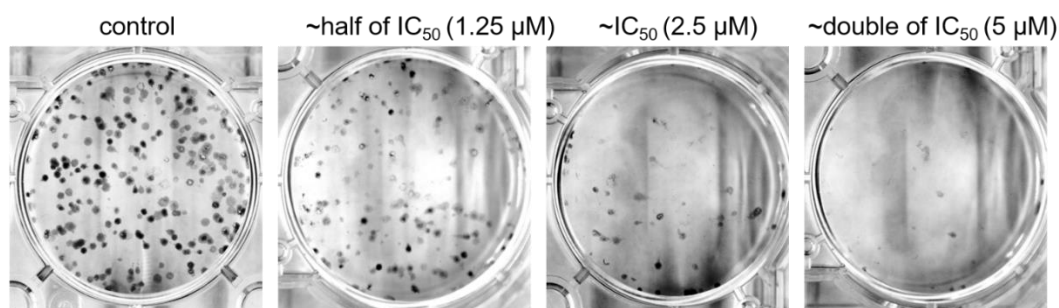


Figure S25: Representative images of the colony formation assay with compounds 1 and 2, which reduced the long-term proliferation capacity of SW480 cells *in vitro*. Chosen concentrations are based on 96-h IC_{50} values in the MTT assay.

Complex 3 IC_{50} (SW480) $2.81 \pm 0.73 \mu M$



Complex 4 IC_{50} (SW480) $0.52 \pm 0.09 \mu M$

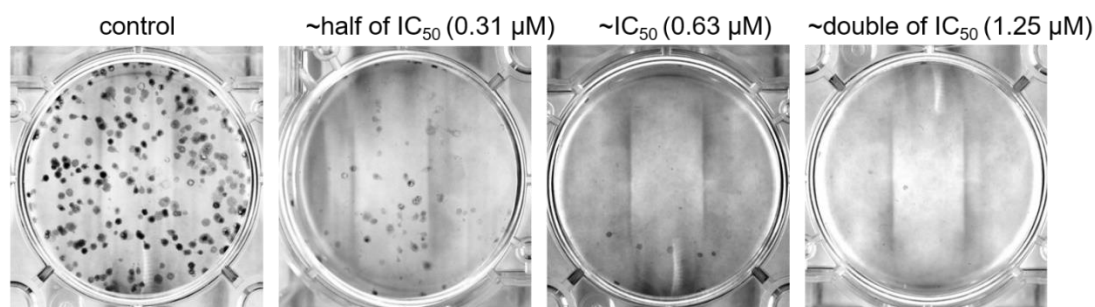
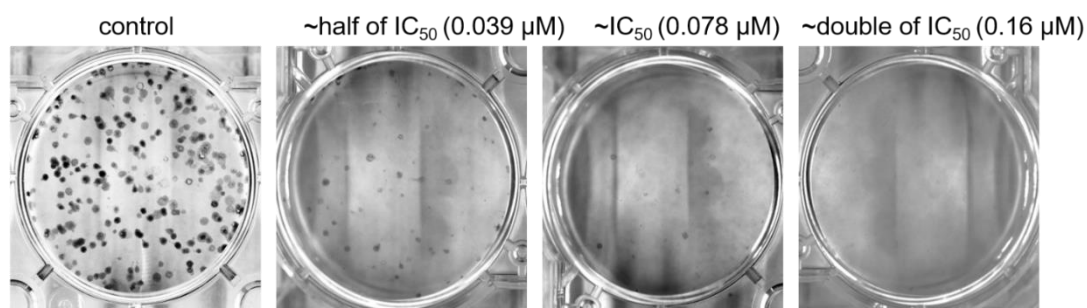


Figure S26: Representative images of the colony formation assay with compounds **3** and **4**, which reduced the long-term proliferation capacity of SW480 cells *in vitro*. Chosen concentrations are based on 96-h IC_{50} values in the MTT assay.

Complex 5 IC_{50} (SW480) $0.072 \pm 0.019 \mu M$



Complex 6 IC_{50} (SW480) $0.27 \pm 0.06 \mu M$

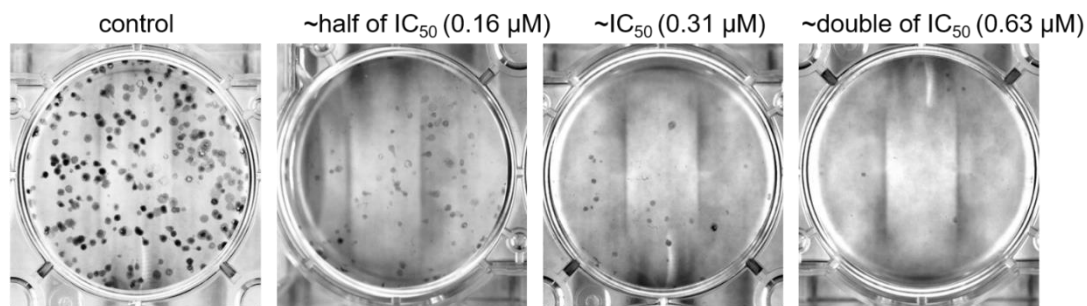


Figure S27: Representative images of the colony formation assay with compounds **5** and **6**, which reduced the long-term proliferation capacity of SW480 cells *in vitro*. Chosen concentrations are based on 96-h IC_{50} values in the MTT assay.

Cellular accumulation

Table S8: Cellular accumulation in SW480 cells after 2 h of incubation with the tested complexes 1–6 at 37 °C and two different concentrations (2 μ M and 5 μ M).

Compound	Concentration [μ M]	Ru [102]/cell [fg]
1	2	97 ± 8
	5	299 ± 54
2	2	4.5 ± 0.3
	5	11 ± 2
3	2	15 ± 2
	5	33 ± 10
4	2	44 ± 3
	5	100 ± 15
5	2	107 ± 14
	5	336 ± 10
6	2	100 ± 15
	5	206 ± 32

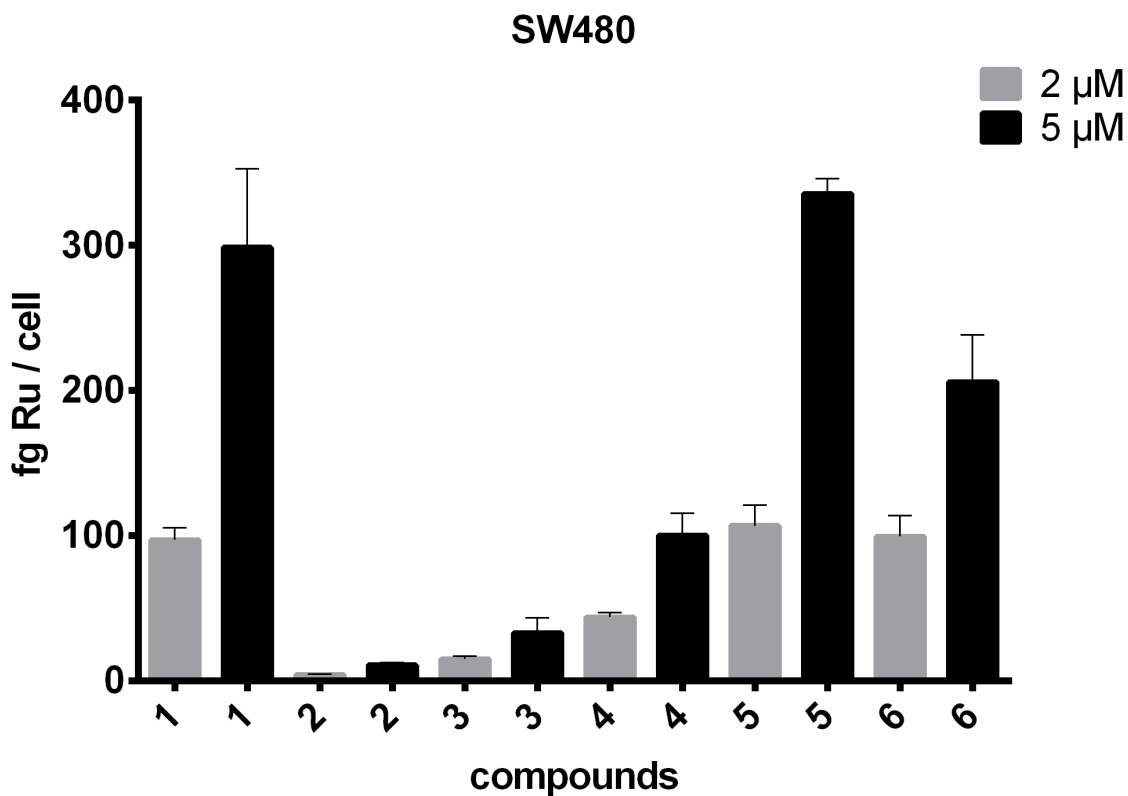


Figure S28: Cellular ruthenium accumulation of compounds 1–6 in fg Ru/cell, measured by ICP-MS. SW480 cells were treated for 2 h with 2 μ M and 5 μ M of test solutions. Values are means $\pm$ SDs from at least three independent measurements performed in triplicates.

Table S9: Instrumental parameters for the determination of the ruthenium content using ICP-MS.

RF power [W]	1550
Nebulizer	MicroMist
Spray chamber	Scott double-pass
Spray chamber temp. [°C]	2
Cone materials	Ni
Plasma gas flow (Ar) [L min ⁻¹]	15.0
Auxiliary gas flow (Ar) [L min ⁻¹]	0.90
Nebulizer gas flow (Ar) [L min ⁻¹]	1.03
Monitored Isotopes	¹⁰² Ru, ¹¹⁵ In
Integration time [s]	0.1

Cell cycle distribution

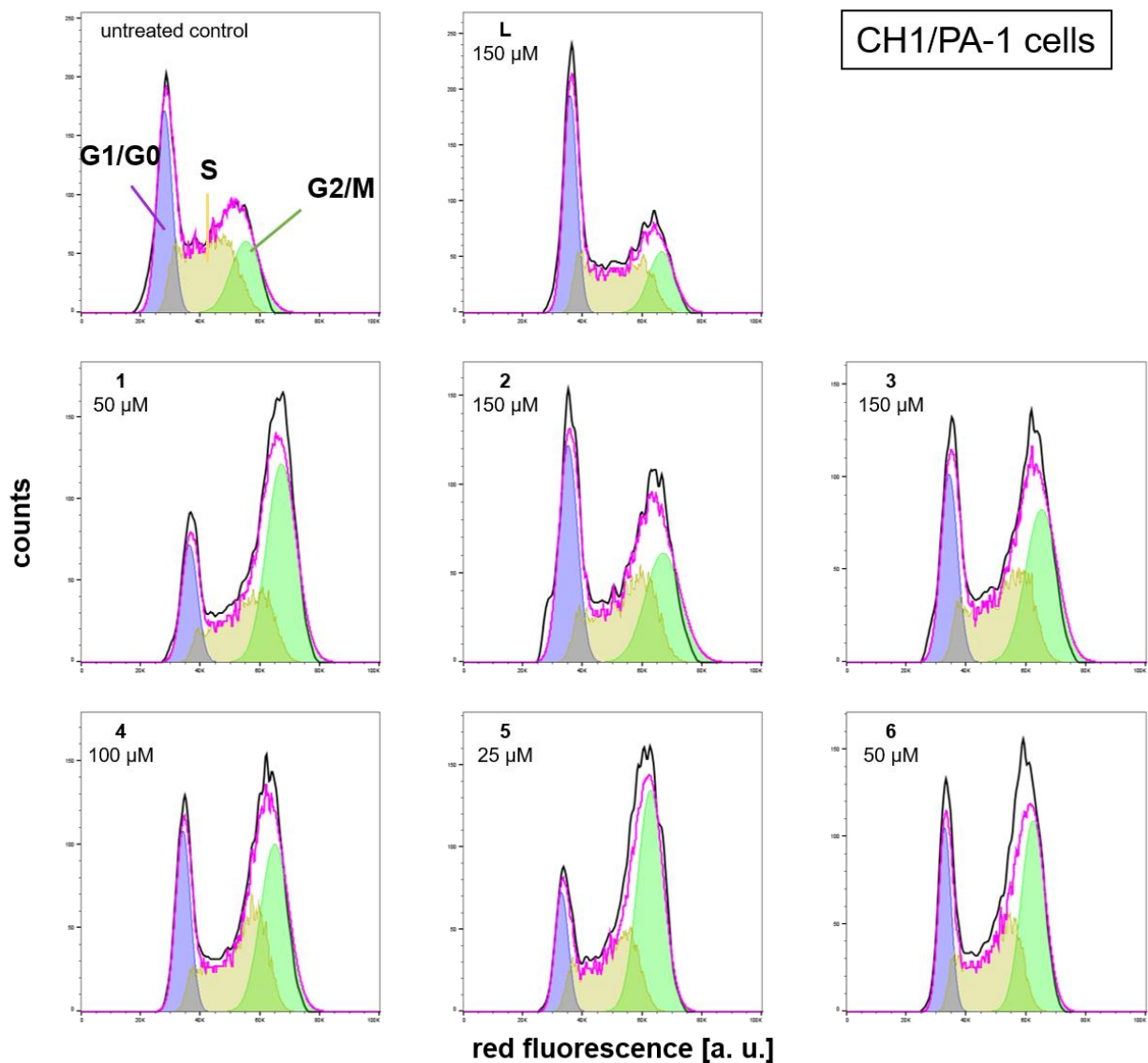


Figure S29: Histograms of cell cycle phase distribution in CH1/PA-1 cells after 24 h drug exposure. The cells were exposed to **L** and complexes **1–6** in a subcytotoxic concentration (around IC_{50} values from 96 h MTT assays). The top left histogram represents an untreated control. All histograms are from one representative experiment out of at least three repetitions per tested concentration.

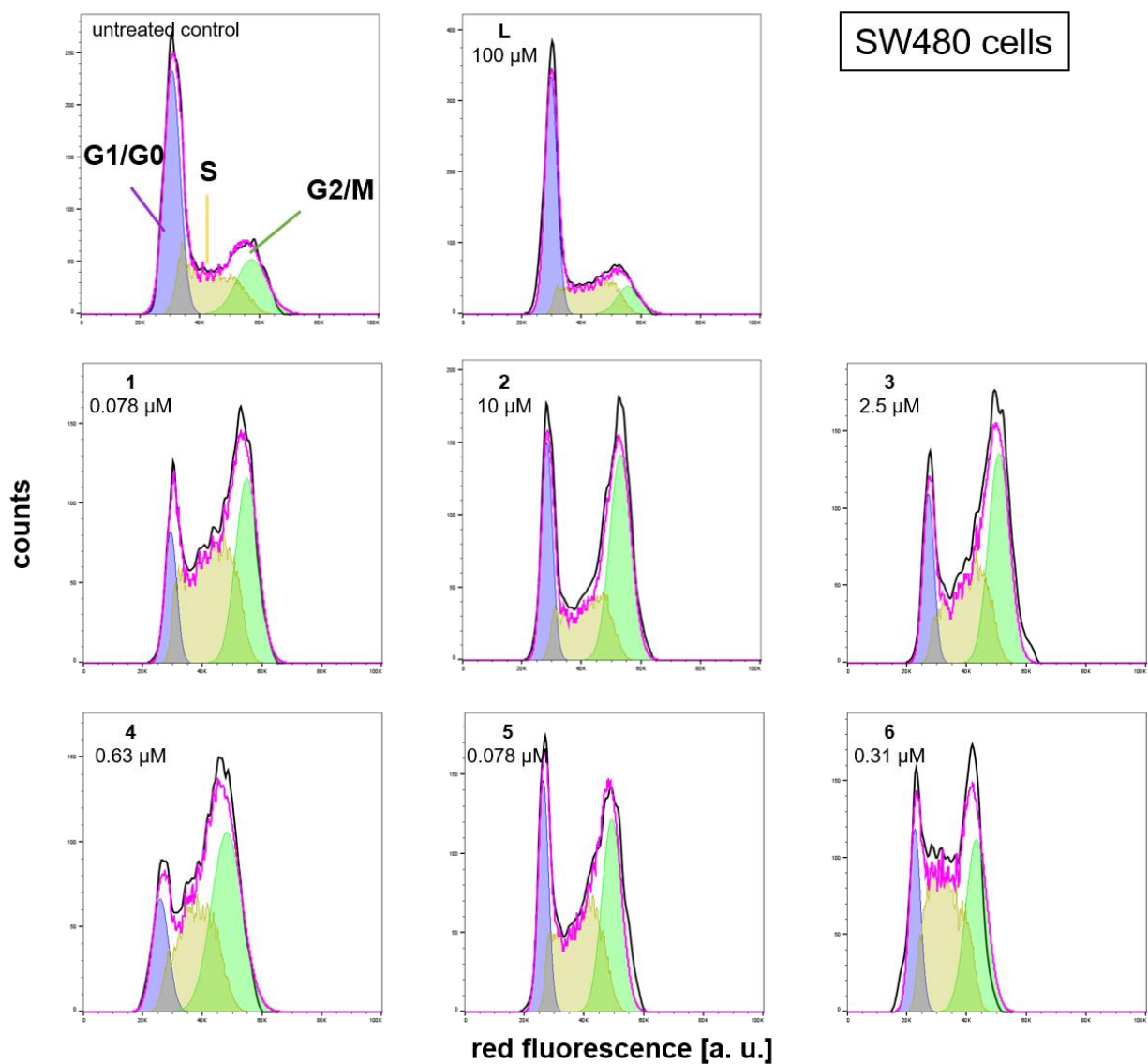


Figure S30: Histograms of cell cycle phase distribution in SW480 cells after 24 h drug exposure. The cells were exposed to L and complexes 1–6 in a subcytotoxic concentration (around IC_{50} values from 96 h MTT assays). The top left histogram represents an untreated control. All histograms are from one representative experiment out of at least three repetitions per tested concentration.

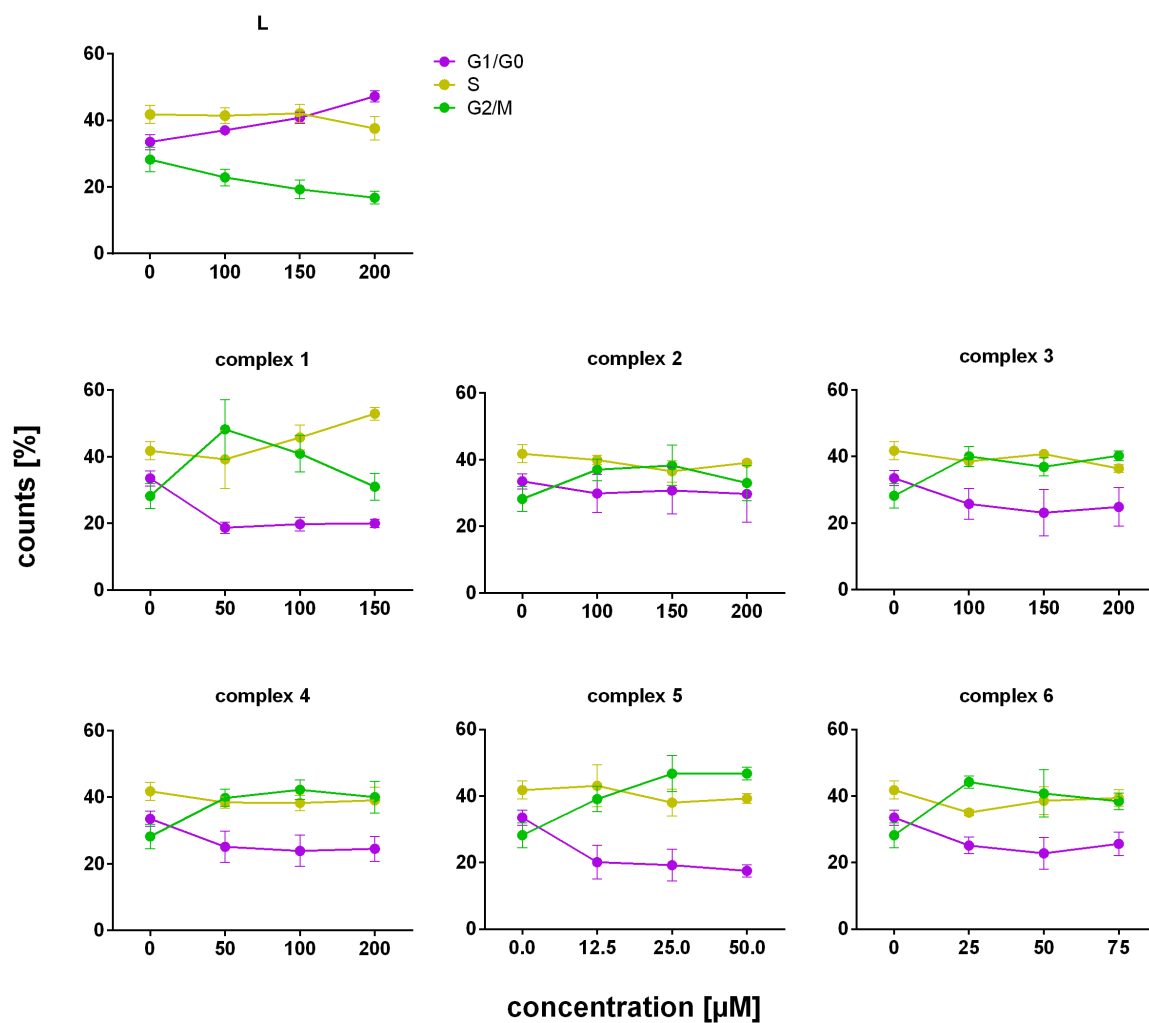


Figure S31: Impact of **L** and complexes **1–6** on cell cycle distribution in CH1/PA-1 cells. Graphs indicate the percentages (means $\pm$ SDs) of cells in G1/G0, S and G2/M phases of the cell cycle.

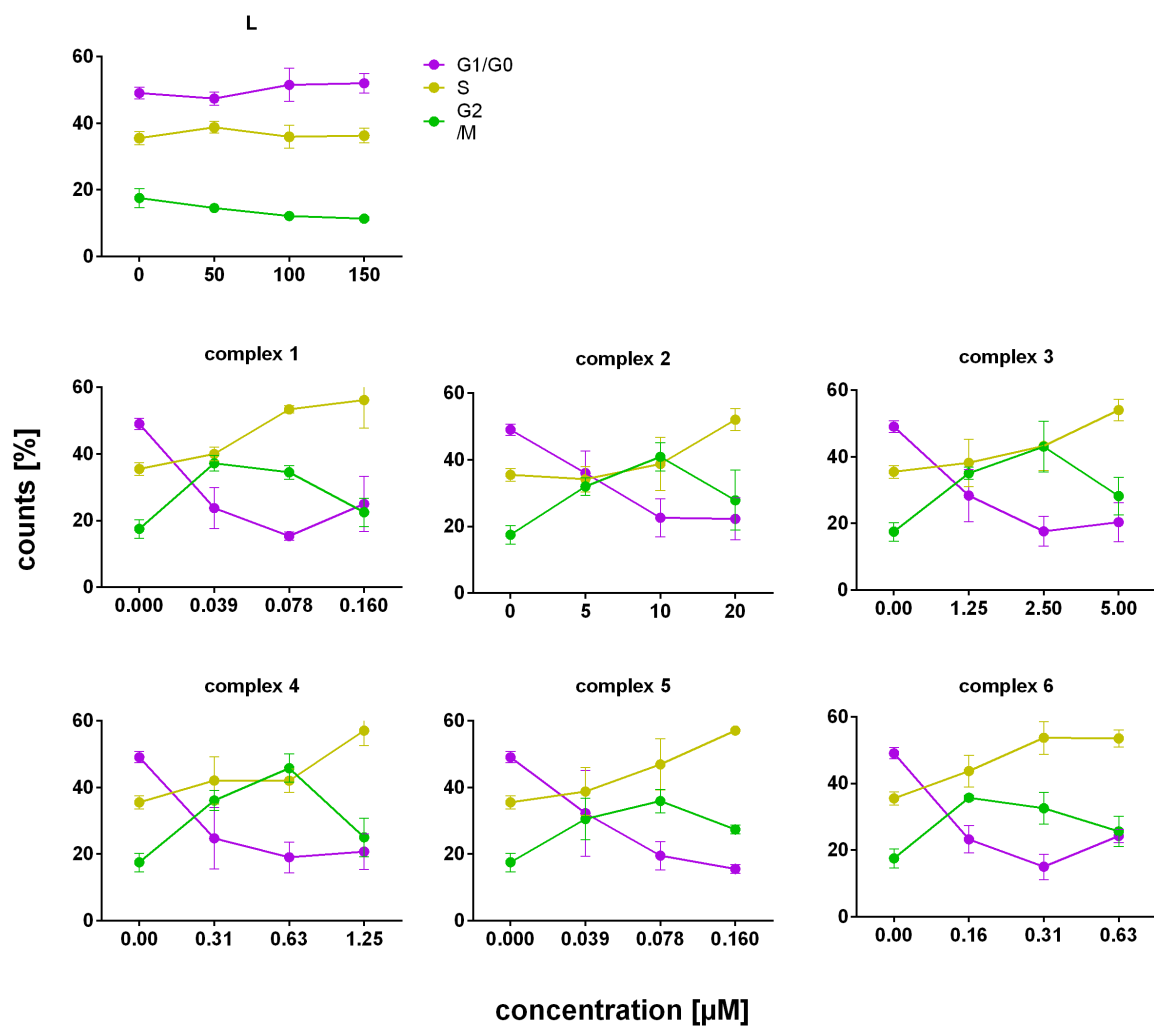


Figure S32: Impact of **L** and complexes **1–6** on cell cycle distribution in SW480 cells. Graphs indicate the percentages (means $\pm$ SDs) of cells in G1/G0, S and G2/M phases of the cell cycle.

Table S10: Cell cycle alterations in CH1/PA-1 cells treated with compounds **L** and **1–6** at three different concentrations (around the IC₅₀ values based on 96 h MTT assays). Data are mean percentages $\pm$ SDs of at least three experiments.

CH1/PA-1		Cell cycle phase		
Compound	Concentration [μ M]	G1/G0	S	G2/M
Control	0	33.5 $\pm$ 2.3	41.8 $\pm$ 2.7	28.2 $\pm$ 2.5
L	100	37.0 $\pm$ 0.3	41.4 $\pm$ 2.4	22.9 $\pm$ 2.8
	150	40.8 $\pm$ 1.7	42.1 $\pm$ 2.7	19.3 $\pm$ 1.9
	200	47.2 $\pm$ 1.7	37.6 $\pm$ 3.5	16.8 $\pm$ 8.9
1	50	18.7 $\pm$ 1.7	39.2 $\pm$ 8.7	48.2 $\pm$ 5.5
	100	19.8 $\pm$ 2.1	45.8 $\pm$ 3.8	40.9 $\pm$ 4.0
	150	20.0 $\pm$ 1.3	52.9 $\pm$ 1.9	31.0 $\pm$ 3.2
2	100	29.9 $\pm$ 5.7	39.9 $\pm$ 1.4	37.0 $\pm$ 6.1
	150	30.8 $\pm$ 7.1	36.5 $\pm$ 3.3	38.3 $\pm$ 5.3
	200	29.7 $\pm$ 8.5	39.1 $\pm$ 0.8	33.0 $\pm$ 3.0
3	100	25.8 $\pm$ 4.6	38.6 $\pm$ 1.5	40.1 $\pm$ 2.7
	150	23.1 $\pm$ 7.0	40.8 $\pm$ 0.6	36.9 $\pm$ 1.5
	200	24.9 $\pm$ 5.8	36.4 $\pm$ 1.2	40.3 $\pm$ 2.6
4	50	25.1 $\pm$ 4.7	38.4 $\pm$ 1.7	39.8 $\pm$ 2.9
	100	23.9 $\pm$ 4.7	38.3 $\pm$ 2.3	42.2 $\pm$ 4.7
	200	24.5 $\pm$ 3.8	39.1 $\pm$ 3.9	40.0 $\pm$ 3.8
5	12.5	20.1 $\pm$ 5.0	43.1 $\pm$ 6.3	39.1 $\pm$ 5.4
	25	19.2 $\pm$ 4.8	38.0 $\pm$ 4.0	46.7 $\pm$ 1.9
	50	17.5 $\pm$ 1.8	39.3 $\pm$ 1.4	46.8 $\pm$ 1.8
6	25	25.1 $\pm$ 2.5	35.0 $\pm$ 0.9	44.2 $\pm$ 7.1
	50	22.8 $\pm$ 4.7	38.6 $\pm$ 4.2	40.8 $\pm$ 2.5
	75	25.6 $\pm$ 3.5	39.4 $\pm$ 2.5	38.4 $\pm$ 3.7

Table S11: Cell cycle alterations in SW480 cells treated with compounds **L** and **1–6** at three different concentrations (around the IC₅₀ values based on 96 h MTT assays). Data are mean percentages $\pm$ SDs of at least three experiments.

SW480		Cell cycle phase		
Compound	Concentration [μ M]	G1/G0	S	G2/M
Control	0	49.1 $\pm$ 1.7	35.5 $\pm$ 1.9	17.5 $\pm$ 2.8
L	50	47.4 $\pm$ 2.0	38.8 $\pm$ 1.7	14.5 $\pm$ 0.5
	100	51.5 $\pm$ 5.0	35.9 $\pm$ 3.4	12.1 $\pm$ 0.4
	150	52.0 $\pm$ 2.9	36.3 $\pm$ 2.2	11.3 $\pm$ 0.5
1	0.039	23.8 $\pm$ 6.1	40.0 $\pm$ 2.1	37.2 $\pm$ 2.3
	0.078	15.4 $\pm$ 1.2	53.4 $\pm$ 1.1	34.5 $\pm$ 2.1
	0.16	25.0 $\pm$ 8.2	56.2 $\pm$ 8.4	22.5 $\pm$ 4.3
2	5	36.0 $\pm$ 6.7	34.2 $\pm$ 3.8	32.0 $\pm$ 2.6
	10	22.6 $\pm$ 5.7	38.8 $\pm$ 7.9	40.9 $\pm$ 4.2
	20	22.3 $\pm$ 6.3	52.1 $\pm$ 3.3	27.9 $\pm$ 9.0
3	1.25	28.4 $\pm$ 7.8	38.2 $\pm$ 7.1	35.1 $\pm$ 1.8
	2.5	17.6 $\pm$ 4.5	43.3 $\pm$ 7.4	43.1 $\pm$ 7.6
	5	20.4 $\pm$ 5.9	54.1 $\pm$ 3.2	28.2 $\pm$ 5.6
4	0.31	24.7 $\pm$ 9.2	42.1 $\pm$ 7.1	36.1 $\pm$ 3.0
	0.63	19.0 $\pm$ 4.6	42.0 $\pm$ 3.5	45.8 $\pm$ 4.2
	1.25	20.7 $\pm$ 5.3	57.1 $\pm$ 4.5	25.0 $\pm$ 5.8
5	0.039	32.3 $\pm$ 12.9	38.8 $\pm$ 7.2	30.5 $\pm$ 6.2
	0.078	19.5 $\pm$ 4.3	46.9 $\pm$ 7.7	35.9 $\pm$ 3.5
	0.16	15.5 $\pm$ 1.3	57.1 $\pm$ 0.1	27.4 $\pm$ 1.3
6	0.16	23.2 $\pm$ 4.1	43.7 $\pm$ 4.8	35.7 $\pm$ 0.7
	0.31	15.0 $\pm$ 3.8	53.7 $\pm$ 4.9	32.6 $\pm$ 4.7
	0.63	24.2 $\pm$ 2.0	53.5 $\pm$ 2.6	25.6 $\pm$ 4.5

Apoptosis assay

Table S12: Apoptosis and necrosis induction, examined by annexin V-FITC/PI staining. SW480 colon cancer cells were exposed to compounds **L** and **1–6** at the indicated concentrations for 24 h and cell populations were analyzed by flow cytometry. Results are mean percentages $\pm$ standard deviations of at least three independent experiments.

Compound	Concentration (μ M)		mean $\pm$ SD
Control	0	necrotic cells [%]	2.0 $\pm$ 1.6
		late apoptotic cells [%]	3.5 $\pm$ 1.5
		early apoptotic cells [%]	2.9 $\pm$ 2.3
		viable cells [%]	92 $\pm$ 4
1	0.1	necrotic cells [%]	0.77 $\pm$ 0.33
		late apoptotic cells [%]	3.6 $\pm$ 2.6
		early apoptotic cells [%]	3.7 $\pm$ 3.1
		viable cells [%]	92 $\pm$ 6
	1	necrotic cells [%]	1.2 $\pm$ 0.8
		late apoptotic cells [%]	4.9 $\pm$ 2.8
		early apoptotic cells [%]	1.3 $\pm$ 0.5
		viable cells [%]	93 $\pm$ 4
	10	necrotic cells [%]	6.7 $\pm$ 3.1
		late apoptotic cells [%]	64 $\pm$ 6
		early apoptotic cells [%]	16 $\pm$ 1
		viable cells [%]	13 $\pm$ 3
2	1	necrotic cells [%]	1.4 $\pm$ 0.8
		late apoptotic cells [%]	2.0 $\pm$ 0.2
		early apoptotic cells [%]	1.1 $\pm$ 0.4
		viable cells [%]	95 $\pm$ 1
	10	necrotic cells [%]	1.5 $\pm$ 0.8
		late apoptotic cells [%]	2.0 $\pm$ 0.3
		early apoptotic cells [%]	1.0 $\pm$ 0.3
		viable cells [%]	96 $\pm$ 1
3	1	necrotic cells [%]	1.0 $\pm$ 0.5
		late apoptotic cells [%]	2.1 $\pm$ 0.3
		early apoptotic cells [%]	1.0 $\pm$ 0.7
		viable cells [%]	96 $\pm$ 1
	10	necrotic cells [%]	0.72 $\pm$ 0.49
		late apoptotic cells [%]	2.1 $\pm$ 0.7
		early apoptotic cells [%]	0.7 $\pm$ 0.3
		viable cells [%]	97 $\pm$ 1

4	0.1	necrotic cells [%]	1.7 ± 1.1
		late apoptotic cells [%]	2.1 ± 0.4
		early apoptotic cells [%]	1.1 ± 0.5
		viable cells [%]	95 ± 2
	1	necrotic cells [%]	0.93 ± 0.65
		late apoptotic cells [%]	2.4 ± 1.2
		early apoptotic cells [%]	1.1 ± 0.7
		viable cells [%]	96 ± 2
	10	necrotic cells [%]	3.4 ± 4.0
		late apoptotic cells [%]	8.3 ± 4.9
		early apoptotic cells [%]	1.0 ± 0.4
		viable cells [%]	87 ± 8
5	0.1	necrotic cells [%]	1.0 ± 0.5
		late apoptotic cells [%]	2.9 ± 1.5
		early apoptotic cells [%]	3.0 ± 2.2
		viable cells [%]	93 ± 4
	1	necrotic cells [%]	0.85 ± 0.48
		late apoptotic cells [%]	3.3 ± 1.3
		early apoptotic cells [%]	1.0 ± 0.5
		viable cells [%]	95 ± 2
	10	necrotic cells [%]	6.3 ± 2.2
		late apoptotic cells [%]	40 ± 6
		early apoptotic cells [%]	25 ± 7
		viable cells [%]	29 ± 11
6	0.1	necrotic cells [%]	17 ± 27
		late apoptotic cells [%]	4.7 ± 2.8
		early apoptotic cells [%]	1.0 ± 0.7
		viable cells [%]	77 ± 29
	1	necrotic cells [%]	1.0 ± 1.3
		late apoptotic cells [%]	2.0 ± 0.7
		early apoptotic cells [%]	1.1 ± 0.7
		viable cells [%]	96 ± 2
	10	necrotic cells [%]	0.94 ± 0.52
		late apoptotic cells [%]	3.7 ± 0.1
		early apoptotic cells [%]	1.1 ± 0.3
		viable cells [%]	94 ± 0
L	10	necrotic cells [%]	0.61 ± 0.44
		late apoptotic cells [%]	1.6 ± 0.2
		early apoptotic cells [%]	1.0 ± 0.5
		viable cells [%]	97 ± 1
	100	necrotic cells [%]	0.86 ± 0.33
		late apoptotic cells [%]	1.9 ± 0.2
		early apoptotic cells [%]	1.0 ± 0.2
		viable cells [%]	96 ± 0

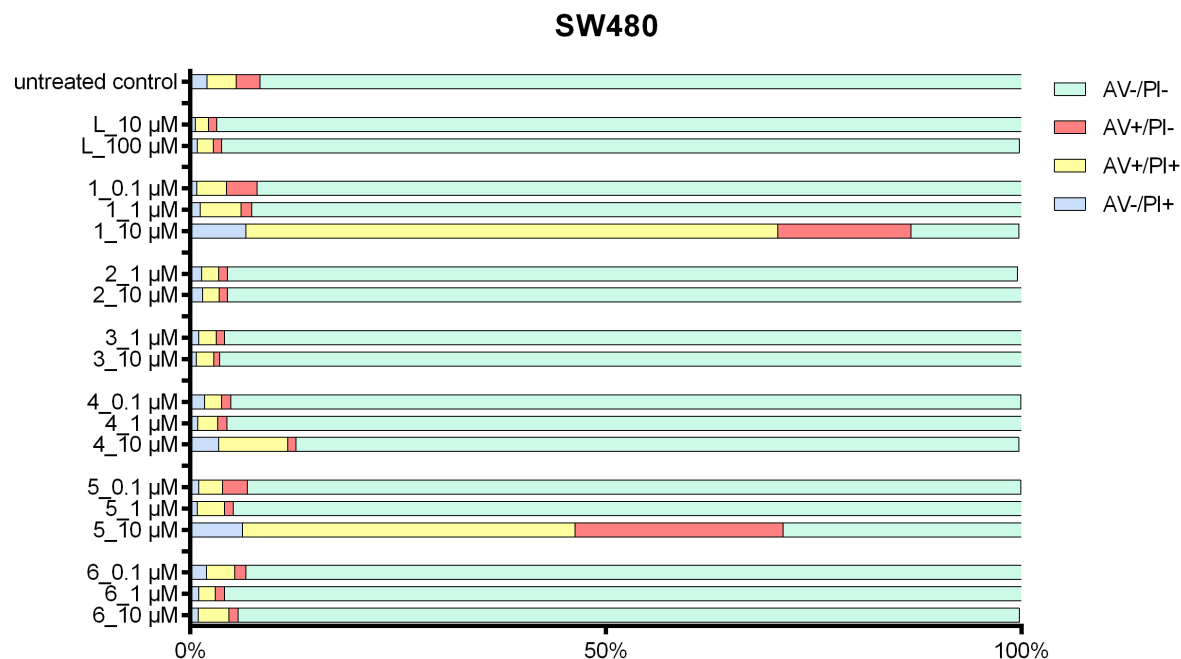


Figure S33: Flow-cytometric analysis of apoptosis/necrosis induction in SW480 cells incubated with different concentrations of **L** and **1–6** for 24 h. Percentages of necrotic (AV-/PI+), late apoptotic (AV+/PI+), early apoptotic (AV+/PI-) and viable (AV-/PI-) cells were determined by double-staining with annexin V-FITC/PI.

ROS assay

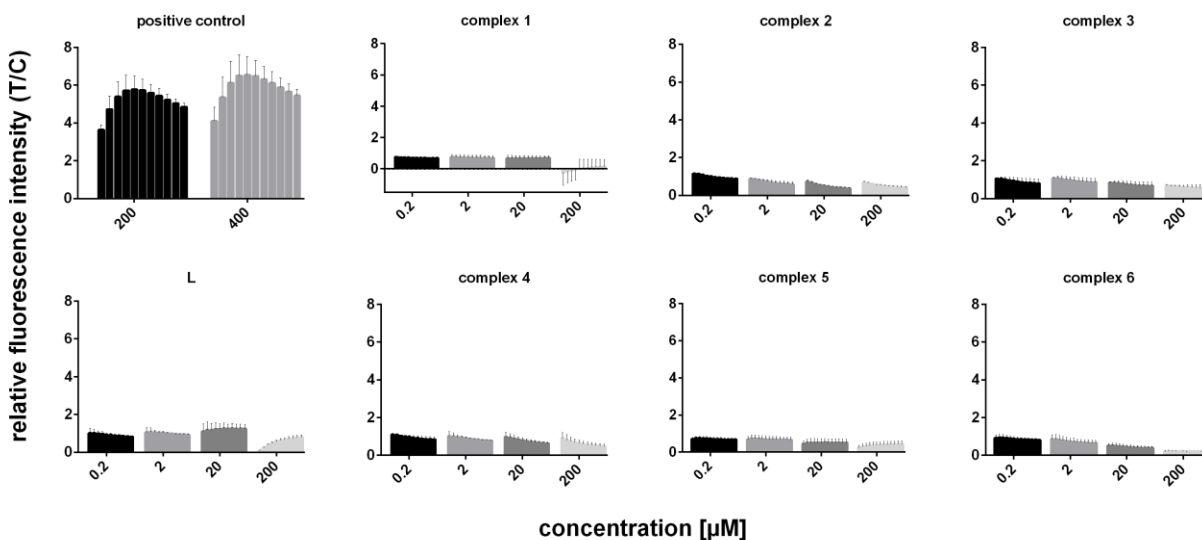


Figure S34: ROS induction in CH1/PA-1 cells by **L** and complexes **1–6** in four different (0.2–200 μM) concentrations and by a positive control (200 and 400 μM tert-butylhydroperoxide) over time for up to 2 h. The values are normalized to the untreated control.

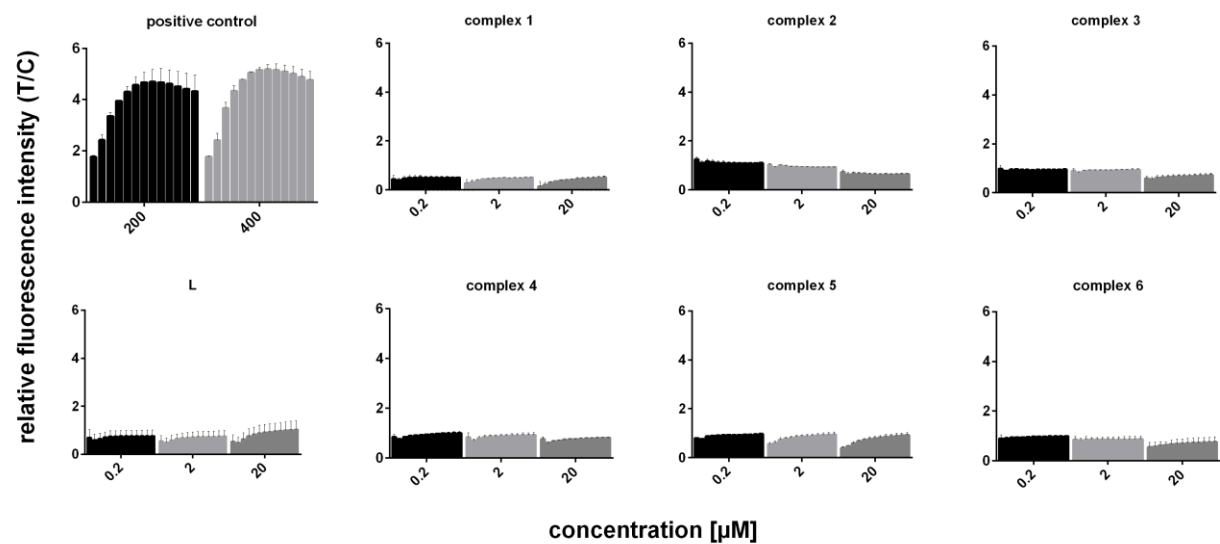


Figure S35: ROS induction in SW480 cells by **L** and complexes **1–6** in four different (0.2–20 μM) concentrations and by a positive control (200 and 400 μM tert-butylhydroperoxide) over time for up to 2 h. The values are normalized to the untreated control.

dsDNA plasmid assay

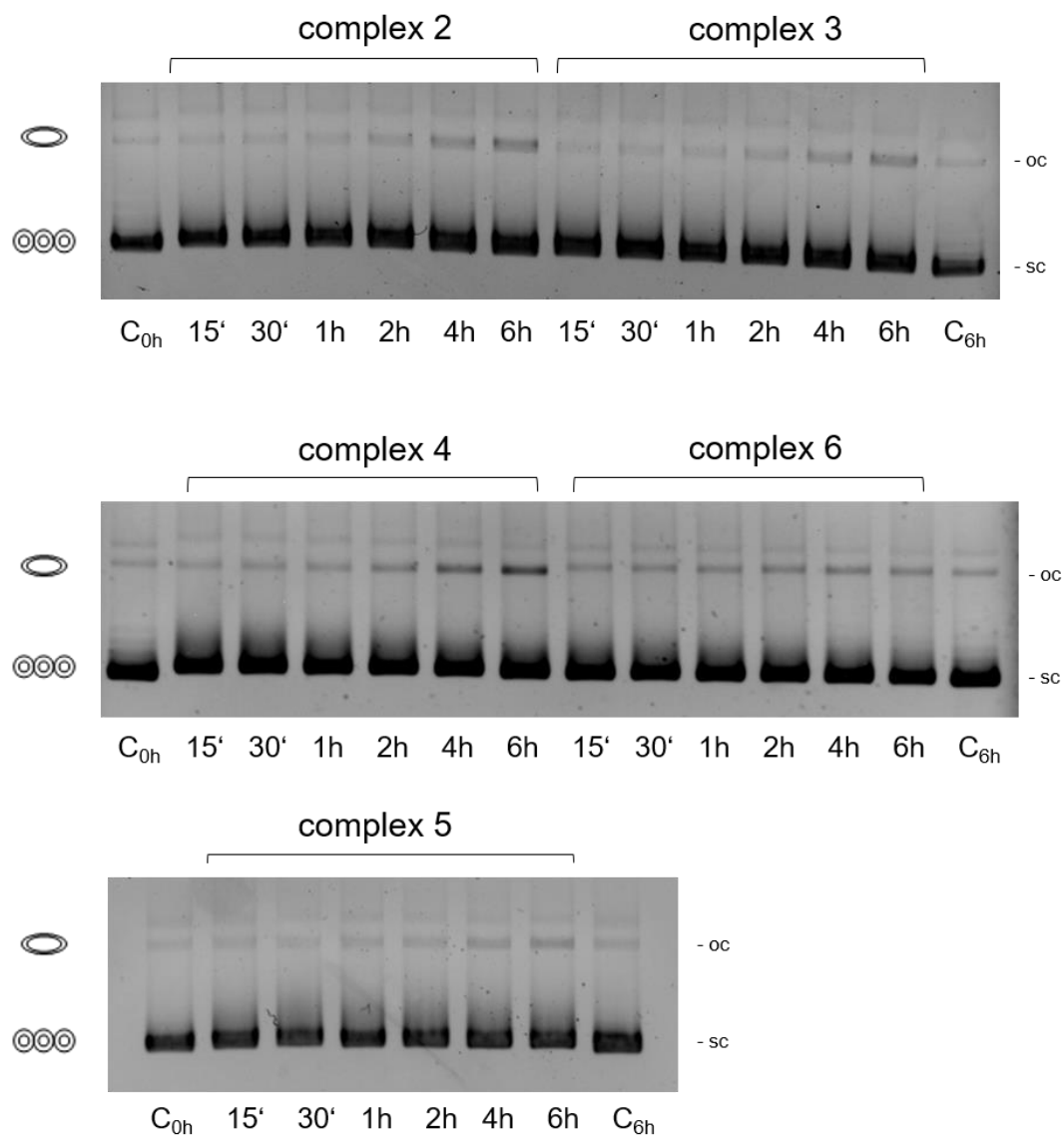


Figure S36: Electropherograms of dsDNA plasmid (pUC19) interactions with 50 μ M of compounds 2–6. The pUC19 plasmid was incubated with the complexes for different time periods (from 15 min to 6 h) at 37 °C and the different plasmid forms were separated by agarose gel electrophoresis. 'C_{0h}' corresponds to an untreated, non-preincubated control at the time point of gel loading, while 'C_{6h}' corresponds to an untreated, 6 h incubated control; oc, open circular; sc, supercoiled are forms of the plasmid DNA (Electropherograms of 1 and L were published previously [8] (as complex 2a and ligand 2 in Figure S79).

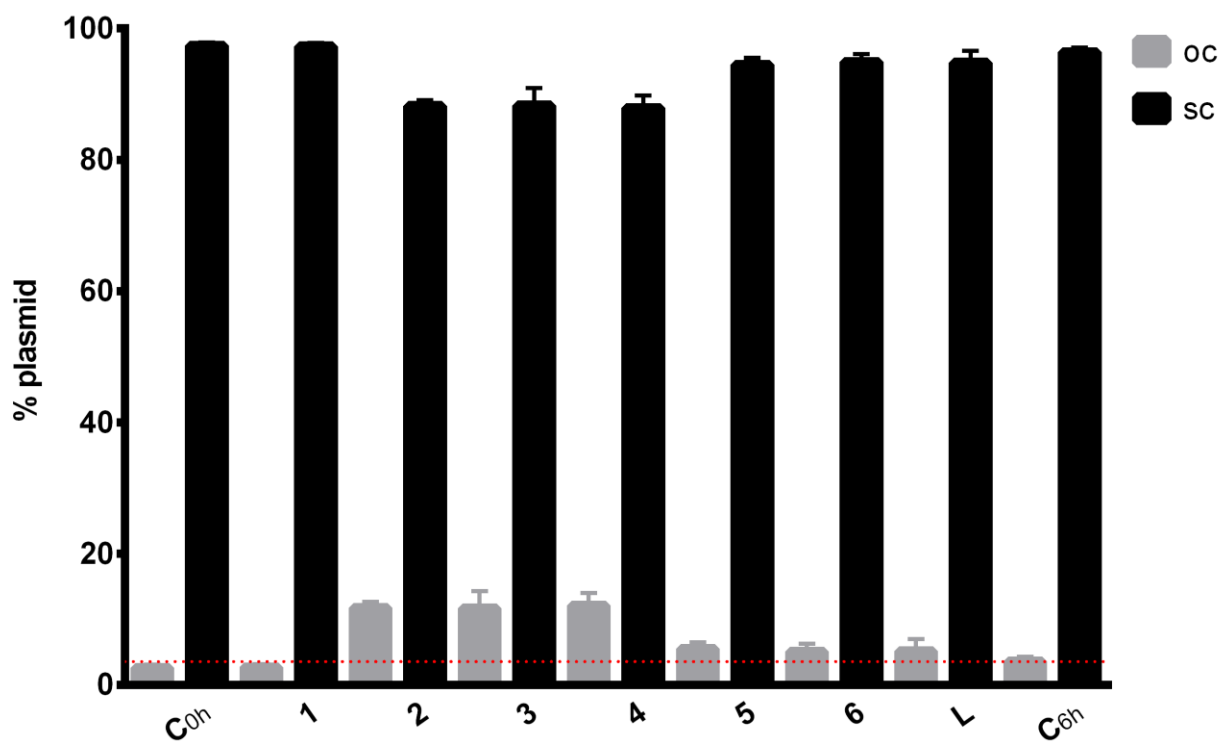


Figure S37: Quantified data from the electrophoretic plasmid DNA interaction studies shown in Figure S36. The grey columns correspond to the open-circular (oc) form and black columns to the supercoiled (sc) form of the pUC19 plasmid after 6 h of incubation and the controls (C0h and C6h). Data for complexes **1** and **L** are taken from [8].

NQO1 activity assay

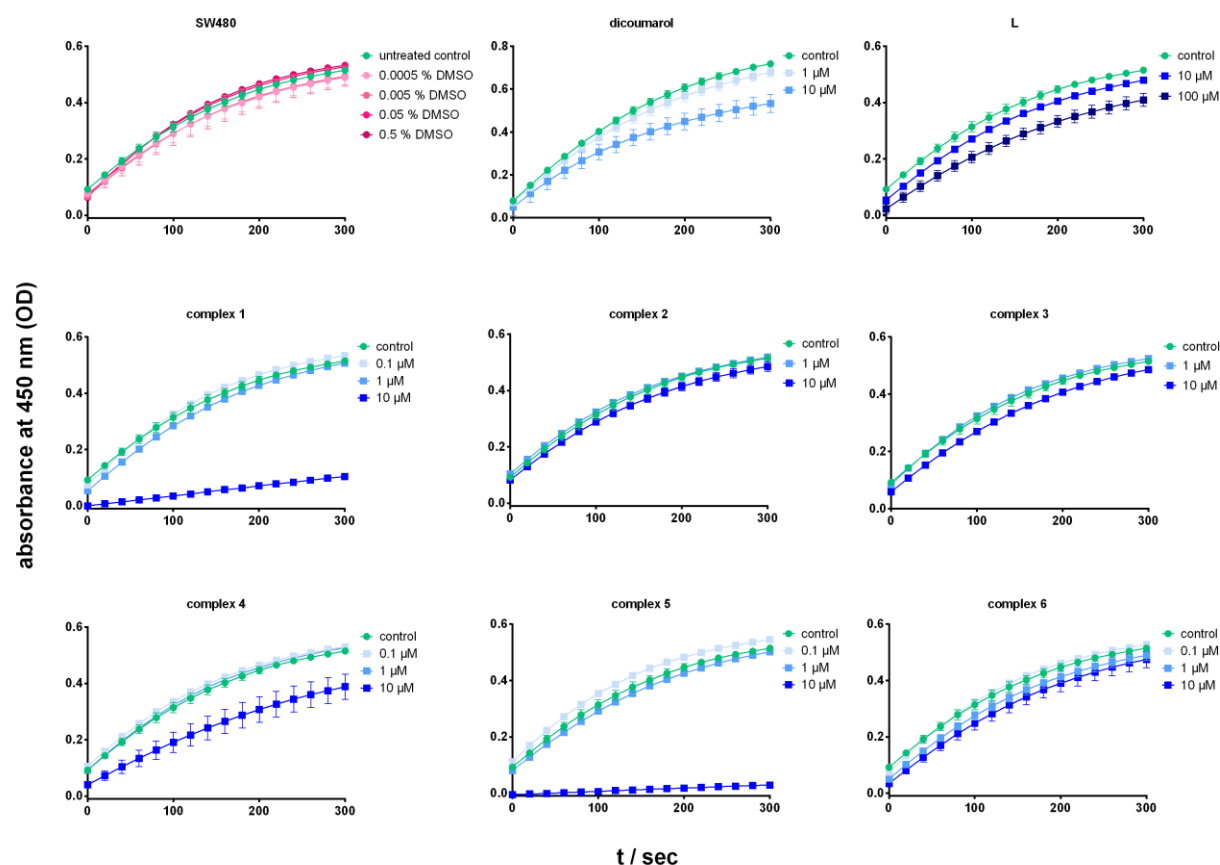


Figure S38: Time-dependent impact on NQO1 activity in SW480 cell extracts after treatment with compounds L and 1–6 or dicoumarol (positive control) compared to negative controls with and without DMSO (concentrations corresponding to the content in solutions of the test compounds). Data are expressed as OD (450 nm) per 100 μ g/mL of total cellular protein.

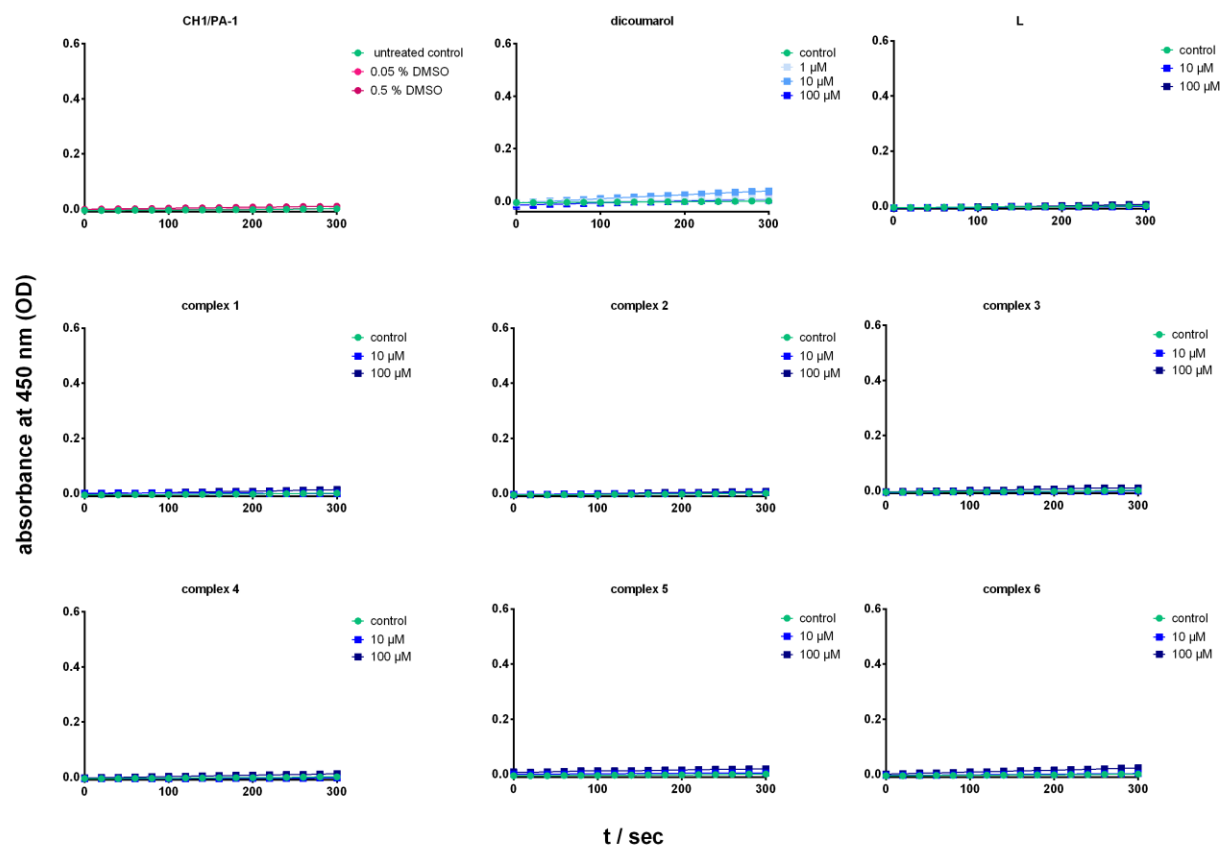


Figure S39: Time-dependent impact on NQO1 activity in CH1/PA-1 cell extracts after treatment with compounds L and 1–6 or dicoumarol (positive control) compared to negative controls with and without DMSO (concentrations corresponding to the content in solutions of the test compounds). Data are expressed as OD (450 nm) per 100 $\mu\text{g/mL}$ of total cellular protein.

References

1. AXS, B. *Bruker SAINT* v.838B Copyright(C); Bruker AXS, 2005-2019.
2. Sheldrick, G.M. *Sadabs. University of Göttingen, Germany* **1996**.
3. Dolomanov, O.V.; Bourhis, L.J.; Gildea, R.J.; Howard, J.A.K.; Puschmann, H. OLEX2: a complete structure solution, refinement and analysis program. *Journal of Applied Crystallography* **2009**, *42*, 339-341, doi:doi:10.1107/S0021889808042726.
4. Hubschle, C.B.; Sheldrick, G.M.; Dittrich, B. ShelXle: a Qt graphical user interface for SHELXL. *Journal of Applied Crystallography* **2011**, *44*, 1281-1284, doi:doi:10.1107/S0021889811043202.
5. Sheldrick, G. SHELXS v 2016/4. **2015**.
6. Sheldrick, G. Crystal structure refinement with SHELXL. *Acta Crystallographica Section C* **2015**, *71*, 3-8, doi:doi:10.1107/S2053229614024218.
7. Spek, A. Structure validation in chemical crystallography. *Acta Crystallographica Section D* **2009**, *65*, 148-155, doi:doi:10.1107/S090744490804362X.
8. Geisler, H.; Westermayr, J.; Cseh, K.; Wenisch, D.; Fuchs, V.; Harringer, S.; Plutzar, S.; Gajic, N.; Hejl, M.; Jakupc, M.A.; et al. Tridentate 3-Substituted Naphthoquinone Ruthenium Arene Complexes: Synthesis, Characterization, Aqueous Behavior, and Theoretical and Biological Studies. *Inorg. Chem.* **2021**, *60*, 9805-9819, doi:10.1021/acs.inorgchem.1c01083.



Article

Anticancer Tungstenocenes with a Diverse Set of (O,O–), (O,S–) and (O,N–) Chelates—A Detailed Biological Study Using an Improved Evaluation via 3D Spheroid Models

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Abstract: The synthesis, characterization and biological activity of tungstenocenes with varying biologically active (O,O–), (S,O–) and (N,O–) chelates are described. Complexes were characterized by ¹H and ¹³C NMR, elemental analysis, ESI-mass spectrometry, FT-IR spectroscopy and X-ray diffraction analysis. The aqueous stability was studied by UV/Vis spectroscopy and the W^{IV} to W^V process by cyclic voltammetry. The cytotoxicity was determined by the MTT assay in A549, CH1/PA-1 and SW480 cancer cells as well as in IMR-90 human fibroblasts. Extensive biological evaluation was performed in three other human cancer cell lines (HCT116, HT29 and MCF-7) in monolayer and multicellular tumor spheroid cultures to better understand the mode of action. Lead compounds showed promising in vitro anticancer activity in all cancer cell lines. Further studies yielded important insights into apoptosis induction, ROS generation, different patterns in metal distribution (detected by LA-ICP-TOF-MS), changes in Ki67 (proliferation marker) expression and DNA interactions. The results based on qualitative and quantitative research designs show that complexes containing (S,O–) chelates are more active than their (O,O–) and (N,O–) counterparts. The most striking results in spheroid models are the high antiproliferative capacity and the different distribution pattern of two complexes differing only in a W–S or W–O bond.

Keywords: metal-based drugs; tungstenocenes; anticancer; 3D cell culture; multicellular tumor spheroids



Citation: Cseh, K.; Berasaluce, I.; Fuchs, V.; Banc, A.; Schweikert, A.; Prado-Roller, A.; Hejl, M.; Wernitznig, D.; Koellensperger, G.; Jakupec, M.A.; et al. Anticancer Tungstenocenes with a Diverse Set of (O,O–), (O,S–) and (O,N–) Chelates—A Detailed Biological Study Using an Improved Evaluation via 3D Spheroid Models. *Pharmaceutics* **2023**, *15*, 1875. <https://doi.org/10.3390/pharmaceutics15071875>

Academic Editor: Yasumasa Ikeda

Received: 10 April 2023

Revised: 15 June 2023

Accepted: 25 June 2023

Published: 3 July 2023



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1. Introduction

Over the past 30 years, the field of bioorganometallic chemistry with advances in catalytic and medicinal applications has risen in importance. A major focus currently is the search for potential cancer drugs with fewer and less severe side effects than those currently employed [1–3]. Our work focuses on group 6 metal complexes containing molybdenum, the only second row transition metal necessary for life, and its heavier analogue tungsten. Molybdenum is easily transported within and excreted from the human body as molybdate. Both excess molybdenum and tungsten are removed in the urine and should be generally less toxic than other metals [4,5]. The group 6 ‘bent’ metallocenes allow for additional ligands to be bound to the metal center and result in a decrease in the angle between the Cp rings. The molybdenocenes and tungstenocenes, therefore, bear a *cis*-dihalido motif like cisplatin, the initial reason for their interest in anticancer research [1]. By replacing

the halides with bioactive chelating ligands, we aim to synthesize anticancer agents which are more tolerable, stable, and ideally would show synergistic effects between the metal and ligand.

Köpf and Köpf–Maier first described the antitumor activity of several metal-based organometallic complexes containing titanium, vanadium, niobium, molybdenum, tungsten and rhenium in 1979 [6–9]. Around two decades later, several research groups reported anticancer activities for neutral and charged metallocene derived compounds of these metals [10–15]. However, tungstenocenes were abandoned due to their lower activity and stability when compared to the other metallocenes. Anticancer research with tungstenocenes, therefore, is very rare; in fact, the only published work in this area is from the Köpf/Köpf–Maier and Meléndez groups [8,16].

While titanocene dichloride exhibited the highest cytotoxic activity in several tumor cell lines, it also showed negative side effects like cisplatin and was abandoned in clinical phase II [17,18]. Molybdenocene dichloride has a unique and potentially useful stability of the metal-cyclopentadienyl bonds under physiological conditions [19]; however, the same stability is not observed in the tungstenocenes. All metallocenes readily lose the chlorido ligands and form aqua, hydroxido or even oxo-bridged dimeric species in aqueous solutions [19,20]. The exchange of the dichlorido motif for chelates to increase stability as well as solubility has been a popular approach explored by various research groups [16,21,22]. The cytotoxic activity of Cp_2WCl_2 bearing 3-hydroxy-4-pyrone ligands was reported by Meléndez and coworkers in 2013 (Figure 1), showing an improved in vitro cytotoxicity of tungstenocenes bearing (O,O-) donor ligands, when compared to Cp_2WCl_2 , in HT29 colon cancer and MCF-7 breast cancer cell lines [16].

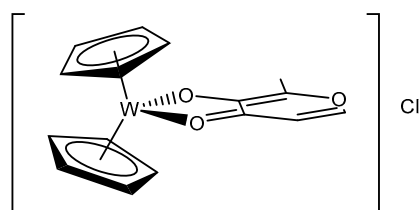


Figure 1. $[\text{Cp}_2\text{W}(\text{maltolato})]\text{Cl}$ complex synthesized by Meléndez and coworkers [16].

Due to the increased stability of the molybdenocenes they have been studied to a greater degree than the more unstable tungsten compounds, the former showing promising in vitro anticancer activity in many different cell lines [21–23]. Nevertheless, with the correct choice of chelating ligands (containing softer sulfur donors) an improved stability of the tungstenocenes can be attained, making this class of compounds more interesting, especially if they exhibit activity against cancer cells.

This paper presents the synthesis, characterization and biological studies of ten tungstenocenes containing an (O,O-), (S,O-) or (N,O-) chelate. A comparison of their cytotoxic activities with previous research and preliminary mode-of-action studies are presented. The bidentate ligand scaffolds were coordinated to the already cytotoxic tungstenocene dichloride, enhancing the cytotoxic activity further as well as increasing the stability in aqueous media [16]. The impact on the aqueous stability of the W–S vs. W–O bonds and counter ion exchange from chloride to hexafluorophosphate is discussed. Previous studies from our research group reported that attempts to synthesize molybdenocene derivatives with chloride as counter ion did not yield any product; however, exchanging the chloride for PF_6^- (hexafluorophosphate anion) allowed for the successful isolation of the products [21]. This anion exchange allowed for a more straightforward purification of the complexes, avoiding size-exclusion column chromatography and resulted in improved yields.

A broad range of ligand scaffolds were utilized in this work, including thiol derivatives of 3-hydroxy-4-pyrones, demonstrated to improve the stability as well as the cytotoxic activity in vitro [21,24]. We have made use of maltol (3-hydroxy-2-methyl-4-(1H)-pyrone), a natural product which is commonly utilized as a ligand with a broad range of biologi-

cally relevant metals because of its potentially beneficial effect on bioavailability [25–30]. Allomaltol and thioallomaltol are interesting since we have shown that this small change in the methyl group from the 3-position in maltol and thiomaltol to the 5-position has a significant effect on the biological activity of the resultant complexes [31]. Ethylmaltol, already employed by Meléndez and coworkers [16] as a ligand for tungstenocene, was also used for comparison purposes as well as its thioethylmaltol analogue. Besides pyrones, flavonoids also possess antioxidant and antiproliferative properties [32], and their thiol derivatives have also been employed as ligands in our work [31]. Picolinic acid has demonstrated many positive effects in the human body, such as immunological, neuroprotective and antiproliferative effects [33], while deferiprone has been shown to selectively kill tumor cells over normal cells [34]. We attempted to determine which chelating motif is most promising as well as to identify structure-activity relationships for the maltol-based complexes.

2. Materials and Methods

2.1. Experimental Part

All utilized solvents, purchased from commercial sources, were of HPLC grade and used without any further purification, except for methanol, which was dried over molecular sieves (3 Å) prior to use [35]. Bis(cyclopentadienyl)tungsten dichloride (99%, Abcr, Karlsruhe, Germany), sodium methoxide (~95%, Fluka), ammonium hexafluorophosphate (99%, Sigma-Aldrich, Vienna, Austria), Lawesson's reagent (99%, Acros Organics, Geel, Belgium), PBS (sterile filtered, Sigma Life Science, Vienna, Austria), maltol (**L1**) (99%, Sigma-Aldrich), picolinic acid (**L5**) (99%, Sigma-Aldrich), deferiprone (**L6**) (98%, Sigma-Aldrich) and ethylmaltol (**L9**) (99%, Sigma-Aldrich) were purchased from the respective commercial source and used as obtained. Thiomaltol (**L2**) [36], 3-hydroxy-2-(4'-chlorophenyl)chromen-4-one (**L3**) [37] and allomaltol [26] were synthesized according to the literature procedures and 3-hydroxy-2-(4'-chlorophenyl)-chromen-4-thione (**L4**), thioallomaltol (**L8**) [38], thioethylmaltol (**L10**) [39] were synthesized using Lawesson's reagent [36] (see Supporting Information for synthesis and characterization). The synthesis of Bis(η^5 -cyclopentadienyl)[2-methyl-3-(oxo- κ O)-4-(1*H*)-pyron-4-ato- κ O]tungsten(IV) chloride (**1A**) was modified compared with the one in the literature [16].

NMR spectra were recorded with a Bruker FT-NMR Avance IIITM 500 MHz spectrometer at 500.10 (¹H), and 125.75 MHz (¹³C) using DMSO-*d*₆. 2D-NMR measurements were recorded utilizing standard pulse programs COSY, HSQC and HMBC. The Microanalytical Laboratory of the University of Vienna performed the elemental analyses with a Eurovector EA 3000(2009) equipped with a high temperature pyrolysis furnace (HT, Hekatech, Germany, 2009). Elemental analyses' samples were weighed (weights from 1–3 mg were used) on a Sartorius SEC 2 ultra-micro balance with ± 0.1 μ g resolution. Two NIST-certified reference materials were used for calibration: sulfanilamide (C₆H₈N₂O₂S) and BBOT (2,5-bis-(5-tert-butyl-2-benzoxazol-2-yl)-thiophenone, C₂₆H₂₆N₂O₂S). The limit of quantification (LOQ) was 0.05 w% for C, H, N and 0.02 w% for S. For samples without N and/or S, the content of these elements was determined and verified to be below the LOQ. X-ray diffraction analyses were carried out on a Bruker X8 APEX II CCD diffractometer at 100 K. ESI-MS were recorded on a Bruker AmaZon SL ion trap mass spectrometer (Bruker Daltonics GmbH). Data were obtained and processed with Compass 1.3 and Data Analysis 4.0 (Bruker Daltonics GmbH). Infrared spectra were attained on a Bruker Vertex 70 FT-IR-spectrometer with an ATR-unit (attenuated total reflection unit) in the range of 4000–600 cm⁻¹. Intensities of the reported bands are described with w for weak, m for medium and s for strong; broad signals are additionally specified with the letter b in front of these abbreviations. Cyclic voltammograms (CVs) were measured in a three-electrode cell using 2 mm diameter glassy carbon disk working electrode, a platinum auxiliary electrode and an Ag|Ag⁺ reference electrode containing 0.1 M AgNO₃. Measurements were carried out at room temperature using an EG&G PARC potentiostat/galvanostat 273A. The solutions were deoxygenized by purging a stream of argon for 3 min through the solution and the experiment was performed under an argon atmosphere. The potentials

were measured in a freshly prepared solution of (*n* Bu₄N)[BF₄] (0.1 M) in acetonitrile using ferrocene (Fe(η^5 -C₅H₅)₂) ($E_{1/2}$ = +0.72 V vs. NHE) [40] as an internal standard and are quoted relative to the normal hydrogen electrode (NHE).

Stock solutions in DMSO of compounds 1–10 were utilized to determine the solubility in PBS by adding 1 vol% of the stock solution to a suitable amount of PBS. The solubility in aqueous media of compounds 1–10 was sufficient (0.01–1.20 mg/mL) for biological experiments. UV/Vis data were recorded on a Perkin Elmer Lambda 650 UV/Vis Spectrophotometer with a Peltier element for temperature control.

2.2. Synthesis

2.2.1. General Complexation Synthesis A

Bis(cyclopentadienyl)tungsten dichloride (1 eq.), ligand (1–1.1 eq.) and sodium methoxide (1.1 eq.) were dissolved in 20–25 mL dry methanol (over molecular sieves 3Å) and stirred for 4–72 h at room temperature or reflux under argon. After any solid impurities were removed by filtration, ammonium hexafluorophosphate (1.1–4.5 eq.) was added and stirred for a further 2–4 h. The formed precipitate was filtered, washed with methanol, for some samples taken up into dichloromethane to filter off impurities and, subsequently, the solvent was removed and then dried in vacuo.

2.2.2. General Complexation Synthesis B

Ligand (1.2 eq.) and sodium methoxide (1.4 eq.) were dissolved in 24 mL dry methanol (dried over molecular sieves 3Å) and stirred at room temperature under argon for 10 min. In a separate Schlenk flask, bis(cyclopentadienyl)tungsten dichloride (130 mg, 0.33 mmol, 1 eq.) was dissolved in dry dichloromethane (8 mL), the prior solution was added via cannula and the resulting mixture was stirred at room temperature for 20 h. Sodiumhexafluorophosphate (2 eq.) was added, and the mixture was stirred for another 2 h.

Bis(η^5 -cyclopentadienyl)[2-methyl-3-(oxo- κ O)-4-(1H)-pyran-4-ato- κ O]tungsten(IV) hexafluorophosphate (1)

The synthesis was performed according to the general procedure A using bis(cyclopentadienyl)tungsten dichloride (135 mg, 350 μ mol), 3-hydroxy-2-methyl-4H-pyran-4-one (48 mg, 385 μ mol) and sodium methoxide (21 mg, 385 μ mol) with a reaction time of 16 h. Ammonium hexafluorophosphate (63 mg, 385 μ mol) was added and stirred for 2 h. The formed precipitate was filtered and washed with methanol. Yield: 119 mg (58%), brown powder. Solubility in PBS (1% DMSO): 0.23 mg/mL. ¹H-NMR (DMSO-*d*₆) δ = 2.35 (s, H, CH₃) 5.90 (s, 10H, Cp), 7.03 (d, ³J(H, H) = 5.0 Hz, H, C5-H), 8.34 (d, ³J(H, H) = 5.0 Hz, H, C6-H) ppm. ¹³C-NMR (DMSO-*d*₆) δ = 14.5 (CH₃), 98.1 (Cp), 110.4 (C5), 156.6 (C6), 157.5 (C2), 161.5 (C3), 187.0 (C4=O) ppm. Elemental analysis: Calculated for C₁₆H₁₅F₆O₃PW: C, 32.90; H, 2.59; P, 5.30%. Found: C, 32.79; H, 2.63; P, 5.07%. (ESI⁺) *m/z*: 439.05 [Cp₂W(maltolate)]⁺. IR: ν = 3128 w (ν_{C-H} , Cp), 1604, 1547 m ($\nu_{C=O}$), 1476, 1429 m ($\nu_{C=C}$), 820 s (ν_{C-H}) cm^{−1}.

Bis(η^5 -cyclopentadienyl)[2-methyl-3-(oxo- κ O)-4-(1H)-pyran-4-ato- κ O]tungsten(IV) chloride (1A)

Bis(cyclopentadienyl)tungsten dichloride (128 mg, 330 μ mol), 3-hydroxy-2-methyl-4H-pyran-4-one (42 mg, 330 μ mol) and sodium methoxide (18 mg, 330 μ mol) were dissolved in 20 mL methanol (dried over molecular sieves 3Å) and stirred for 16 h at room temperature under an argon atmosphere. The solvent was removed in vacuo and the remaining solids were dissolved in dichloromethane. Afterwards, the mixture was filtered leaving a blue insoluble precipitate behind. Dichloromethane was removed under reduced pressure and the product was washed several times with THF, followed by diethyl ether. Yield: 60 mg (42%), brown powder. Solubility in PBS (1% DMSO): 5.00 mg/mL. Elemental analysis: Calculated for C₁₆H₁₅ClO₃W·0.75H₂O: C, 39.37; H, 3.41%. Found: C, 39.48; H, 3.68%. (ESI⁺) *m/z*: 439.03 [Cp₂W(maltolate)]⁺.

Bis(η^5 -cyclopentadienyl)[2-methyl-3-(oxo- κ O)-4-(1H)-pyran-4-thionato- κ S]tungsten(IV) hexafluorophosphate (2)

The synthesis was performed according to the general procedure A using bis(cyclopentadienyl)tungsten dichloride (135 mg, 350 μ mol), 3-hydroxy-2-methyl-4H-pyran-4-thione (50 mg, 350 μ mol) and sodium methoxide (19 mg, 350 μ mol) with a reaction time of 48 h. Ammonium hexafluorophosphate (118 mg, 700 μ mol) was added and stirred for 3 h. The formed precipitate was filtered and washed with methanol. Yield: 108 mg (52%), red powder. Solubility in PBS (1% DMSO): 0.25 mg/mL. $^1\text{H-NMR}$ (DMSO- d_6) δ = 2.34 (s, H, CH₃) 5.70 (s, 10H, Cp), 7.83 (d, $^3J(\text{H}, \text{H})$ = 4.6 Hz, H, C5-H), 8.21 (d, $^3J(\text{H}, \text{H})$ = 4.6 Hz, H, C6-H) ppm. $^{13}\text{C-NMR}$ (DMSO- d_6) δ = 15.3 (CH₃), 96.3 (Cp), 119.4 (C5), 149.7 (C6), 156.7 (C2), 171.4 (C3), 181.5 (C4=S) ppm. Elemental analysis: Calculated for C₁₆H₁₅F₆O₂PSW: C, 32.02; H, 2.52, S, 5.34%. Found: C, 32.28; H, 2.45; S, 5.01%. (ESI⁺) m/z : 455.17 [Cp₂W(thiomaltolate)]⁺. IR: ν = 3124 m ($\nu_{\text{C-H}}$, Cp), 1583, 1505 m ($\nu_{\text{C=O}}$), 1470, 1424 m ($\nu_{\text{C=C}}$), 822 s ($\nu_{\text{C-H}}$) cm⁻¹.

Bis(η^5 -cyclopentadienyl)[2-(4-chlorophenyl)-3-(oxo- κ O)-4(H)-chromen-4-oato- κ O]tungsten(IV) hexafluorophosphate (3)

The synthesis was performed according to the general procedure A using bis(cyclopentadienyl)tungsten dichloride (128 mg, 300 μ mol), 3-hydroxy-2-(4'-chlorophenyl)chromen-4-one (90 mg, 330 μ mol) and sodium methoxide (18 mg, 330 μ mol) with a reaction time of 72 h at 40 °C. Ammonium hexafluorophosphate (270 mg, 1.50 mmol) was added and stirred for 2 h. After washing with MeOH the precipitate was dissolved in DCM and the remaining solids were filtered off. Yield: 69 mg (32%), green powder. Solubility in PBS (1% DMSO): 0.03 mg/mL. $^1\text{H-NMR}$ (DMSO- d_6) δ = 6.06 (s, 10H, Cp), 7.64 (m, 1H, C7-H), 7.69 (d, $^3J(\text{H}, \text{H})$ = 8.7 Hz, 2H, C3'-H' and C5'-H), 7.97–8.02 (m, 1H, C8-H), 8.03 (d, $^3J(\text{H}, \text{H})$ = 8.0 Hz, 1H, C6-H), 8.08 (d, $^3J(\text{H}, \text{H})$ = 8.0 Hz, 1H, C9-H), 8.37 (d, $^3J(\text{H}, \text{H})$ = 8.7 Hz, 2H, C2'-H and C6'-H) ppm. $^{13}\text{C-NMR}$ (DMSO- d_6) δ = 98.5 (Cp), 118.0 (C5), 118.4 (C6-H), 123.9 (C9-H), 126.2 (C7-H), 129.0 (C3'-H and C5'-H), 129.2 (C1'), 129.3 (C2'-H and C6'-H), 135.3 (C8-H), 136.0 (C4'), 150.7 (C2), 154.2 (C10), 158.4 (C3), 188.8 (C4=O) ppm. Elemental analysis: Calculated for C₂₅H₁₈ClF₆O₃PW: C, 41.09; H, 2.48%. Found: C, 41.22; H, 2.55%. (ESI⁺) m/z : 585.05 [Cp₂W(flavonolate)]⁺. IR: ν = 3121 w ($\nu_{\text{C-H}}$, Cp), 1587, 1536 w ($\nu_{\text{C=O}}$), 1489, 1430 s ($\nu_{\text{C=C}}$), 823 s ($\nu_{\text{C-H}}$) cm⁻¹.

Bis(η^5 -cyclopentadienyl)[2-(4-chlorophenyl)-3-(oxo- κ O)-4(H)-chromen-4-thionato- κ S]tungsten(IV) hexafluorophosphate (4)

The synthesis was performed according to the general procedure A using bis(cyclopentadienyl)tungsten dichloride (128 mg, 300 μ mol), 3-hydroxy-2-(4'-chlorophenyl)chromen-4-thione (94 mg, 330 μ mol) and sodium methoxide (18 mg, 330 μ mol) with a reaction time of 48 h under reflux. Ammonium hexafluorophosphate (54 mg, 330 μ mol) was added and stirred for 4 h. The formed precipitate was filtered off, dissolved in dichloromethane and filtered off in order to remove insoluble materials. Yield: 94 mg (39%), brown powder. Solubility in PBS (1% DMSO): 0.01 mg/mL. $^1\text{H-NMR}$ (DMSO- d_6) δ = 5.85 (s, 10H, Cp), 7.69 (d, $^3J(\text{H}, \text{H})$ = 8.7 Hz, 2H, C3'-H and C5'-H), 7.70–7.76 (m, 1H, C7-H), 7.96–8.01 (m, 1H, C8-H), 8.14 (d, $^3J(\text{H}, \text{H})$ = 8.0 Hz, 1H, C9-H), 8.33 (d, $^3J(\text{H}, \text{H})$ = 8.0 Hz, 1H, C6-H), 8.42 (d, $^3J(\text{H}, \text{H})$ = 8.7 Hz, 2H, C2'-H and C6'-H) ppm. $^{13}\text{C-NMR}$ (DMSO- d_6) δ = 96.5 (Cp), 118.7 (C9-H), 124.7 (C5), 125.7 (C6-H), 127.6 (C7-H), 129.1 (C3'-H and C5'-H), 129.1 (C1'), 130.5 (C2'-H and C6'-H), 134.4 (C8-H), 136.7 (C4'), 148.3 (C2), 149.3 (C10), 169.4 (C3), 187.2 (C4=S) ppm. Elemental analysis: Calculated for C₂₅H₁₈ClF₆O₂SPW: C, 40.21; H, 2.43; S, 4.29%. Found: C, 39.95; H, 2.71; S, 4.26%. (ESI⁺) m/z : 601.02 [Cp₂W(thioflavonolate)]⁺. IR: ν = 3124 w ($\nu_{\text{C-H}}$, Cp), 1580 w ($\nu_{\text{C=O}}$), 1479, 1426 m ($\nu_{\text{C=C}}$), 817 s ($\nu_{\text{C-H}}$) cm⁻¹.

Bis(η^5 -cyclopentadienyl)[2-(carboxylato- κ O)-pyridine- κ N]tungsten(IV) hexafluorophosphate (5)

The synthesis was performed according to the general procedure B using bis(cyclopentadienyl)tungsten dichloride (130 mg, 330 μ mol), picolinic acid (50 mg, 400 μ mol) and sodium methoxide (26 mg, 460 μ mol). Afterwards, sodium hexafluorophosphate (112 mg, 660 μ mol) was added and stirred for 2 h. The resulting solids were filtered off, redissolved in water and filtered through a folded filter. The filtrate was concentrated to 5 mL and stored at 4 °C overnight. The formed precipitate was filtered off, washed with THF and n-hexane and dried in vacuo. Yield: 52 mg (27%), red powder. Solubility in PBS (1% DMSO): 0.18 mg/mL. $^1\text{H-NMR}$ (DMSO- d_6) δ = 6.01 (s, 10H, Cp), 7.85–7.91 (m, 1H, C5-H), 8.16 (d, $^3J(\text{H}, \text{H})$ = 7.7 Hz, 1H, C3-H), 8.46–8.52 (m, 1H, C4-H), 9.09 (d, $^3J(\text{H}, \text{H})$ = 5.3 Hz, 1H, C6-H) ppm. $^{13}\text{C-NMR}$ (DMSO- d_6) δ = 97.2 (Cp), 126.5 (C3-H), 129.8 (C5-H), 143.4 (C4-H), 150.4 (C6-H), 160.0 (C2), 178.8 (C7=O) ppm. Elemental analysis: Calculated for $\text{C}_{16}\text{H}_{14}\text{F}_6\text{NO}_2\text{PW} \cdot 0.5\text{H}_2\text{O}$: C, 32.57; H, 2.56; N, 2.37%. Found: C, 32.54; H, 2.45; N, 2.33%. (ESI $^+$) m/z : 436.05 [$\text{Cp}_2\text{W}(\text{picolinato})$] $^+$. IR: ν = 3121 m ($\nu_{\text{C-H}}$, Cp), 1684, 1610 s ($\nu_{\text{C=O}}$), 1484, 1438 m ($\nu_{\text{C=C}}$), 817 s ($\nu_{\text{C-H}}$) cm^{-1} .

Bis(η^5 -cyclopentadienyl)[1,2-dimethyl-3-(oxo- κ O)-4-(1H)-pyridonato- κ O]tungsten(IV) hexafluorophosphate (6)

The synthesis was performed according to the general procedure A using bis(cyclopentadienyl)tungsten dichloride (128 mg, 300 μ mol), 3-hydroxy-1,2-dimethyl-4-(1H)-pyridone (46 mg, 330 μ mol) and sodium methoxide (18 mg, 330 μ mol) with a reaction time of 4 h. Ammonium hexafluorophosphate (54 mg, 330 μ mol) was added and stirred for 2 h. The formed precipitate was filtered off and dissolved in dichloromethane. Afterwards, the insoluble materials were removed by filtration. Yield: 95 mg (54%), dark brown powder. Solubility in PBS (1% DMSO): 1.20 mg/mL. $^1\text{H-NMR}$ (DMSO- d_6) δ = 2.28 (s, 3H, 2-CH $_3$), 3.84 (s, 3H, N-CH $_3$), 5.78 (s, 10H, Cp), 6.68 (d, $^3J(\text{H}, \text{H})$ = 6.0 Hz, 1H, C5-H), 7.73 (d, $^3J(\text{H}, \text{H})$ = 6.0 Hz, 1H, C6-H) ppm. $^{13}\text{C-NMR}$ (DMSO- d_6) δ = 11.7 (2-CH $_3$), 42.4 (N-CH $_3$), 97.6 (Cp), 108.4 (C5-H), 135.7 (C6-H), 136.4 (C2), 162.6 (C3), 176.4 (C4=O) ppm. Elemental analysis: Calculated for $\text{C}_{17}\text{H}_{18}\text{F}_6\text{NO}_2\text{PW}$: C, 34.19; H, 3.04; N, 2.35%. Found: C, 34.08; H, 3.10; N, 2.35%. (ESI $^+$) m/z : 452.08 [$\text{Cp}_2\text{W}(\text{deferipronato})$] $^+$. IR: ν = 3125 w ($\nu_{\text{C-H}}$, Cp), 1610, 1555 w ($\nu_{\text{C=O}}$), 1496, 1462 m ($\nu_{\text{C=C}}$), 825 s ($\nu_{\text{C-H}}$) cm^{-1} .

Bis(η^5 -cyclopentadienyl)[2-methyl-5-(oxo- κ O)-4-(1H)-pyran-4-ato- κ O] tungsten(IV) hexafluorophosphate (7)

The synthesis was performed according to the general procedure A using bis(cyclopentadienyl)tungsten dichloride (135 mg, 350 μ mol), 5-hydroxy-2-methyl-4H-pyran-4-one (44 mg, 350 μ mol) and sodium methoxide (21 mg, 385 μ mol) with a reaction time of 72 h. Ammonium hexafluorophosphate (118 mg, 700 μ mol) was added and stirred for 4 h. Yield: 78 mg (38%), brown powder. Solubility in PBS (1% DMSO): 0.35 mg/mL. $^1\text{H-NMR}$ (d_6 -DMSO): δ = 2.48 (s, 3H, H7), 5.91 (s, 10H, H $_{\text{Cp}}$), 6.98 (s, 1H, H3), 8.31 (s, 1H, H6). $^{13}\text{C-NMR}$ (d_6 -DMSO): δ = 19.83 (C7), 98.53(Cp), 109.61 (C3), 144.72 (C6), 163.61 (C5), 169.95 (C2), 190.41 (C4=O). Elemental analysis: Calculated for $\text{C}_{16}\text{H}_{15}\text{F}_6\text{O}_3\text{PW}$: C, 32.90; H, 2.59%. Found: C, 32.72; H, 2.58%. (ESI $^+$) m/z : 439.02 [$\text{Cp}_2\text{W}(\text{allomaltolato})$] $^+$. IR: ν = 3118 w ($\nu_{\text{C-H}}$, Cp), 1606, 1551 m ($\nu_{\text{C=O}}$), 1472, 1433 m ($\nu_{\text{C=C}}$), 819 s ($\nu_{\text{C-H}}$) cm^{-1} .

Bis(η^5 -cyclopentadienyl)[2-methyl-5-(oxo- κ O)-pyran-4-(1H)-thionato- κ S] tungsten(IV) hexafluorophosphate (8)

The synthesis was performed according to the general procedure A using bis(cyclopentadienyl)tungsten dichloride (135 mg, 350 μ mol), 5-hydroxy-2-methyl-4H-pyran-4-thione (50 mg, 350 μ mol) and sodium methoxide (21 mg, 385 μ mol) with a reaction time of 72 h. Ammonium hexafluorophosphate (114 mg, 700 μ mol) was added and stirred for 4 h. The flask was stored at 4 °C overnight. The formed precipitate was filtered off and dissolved in dichloromethane and potential salt rests were filtered off. Yield: 88 mg

(42%), red powder. Solubility in PBS (1% DMSO): 0.41 mg/mL. $^1\text{H-NMR}$ (d_6 -DMSO): δ = 2.48 (s, 3H, H7), 5.70 (s, 10H, H_{Cp}), 7.86 (s, 1H, H3), 8.15 (s, 1H, H6). $^{13}\text{C-NMR}$ (d_6 -DMSO): δ = 18.97 (C7), 96.61 (Cp), 119.36 (C3), 142.92 (C6), 162.85 (C5), 172.97 (C2), 187.96 (C4=S). Calculated for $\text{C}_{16}\text{H}_{15}\text{F}_6\text{O}_2\text{PSW}$: C, 32.02; H, 2.52; S, 5.34%. Found: C, 31.68; H, 2.48; S, 5.57%. (ESI^+) m/z : 454.98 [$\text{Cp}_2\text{W}(\text{thioallomaltolato})$] $^+$. IR: ν = 3126 m ($\nu_{\text{C-H}}$, Cp), 1587, 1510 m ($\nu_{\text{C=S}}$), 1439 m ($\nu_{\text{C=C}}$), 826 s ($\nu_{\text{C-H}}$) cm^{-1} .

Bis(η^5 -cyclopentadienyl)[2-ethyl-3-(oxo- κO)-4-(1H)-pyran-4-ato- κO] tungsten(IV) hexafluorophosphate (**9**)

The synthesis was performed according to the general procedure B using bis(cyclopentadienyl)tungsten dichloride (130 mg, 330 μmol), 2-ethyl-3-hydroxy-4H-pyran-4-one (56 mg, 400 μmol) and sodium methoxide (26 mg, 460 μmol). Sodium hexafluorophosphate (112 mg, 660 μmol) was added and stirred for 2 h. The solvents were removed, the residue was taken up into dichloromethane, washed with water (3×20 mL), dried over magnesium sulfate and filtered through a folded filter. The solution was concentrated to 5 mL and the product was precipitated through the addition of diethyl ether, filtered, washed with n-hexane and dried in vacuo. Yield: 73 mg (37%), brown powder. Solubility in PBS (1% DMSO): 0.55 mg/mL. $^1\text{H-NMR}$ (d_6 -DMSO): δ = 1.14 (t, 3H, 3J (H, H) = 7.6 Hz, H8), 2.71 (q, 2H, 3J (H, H) = 7.6 Hz, H7), 5.91 (s, 10H, H_{Cp}), 7.06 (d, 1H, 3J (H, H) = 5.0 Hz, H5), 8.39 (d, 1H, 3J (H, H) = 5.0 Hz, H6). $^{13}\text{C-NMR}$ (d_6 -DMSO): δ = 11.3 (C8), 21.95 (C7), 98.57 (Cp), 110.88 (C5), 157.16 (C6), 161.3 (C2), 161.9 (C3), 187.74 (C4=O). Calculated for $\text{C}_{17}\text{H}_{17}\text{F}_6\text{O}_3\text{PW}$: C, 34.14; H, 2.86%. Found: C, 34.06; H, 2.87%. (ESI^+) m/z : 452.97 [$\text{Cp}_2\text{W}(\text{ethylmaltolato})$] $^+$. IR: ν = 3125 w ($\nu_{\text{C-H}}$, Cp), 1597, 1522 m ($\nu_{\text{C=O}}$), 1475, 1429 m ($\nu_{\text{C=C}}$), 998, 945, s ($\nu_{\text{C-C}}$), 819 s ($\nu_{\text{C-H}}$), 725 s (ν_{CH_2}) cm^{-1} .

Bis(η^5 -cyclopentadienyl)[2-ethyl-3-(oxo- κO)-4-(1H)-pyran-4-thionato- κS] tungsten(IV) hexafluorophosphate (**10**)

The synthesis was performed according to the general procedure B using bis(cyclopentadienyl)tungsten dichloride (130 mg, 330 μmol), 2-ethyl-3-hydroxy-4H-pyran-4-thione (62 mg, 400 μmol) and sodium methoxide (26 mg, 460 μmol). Sodium hexafluorophosphate (112 mg, 660 μmol) was added and stirred for 2 h. The solvents were removed, the residue was taken up into dichloromethane, washed with water (3×20 mL), dried over magnesium sulfate and filtered through a folded filter. The solution was concentrated to 5 mL and the product was precipitated through the addition of diethyl ether, filtered, washed with n-hexane and dried in vacuo. Yield: 126 mg (62%), dark green powder. Solubility in PBS (1% DMSO): 0.32 mg/mL. $^1\text{H-NMR}$ (d_6 -DMSO): δ = 1.12 (t, 3H, 3J (H, H) = 7.6 Hz, CH_3), 2.70 (q, 2H, 3J (H, H) = 7.5 Hz, CH_2), 5.71 (s, 10H, H_{Cp}), 7.86 (d, 1H, 3J (H, H) = 4.6 Hz, H5), 8.25 (d, 1H, 3J (H, H) = 4.6 Hz, H6). $^{13}\text{C-NMR}$ (d_6 -DMSO): δ = 10.94 (C8), 22.57 (C7), 96.74 (Cp), 119.89 (C5), 150.25 (C6), 160.77 (C2), 171.30 (C3), 182.38 (C4=S). Calculated for $\text{C}_{17}\text{H}_{17}\text{F}_6\text{O}_2\text{PSW}$: C, 33.24; H, 2.79; S, 5.22%. Found: C, 33.21; H, 2.80; S, 5.23%. (ESI^+) m/z : 468.93 [$\text{Cp}_2\text{W}(\text{thioethylmaltolato})$] $^+$. IR: ν = 3126 w ($\nu_{\text{C-H}}$, Cp), 1571 m ($\nu_{\text{C=S}}$), 1499, 1408 m ($\nu_{\text{C=C}}$), 815 s ($\nu_{\text{C-H}}$) cm^{-1} .

2.3. Cell Lines and Culture Conditions

CH1/PA-1 (ovarian teratocarcinoma), SW480 (colon carcinoma) and A549 (non-small cell lung cancer) cells were maintained as described in the reference [41]. IMR-90 human fetal lung fibroblasts (from the European Collection of Authenticated Cell Cultures, Porton Down, Salisbury, UK) were cultured in Stable CellTM DMEM/F12 supplemented with 0.01 mg/mL human recombinant insulin (both purchased from Sigma-Aldrich) and 10% fetal bovine serum (FBS, BioWest, Nuaille, France). Human colon carcinoma cell lines HCT116 and HT29 (both kindly provided by the Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Austria) were cultured in McCoy's 5a medium supplemented with 4 mM L-glutamine (both purchased from Sigma-Aldrich) and 10% FBS. MCF-7 human breast adenocarcinoma cells (kindly provided by M. Ogris, Department of

Pharmaceutical Sciences, University of Vienna, Austria) were maintained in DMEM (high glucose) supplemented with 4 mM L-glutamine, 0.1 mg/mL human insulin (all purchased from Sigma-Aldrich) and 10% FBS. All cell lines were grown as monolayers in 75 cm² culture flasks (CytoOne, Starlab, Hamburg, Germany) at 37 °C in a humidified atmosphere containing 5% CO₂.

2.4. Spheroid Formation and Growth

For growing multicellular tumor spheroids, 400 HCT116, 400 HT29 or 800 MCF-7 cells per well were plated into round-bottom ultra-low attachment 96-well plates (Corning, Glendale, AZ, USA) in the media described above and incubated at 37 °C for 96 h. Spheroids were treated with 10 µM of the two most cytotoxic compounds **3** and **4** for another 96 h. The diameters of the spheroids were measured with Cell^F software under a CKX41 Olympus microscope before treatment and in constant daily intervals over the 96 h treatment period. For statistical analysis the *t*-test with Welch's correction was implemented in GraphPad Prism software (Version 6.01).

2.5. Cell Viability (MTT Assay)

Exponentially growing cells were plated into 96-well microculture plates (CytoOne, tissue culture treated, Starlab, Hamburg, Germany) in densities of 1×10^3 CH1/PA-1, 2×10^3 SW480, 3×10^3 A549 and 7×10^3 IMR-90 cells per well (each in 100 µL). After a 24 h preincubation, drugs were added in serial dilution to give a final volume of 200 µL/well and exposed for 96 h. At the end of the exposure, the drug solutions were replaced by 100 µL RPMI 1640/MTT mixture (7:1 ratio). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide), (Acros Organics, Geel, Belgium) solution was prepared in phosphate-buffered saline (5 mg/mL) and mixed with RPMI1640 medium supplemented with 4 mM L-glutamine (both from Sigma-Aldrich) and 10% FBS. After 4 h incubation, the formazan crystals were solubilized in 150 µL of DMSO per well. Absorbance was measured at 550 nm (and at 690 nm as a reference) with a microplate reader (ELx808, BioTek, Winoosky, VT, USA) and Gen5TM 3.08 software (BioTek). The 50% inhibitory concentrations (IC₅₀) relative to untreated controls were interpolated from concentration-effect curves. At least three biologically and technically independent experiments were performed.

2.6. Cell Viability (Resazurin Assay)

For the resazurin assay in monolayer cultures, cells were seeded into 96-well microculture plates (CytoOne, tissue culture treated, Starlab, Hamburg, Germany) in densities of 2×10^3 HCT116, 2×10^3 HT29 and 4×10^3 MCF-7 cells per well (each in 100 µL). For tests in 3D cultures, spheroids were grown as described above. After 24 h (for monolayers) or 4 days (for spheroids) the test compounds in the appropriate medium were added in serial dilution to the plates to obtain a total volume of 200 µL/well. Wells without cells, containing medium and resazurin (Alfa Aesar, Thermo Fisher, Kandel, Germany) solution were used as blanks. After 96 h exposure 20 µL of the resazurin solution (440 µM) were added to each well, followed by a 4 h (2D) or 12 h (3D) incubation period. Resorufin fluorescence was quantified at 530 nm (and a reference wavelength of 620 nm) by using a microplate reader (Synergy HT, BioTek) with Gen5TM 3.10 software (BioTek).

2.7. Apoptosis Studies

Apoptosis and necrosis induction upon drug treatment was analyzed by double staining with FITC-conjugated annexin V (eBioscience, San Diego, CA, USA) and propidium iodide (PI, 1.0 mg/mL, Sigma-Aldrich) via flow cytometry. In brief, HCT116 and HT29 cells (7×10^4 cells/well) as well as MCF-7 cells (9×10^4 cells/well) were seeded into 24-well plates in 600 µL MEM and incubated overnight for adherence. After resuming exponential growth, cells were exposed to different concentrations of the test compounds for further 24 h. Positives controls were treated with KP1998 (compound **7** in reference [42]). Following treatment, the drug-containing medium was collected, cells were washed once with PBS

and harvested. After trypsinization, the cell suspension was added to the pre-collected medium and cells were pelleted by centrifugation ($300 \times g$, 3 min). The supernatant was removed, and cells were stained with FITC-conjugated annexin V ($0.4 \mu\text{g/mL}$) in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2) and incubated at 37°C for 15 min in the dark. Propidium iodide ($1.6 \mu\text{g/mL}$) was added shortly before flow cytometry analysis. Samples were analyzed with a Millipore Guava easyCyte™ 8HT flow cytometer (Merck Millipore, Burlington, MA, USA) with InCyte software. Five thousand events were collected for each experiment, and at least three independent experiments were performed. The recorded results were analyzed with FlowJo software 10.6.1 (TreeStar, Ashland, OR, USA).

2.8. Electrophoretic dsDNA Plasmid Assay

An amount of 400 ng of the pUC19 plasmid (2686 bp, New England Biolabs, Ipswich, MA, USA) was incubated with the test compounds ($50 \mu\text{M}$). Stock solutions of the test compounds were prepared in MilliQ water. The samples were incubated for different intervals (15 min to 6 h) with continuous shaking at 37°C . Following the incubation time, $4 \mu\text{L}$ aliquots of $6 \times$ DNA loading dye (Thermo Fisher, Kandel, Germany) were added to the $20 \mu\text{L}$ DNA samples. Electrophoresis was performed in a 1% agarose gel in $1 \times$ TBE buffer, started at 60 V for 5 min and continued at 120 V for 90 min. Ethidium bromide (EtBr, Serva Electrophoresis GmbH, Heidelberg, Germany) staining was conducted in $1 \times$ TBE ($0.75 \mu\text{g/mL}$) for 20 min. Images were taken by the GelDoc Imaging System Fusion F $\times$ 7 (Vilber Lourmat, Eberhardzell, Germany). For quantification ImageJ/Fiji1.46 was used.

2.9. Reactive Oxygen Species (ROS) Detection

Reactive oxygen species (ROS) generation was measured by means of the DCFH-DA assay. HCT116 and HT29 cells (2.5×10^4 cells/well) as well as MCF-7 cells (3.5×10^4 cells/well) were seeded into 96-well plates ($100 \mu\text{L}$ /well) and incubated overnight for attachment. On the following day, cells were washed with $200 \mu\text{L}$ Hanks' balanced salt solution (HBSS, Sigma-Aldrich, containing 1% FBS) and stained with $100 \mu\text{L}$ of $25 \mu\text{M}$ DCFH-DA (2',7'-dichlorofluorescein diacetate, Sigma-Aldrich) in HBSS (containing 1% FBS) for 45 min at 37°C in the dark. After the incubation period, cells were washed with $200 \mu\text{L}$ HBSS (1% FBS) and loaded with compounds at concentrations of $2 \mu\text{M}$, $20 \mu\text{M}$, $100 \mu\text{M}$ and $200 \mu\text{M}$ in $200 \mu\text{L}$ phenol-red-free MEM (1% FBS) in triplicates. As positive controls, cells were treated with $200 \mu\text{M}$ and $400 \mu\text{M}$ tert-butylhydroperoxide (Sigma-Aldrich). Wells containing only phenol-red-free medium with 1% FBS were used as blanks, and non-drug treated, cell containing wells as reference values. Fluorescence (excitation: $485/20 \text{ nm}$, emission: $516/20 \text{ nm}$) was recorded in 10 min intervals with the Synergy HT reader (BioTek) over 2 h.

To verify interactions of complex 4 with the fluorescence of DCF (2',7'-dichlorofluorescein, Sigma-Aldrich), a fluorescence quenching assay was performed. For this purpose, DCF was dissolved in abs. ethanol (Sigma-Aldrich), diluted in phenol-red-free MEM (1% FBS) to $5 \mu\text{M}$ and complex 4 dissolved in DMF, diluted to 20 and $200 \mu\text{M}$ in the same medium. Furthermore, two combinations of $5 \mu\text{M}$ DCF with 20 and $200 \mu\text{M}$ of complex 4 were prepared. The fluorescence intensity of the substances alone and their combinations were measured (excitation: $485/20 \text{ nm}$, emission: $516/20 \text{ nm}$) in 10 min intervals with the Synergy HT reader (BioTek) over 2 h.

2.10. LA-ICP-TOF-MS Analysis

Totals of 400 HCT116, 400 HT29 and 800 MCF-7 cells per well were seeded into round-bottom ultra-low attachment 96-well plates (Glendale, AZ, USA) for production of spheroids within 4 days. On the fifth day, spheroids were treated with the test compounds at concentrations based on their IC_{50} values (according to the 3D resazurin assay). After 72 h spheroids were collected, embedded in TissueTek (Sakura, Tokyo, Japan) and stored at -80°C until further processing. Samples were cut with a Cryostat CM3050S (Leica

Biosystems, Nussloch, Germany) in 5 μm thin sections and placed on Superfrost slides (Thermo Fisher Scientific, Kandel, Germany). An Iridia 193 nm excimer laser ablation system (Teledyne Photon Machines, Bozeman, MT, USA) was coupled to an icpTOF 2R (TOFWERK AG, Thun, Switzerland) via an aerosol rapid introduction system (ARIS). The LA and ICP-TOF-MS settings were optimized on a daily basis using NIST SRM612 glass certified reference material (National Institute for Standards and Technology, Gaithersburg, MD, USA). Laser ablation sampling was performed in NoGas mode, in fixed dosage mode 2, at a repetition rate of 200 Hz, a fluence of 0.7 J cm^2 and using a 5 μm $\times$ 5 μm square spot. The line scans overlapped one another by 2.5 μm .

2.11. Immunofluorescence Staining

Spheroids were grown and cryosectioned, as described in the section above. For immunofluorescence staining the samples were fixed with 4% PFA (in PBS) for 15 min. The slides were washed with PBS before permeabilization with 0.1% Triton-X (Sigma-Aldrich, Steinheim, Germany) for 10 min. After three times washing with PBS the samples were stained for 45 min with 10% goat serum (G9023, Sigma-Aldrich) and 5% BSA for avoiding non-specific antibody binding. After one washing step a primary antibody against Ki67 (#9129, Cell Signaling Technology) was applied and samples incubated overnight at 4 °C. On the next day, slides were washed with PBS and incubated with goat anti-rabbit Alexa Fluor 594 (#8889 Cell Signaling Technology) for 1 h at room temperature in the dark, followed by two washing steps with PBS. Slides were mounted with ProlongGold Antifade Reagent with DAPI (Invitrogen, Thermo Fisher) and analyzed with a confocal laser scanning microscope Zeiss LSM800 (Zeiss, Jena, Germany). Images were processed with Zen software (Zeiss). For fluorescence signal intensities data extraction ImageJ/Fiji1.46 was used. For quantification, the maximum signal intensity of the KI67⁺ cells was evaluated relative to the untreated control. For statistical analysis the *t*-test with Welch's correction was implemented in GraphPad Prism software (Version 6.01).

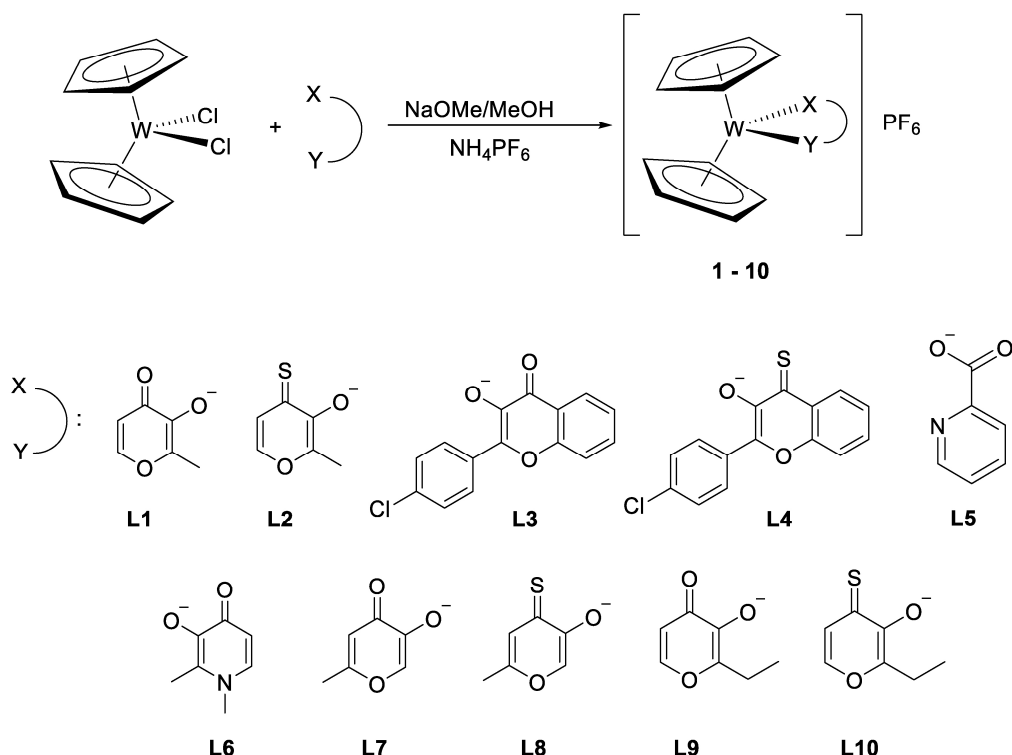
3. Results and Discussion

3.1. Synthesis and Characterisation

The syntheses of the (S,O-) chelates (**L2**, **L8**, **L10**) using Lawesson's reagent and ¹H-NMR spectra can be found in the supporting information (Figures S1–S11 and see Scheme 1 for structures of ligands and complexes). Complexes **1–10** were obtained by exchanging the chlorido ligands of the Cp₂WCl₂ starting material through a standard complexation method, resulting in positively charged complexes. All reactions were performed under inert conditions under an argon atmosphere. Anion exchange reactions, however, were carried out open to air. The general synthetic procedure involved weighing the starting material Cp₂WCl₂, the corresponding ligand **L1–L10** and the base sodium methoxide in a glove-box under an argon atmosphere, adding dry methanol and stirring for 4–72 h at room temperature or under reflux. Unreacted materials, if present, were removed by filtration. Afterwards, ammonium or sodium hexafluorophosphate (PF₆[−]) was added and the solution stirred again for 2–4 h. The formed precipitate was filtered off and washed with methanol or extracted using dichloromethane, yielding **1–10** in low to moderate yields: 27–62%. Complex **1A** also contains the maltolato ligand but has a chloride as counterion, it did not undergo the PF₆[−] exchange reaction described above (for comparison purposes). All compounds are soluble in chlorinated solvents, acetone, ethyl acetate and sparingly soluble in water, methanol and ethanol. The solids are highly stable in air; however, storage for longer periods under argon is recommended.

The IR spectra of complexes **1–10** showed the typical C–H stretch for the Cp moieties (3130–3120 cm^{-1}) as well as the characteristic $\nu_{\text{C=O}}$ and $\nu_{\text{C=C}}$ bands (1610–1505 and 1500–1420 cm^{-1} , respectively) for coordinated complexes. Due to the uncoordinated C=O of compound **5**, the $\nu_{\text{C=O}}$ stretch appears at higher wavenumbers (1684 and 1610 cm^{-1}), which is typical. In addition, $\nu_{\text{C-H}}$ bending is observed in the common wavenumbers

(ca. 820 cm^{-1}) [43]. These values are comparable with the tungstenocenes found in the literature [16].



Scheme 1. Synthesis of complexes 1–10.

An advantage of bent metallocenes is that the ^1H -NMR spectra of each complex (Figures S1–S11) shows just one signal for both cyclopentadienyl rings, since the rings are symmetrically coordinated to tungsten in a η^5 manner and the bidentate ligand is in an ancillary position, located in the plane bisecting the Cp–W–Cp. All NMR spectra were recorded in d_6 -DMSO and all peaks in the ^1H and ^{13}C have been fully assigned. The signal of the Cp ring in complexes 1–10 shifts downfield ($\delta = 6.06$ – 5.70 ppm) in the ^1H -NMR compared with tungstenocene dichloride ($\delta = 5.63$ ppm). Signals for the ^1H -NMR spectrum of compound 1 show exactly the same chemical shifts as the chloride analogue reported in the literature [16]. All (S,O–) chelates show an upfield shift in the Cp signal of 0.20 ppm compared to their (O,O–) analogues.

Compounds 1–10 were dissolved in 1% methanol/water and identified by ESI-MS measurements, where peaks for the $[\text{Cp}_2\text{W}(\text{L})]^+$ fragment were observed in the positive mode. Characteristic tungsten isotope patterns were also found for all complexes. The theoretical and experimental m/z values are listed in Table S1.

3.2. X-ray Diffractometry

X-ray suitable crystals of all compounds were obtained by slow diffusion of *n*-hexane in acetone (compounds 1A, 3–6, and 8–10) or ethanol (compounds 2 and 7) solutions (Figure 2). Compound 1A (with a chloride counterion instead of the hexafluorophosphate) crystallized in the orthorhombic *Cmca* space group, compounds 2 and 9 in the orthorhombic *Pnma* space group, compounds 3, 4 in the monoclinic $P2_1/n$ space group and 8 in the monoclinic $P2_1/c$ space group, compounds 5 and 6 in the orthorhombic *Pca2*₁ and *Pna2*₁ space groups, respectively, compound 7 in the orthorhombic *Pmn2*₁ space group, and compound 10 in the *P-1* triclinic space group. Unfortunately, the structure of 1A contains a disordered maltolato ligand (the two disordered states of the maltolato moiety rotate by 180°) and further discussions of this structure are not useful. We obtained more than one measurable crystal of 1A but in each measurement the disorder appeared. The bond lengths of the

coordinating carbonyl or thione in position 1 are, as expected, very distinctive. W–S bonds are the longest, with values in the range 2.430–2.454 Å, whereas the W–O bonds are the shortest, in the range of 2.067–2.127 Å. In between lies the W–N bond, at 2.157 Å (see Table 1 for selected bond lengths). The bond lengths between the W and the Cp-centroids are found to be in the same range, from 1.964 to 1.983 Å. The ring slippage is not insignificant (0.106–0.173 Å) and indicates a strain on the Cp moieties most likely resulting from their interactions with each other rather than interactions with the bidentate ligands since no discernable trend could be identified. The W–O2 bond lengths in complexes 2–10 do not differ much from each other and are found to be in a narrow range (2.067–2.102 Å).

Crystal data, data collection parameters and structure refinement details are given in Tables S2–S22.

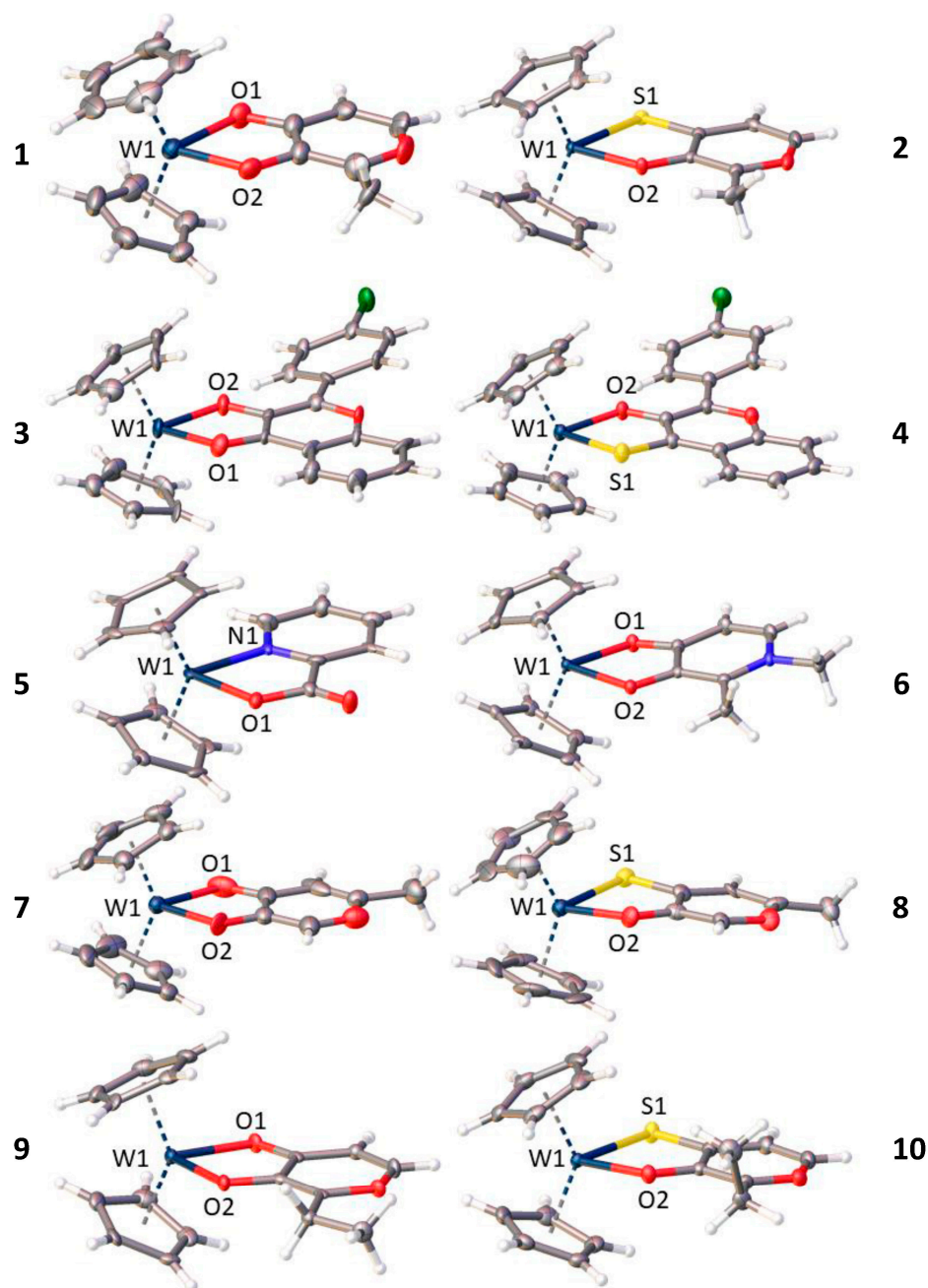


Figure 2. Crystal structures of compounds 1–10, drawn with 50% displacement ellipsoids.

Table 1. Relevant bond distances for compounds **2–10**. Due to the disorder in the structure of **1** it has been omitted. Centroid distances calculated by Platon [44].

Compound	Bond Lengths [Å]				
	W–O2	W–O/S/N	W–C _{pcentroid}	Ring Slippage	C1=O/S1
2	2.067 (2)	2.441 (1)	1.9821 (8)	0.151	1.710 (2)
3	2.072 (7)	2.127 (8)	1.965 (6)–1.979 (6)	0.106–0.147	1.309 (12)
4	2.0812 (15)	2.4298 (5)	1.9776 (11)–1.9824 (12)	0.130–0.173	1.699 (2)
5	2.097 (9)	2.157 (11)	1.966 (3)–1.972 (3)	0.107–0.128	1.35 (2)
6	2.0940 (19)	2.097 (2)	1.9690 (14)–1.9745 (14)	0.143–0.161	1.312 (3)
7	2.102 (8)	2.114 (13)	1.964 (5)	0.143	1.32 (2)
8	2.091 (11)	2.454 (4)	1.974 (10)–1.975 (9)	0.117–0.153	1.709 (15)
9	2.074 (5)	2.118 (4)	1.970 (2)	0.156	1.287 (8)
10	2.082 (1)	2.454 (1)	1.9822 (11)–1.9832 (12)	0.125–0.145	1.706 (2)

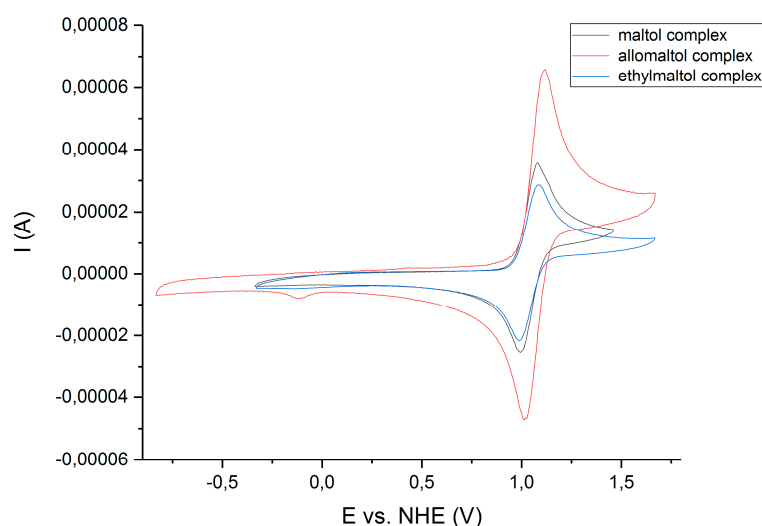
3.3. Cyclic Voltammetry

The electrochemical behavior of all complexes and the precursor Cp_2WCl_2 was studied by cyclic voltammetry with a scan rate of 200 mV/s, from -0.5 to $+1.2$ or $+1.5$ V. Measurements were performed in triplicate. Due to low water solubility of compounds **1–10** acetonitrile (MeCN) was utilized in order to reach the 2 mM concentration. All complexes exhibit a reversible one-electron process, W^{IV} to W^{V} .

The oxidation of W^{IV} to W^{V} for complexes **1–10** takes place in the potential range 0.885 to 1.330 V vs. Normal Hydrogen Electrode (NHE) measured in acetonitrile, whereas for Cp_2WCl_2 it occurs at 0.648 V (Table 2). Hence, the coordination of the bidentate ligands increases the redox stability of the complexes, making them more difficult to oxidize. Due to solvent effects, the values obtained for complexes **1–10** cannot be distinctively compared with physiological significant potentials [40]. The physiological significant potential in the cell is delimited by NADP^+ at -0.320 V ($\text{NADP}^+ + \text{H}^+ + 2\text{e}^- \rightarrow \text{NADPH}$) and by oxygen at $+0.820$ V ($\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O}$). All compounds, except for possibly compound **6** because of its significantly lower oxidation potential of 0.886 V, will most likely, but we cannot say absolutely, show redox activity outside the physiological region. The donor atoms directly influence the redox potential; compounds **1**, **7** and **9**, bearing the (O,O-) donors (Figure 3, for the CV of maltol, allomaltol and ethylmaltol complexes and the supporting information Figures S12–S16 for all other compounds), show lower redox potentials (1.037–1.071 V) than their (S,O-) counterparts (1.047–1.080 V), whereas the (N,O-) donor-atom derivative compound **5** exhibits the highest oxidation potential (1.330 V). Furthermore, the molybdenum counterpart of compound **5** also showed a considerably higher redox potential when compared to the precursor and to other molybdenocene derivatives [21]. The lowest oxidation potential, as already mentioned, belongs to compound **6**, the deferiprone derivative (0.885 V). Compounds **1** and **2**, bearing the maltol and thiomaltol ligands, are more easily oxidized than compounds **3** and **4**, which bear the (O,O-) and (S,O-) flavone moieties, respectively, and exhibit higher oxidation potentials. The shifting to higher redox potentials upon coordination to the bidentate ligands has also been observed in the literature; previous studies with (O,O-) donor ligand scaffolds reveal that upon coordination of the (O,O-) moiety the redox potential increases by 0.35–0.40 V [16]. As we did not observe any color changes during the CV experiments, we are confident that the MeCN does not interact with the complexes. In order to confirm this, we recorded an ^1H NMR in $\text{MeCN}-d_3$ of compound **3**, over the time span of a cyclic voltammetry experiment, and no change in the NMR spectra was observed over 30 min (Figure S17).

Table 2. Cyclic voltammetry data for compounds 1–10 and Cp_2WCl_2 in MeCN. The redox potentials are reported vs. NHE.

Compound	$E_{1/2}$ (V)
1	1.037
2	1.047
3	1.059
4	1.082
5	1.330
6	0.885
7	1.071
8	1.080
9	1.038
10	1.064
Cp_2WCl_2	0.648

**Figure 3.** Cyclic voltammogram of the maltol derivatives ((O,O-) chelates), compounds 1, 7 and 9, in MeCN referenced to the NHE.

3.4. Aqueous Solubility and Stability Measurements

In order to examine the stability of the compounds in aqueous solution, UV/Vis measurements were performed every hour during 24 h at 293 K. Solutions of compounds 1–10 were prepared in PBS (10%) from a previously prepared DMSO stock solution of each compound. The total concentration of DMSO was 1 vol% for the final complex solutions of 1–10.

All complexes were found to be stable in PBS over 24 h, as the peak maxima (λ_{max}) do not shift. However, compounds 3, 4, 7, 8 and 10 slowly precipitate out of the solution over time (Figures S18–S27). Precipitation, however, was not a problem when working at low concentrations in the in vitro experiments. The wavelength of the peak maxima and molar extinction coefficients are reported in the supplementary information Table S23.

Overall, compounds 1–10 showed sufficient aqueous solubility and stability for further biological investigations.

3.5. Cytotoxic Activity In Vitro (MTT Assay)

The cytotoxic activities in vitro of compounds 1–10 as well as the starting material Cp_2WCl_2 and compound 1A (cation of compound 1 with a chloride as counter ion) were investigated by means of the colorimetric MTT assay in the human cancer cell lines A549 (non-small cell lung carcinoma), SW480 (colon carcinoma), CH1/PA-1 (ovarian teratocarcinoma) and in human lung fibroblasts IMR-90. Compounds 2, 3, 4, 10 and Cp_2WCl_2 show a

typical pattern of antiproliferative activities in cancer cell lines, with the highest activity in the cell line CH1/PA-1, which is a broadly chemosensitive cell line [26], moderate activity in the cell line SW480 and the lowest activity in the intrinsically multi-drug resistant A549 cell line (Figures S28 and S29) [45]. Furthermore, compounds **2**, **3**, **4** and **10** show lower cytotoxicity in IMR-90 fibroblasts, which suggests a capacity of these drugs to selectively eliminate malignant cells (Figures S28 and S29). Remarkably, complex **2**, containing the (*S,O*-) donor thiomaltol ligand, shows a moderate non-selective activity in all tested cancer cell lines, which is promising with regard to overcoming multidrug resistance. Compounds **3** and **4**, bearing a flavone and a thioflavone ligand, respectively, show the most promising IC₅₀ values, reaching the low micromolar range in the cell lines CH1/PA-1 and SW480, and compound **4** even in the A549 cell line. The high in vitro anticancer activity of transition metal complexes bearing flavones and their derivatives is known in the literature [37,46,47]. The thioethylmaltol (**10**) derivative shows an enhanced potency in all three cell lines. In the case of the maltolato complexes **1**, bearing PF₆[−] as the anion, and **1A**, bearing Cl[−] as the anion, no activity was observed in the SW480 and A549 cell lines.

The in vitro anticancer activity data of the analogous molybdenocene derivatives synthesized by our group have also been included in Table 3 [21]. Compound **2** shows greater activity in two cells lines (SW480 and A549) than its molybdenum counterpart; however, the IC₅₀ values in the CH1/PA-1 cell line are in the same range. Compounds **3** and **4**, bearing the (thio)flavone ligands, are the most active of the series, mirroring the molybdenum (thio)flavone complexes, which show a very similar in vitro anticancer activity and were also the most active compounds in their series. The picolinic acid (**5**) and deferiprone (**6**) derivatives show negligible activity, while the allomaltol (**7**), thioallomaltol (**8**) and ethylmaltol (**9**) derivatives show similar activities as complex **1** (bearing a maltol ligand). In general, the (*S,O*-) chelates are more active than their (*O,O*-) counterparts and the tungstenocenes are more active than the molybdenocenes with some exceptions. According to Meléndez et al., the enhancement in cytotoxicity of the tungstenocene derivatives is due to their lower oxidation potentials when compared with their molybdenum analogs, resulting in higher redox activity under physiological conditions [16]. Even though the in vitro anticancer activity of Cp₂WCl₂ seems to be quite promising (IC₅₀ values of 38 µM in SW480 and 2.4 µM in CH1/PA-1 cells), this compound is not stable enough when exposed to air and it is known to decompose in the physiological environment in less than 3 h, making the active species unidentifiable [16].

Table 3. In vitro cytotoxicity (IC₅₀ values) in monolayer cultures of three human cancer cell lines (A549, SW480 and CH1/PA-1) and IMR-90 fibroblasts; 50% inhibitory concentrations (mean ± SD), obtained by the MTT assay (exposure time: 96 h); n. d., not determined * [21] ** [41].

Compound	IC ₅₀ Values [µM]			
	A549	SW480	CH1/PA-1	IMR-90
[Cp ₂ W(L1)]PF ₆ (1)	>200	>200	85 ± 41	n. d.
[Cp ₂ W(L1)]Cl (1A)	>200	>200	147 ± 18	n. d.
[Cp ₂ Mo(L1)]PF ₆ *	>200	>200	>200	n. d.
[Cp ₂ W(L2)]PF ₆ (2)	36 ± 6	36 ± 7	24 ± 3	107 ± 30
[Cp ₂ Mo(L2)]PF ₆ *	106 ± 15	55 ± 9	29 ± 10	n. d.
[Cp ₂ W(L3)]PF ₆ (3)	19 ± 3	5.4 ± 1.1	2.7 ± 0.4	38.2 ± 0.4
[Cp ₂ Mo(L3)]PF ₆ *	24 ± 4	5.6 ± 0.2	1.7 ± 0.4	n. d.
[Cp ₂ W(L4)]PF ₆ (4)	6.3 ± 0.5	2.4 ± 0.5	1.6 ± 0.5	11 ± 2
[Cp ₂ Mo(L4)]PF ₆ *	5.7 ± 1.0	1.6 ± 0.2	0.72 ± 0.13	n. d.
[Cp ₂ W(L5)]PF ₆ (5)	>200	147 ± 21	>200	n. d.
[Cp ₂ Mo(L5)]PF ₆ *	>200	>200	>200	n. d.
6	141 ± 36	154 ± 29	>200	n. d.
7	>200	>200	152 ± 16	n. d.
8	>200	129 ± 14	93 ± 15	n. d.

Table 3. Cont.

Compound	IC ₅₀ Values [μM]			
	A549	SW480	CH1/PA-1	IMR-90
9	>200	101 ± 15	>200	n. d.
10	53 ± 8	13 ± 1	21 ± 1	114 ± 33
Cp ₂ WCl ₂	>200	38 ± 21	2.4 ± 0.5	n. d.
Cp ₂ MoCl ₂	>200 *	>200 *	>200 *	n. d.
Cisplatin	3.8 ± 1.0 **	2.3 ± 0.2 **	0.073 ± 0.001 **	14 ± 1

3.6. Drug Sensitivity in 2D and 3D Cultures, Effects on Growth and Morphology

The activity of compounds **1A**, **2–10** and Cp₂WCl₂ was compared in conventional 2D monolayer and 3D multicellular tumor spheroid models. In publications of Meléndez et al., new tungstenocenes and molybdenocenes were studied for their cytotoxic activity in 2D-cultured cell lines such as HT29 (colorectal adenocarcinoma) and MCF-7 (breast adenocarcinoma) [16,22]. In addition to these two cell lines, HCT116 (colorectal adenocarcinoma) cells were selected for analyzing drug sensitivity, effects on growth and morphology, as well as apoptosis and ROS induction by our novel tungstenocenes. To overcome the limitations of monolayer-based assays, 3D cell cultures have been chosen to improve the screening of anticancer drug candidates by increasing the dimensionality with all its implications [48–52]. All three cell lines are applicable for 3D cell culture, which more realistically mimics the biochemical and biophysical environment of solid tumors. For determining the activity of the new tungstenocenes in 2D and 3D cell cultures the resazurin assay was chosen, where metabolically active cells reduce the blue non-fluorescent resazurin to red, highly fluorescent resorufin, enabling the quantification of cell viability by measurement of relative fluorescent units (RFU) [53].

Compounds **3** and **4**, bearing (thio)flavone as chelating ligand, must be highlighted based on their strong antiproliferative activity. Both complexes showed markedly increased activity in the low micromolar range in both monolayers and spheroid cultures in the tested cell lines. These results correlate well with the MTT-based results, where compounds **3** and **4** were found the most cytotoxic ones in CH1/PA-1, SW480 and A549 cell lines (Tables 3 and 4). The ratios of IC₅₀ values in 3D over 2D models were calculated, where compounds **3** and **4** displayed their lowest 3D/2D ratios (2.3 and 2.5, respectively) in HCT116 cells and their highest ratios in MCF-7 cells (7.0) (Table S24). Thiomaltol complex **2** showed nearly equal but modest activity in 3D and 2D (ratio 1.6) in HCT116 cells; furthermore, an IC₅₀ was reached in MCF-7 monolayers (66 ± 12 μM), but otherwise IC₅₀ values were higher than the maximum tested concentration (>200 μM). Complex **10** demonstrated activity in monolayers of the two cell lines, whereas in 3D models IC₅₀ values could only be reached in HCT116 spheroids, but with a remarkably low ratio between 3D and 2D (1.6) (Table S24). Concentration–effect curves for compounds **2–4** and **10** are depicted in Figures S30–S32. The other tested tungstenocenes showed nearly no cytotoxicity in HCT116, HT29 and MCF-7 cells. Obviously, the leaving groups play a crucial role for cytotoxic activity. Generally, compounds with enhanced cytotoxicity bear (S,O–) bidentates in the ancillary position, whereas complex **3** bearing a (O,O–) chelating flavone ligand tends to be similarly cytotoxic as complex **4** in 2D and 3D cell culture models.

The two most cytotoxic compounds **3** and **4** bearing (thio)flavone ligands, were investigated for their impact on long-term proliferation capacity. For better comparison, a concentration of 10 μM was used for both complexes in all three cell lines. Spheroids were grown for 4 days (to average diameters of 304 ± 1 μm (HCT116), 334 ± 6 μm (HT29) and 331 ± 7 μm (MCF-7)) and then treated with the tungstenocenes for a further 4 days. Cell growth and morphology was monitored every day and the respective diameters were determined (Figures 4 and S33–S36). Both compounds were capable of inhibiting spheroid growth with similar magnitude. HCT116 spheroids treated with complex **3** showed a markedly reduced growth ($p < 0.0001$) compared to the untreated control, and complex **4**

completely stopped spheroid growth ($p < 0.0001$) already after 24 h incubation (Figure 4). In the other two cell lines, HT29 and MCF-7, both complexes were able to completely stop spheroid growth (in all cases $p < 0.0001$) already after 24 h treatment (Figures S33–S36). When comparing the morphologies of the three different spheroid models, the compactness and the nice spherical shape of HCT116 and HT29 could be noticed, while MCF-7 demonstrated a less even outer margin. Remarkably, the structural integrity in the proliferating zone of HT29 spheroids under treatment with complex 4 was obviously lower, already after 48 h incubation.

Subsequently, the tungstenocene-induced inhibition of cell proliferation was investigated via the cell cycle regulated protein Ki67, which is widely used as a proliferation marker [54–56]. HCT116 spheroids were grown for 4 days and exposed to complexes 3 and 4 (both 10 μM) for 72 h. The treated spheroids were cryo-sectioned followed by Ki67 immunofluorescence labelling and confocal laser scanning microscopy. The low number of Ki67⁺ cells indicated high antiproliferative activity of the treated sample. In the untreated control, the vast majority of Ki67⁺ cells were detected at the periphery of the multicellular tumor spheroid (MTS) section. In total $38.1 \pm 7.3\%$ of cells in the control were found to be in proliferating state, in contrast to the exposed samples, where the numbers of proliferating cells decreased significantly ($15.8 \pm 2.8\%$ for complex 3 and $19.1 \pm 1.3\%$ for complex 4) (Figure 5, Table S25). While it is apparent that tungstenocene treatment inhibits the proliferation of cells in the outermost layers of the spheroid (Figure 5) Ki67⁺ cells could be observed in deeper regions, showing that proliferation persists in the inner parts of the MTS. A 2.4- and 2-fold reduction in number of Ki67⁺ cells was quantified relative to untreated control, for complex 3 and complex 4, respectively (in both cases $p < 0.05$ (Figure S37)). Interestingly, complex 4 was observed to efficiently inhibit the proliferation of the cells on the edge but barely in the inner of the spheroid (Figure 5). These findings might reflect a limited uptake of complex 4 into the deeper zones of HCT116 spheroids; for this purpose, W distribution was investigated in cryo-sectioned HCT116 spheroids via LA-ICP-TOF-MS (laser ablation (LA) in combination with inductively coupled plasma time-of-flight mass spectrometry (ICP-TOF-MS)).

Table 4. Inhibition of cancer cell growth (IC_{50}) in 2D and 3D cultures of the human cancer cell lines HCT116, HT29 and MCF-7; 50% inhibitory concentrations (mean $\pm$ SD) of Cp_2WCl_2 and ten tungstenocenes, obtained by the resazurin assay (exposure time: 96 h).

Compound	IC_{50} Values [μM]					
	HCT116		HT29		MCF-7	
	2D	3D	2D	3D	2D	3D
1A	>200	>200	>200	>200	>200	>200
2	98 ± 12	158 ± 28	53 ± 9	>200	66 ± 12	>200
3	9.2 ± 1.4	21 ± 4	17 ± 4	86 ± 2	6.5 ± 1.8	44 ± 1
4	6.0 ± 0.9	15 ± 4	5.5 ± 1.0	31 ± 6	2.7 ± 0.7	19 ± 2
5	>200	>200	>200	>200	>200	>200
6	>200	>200	>200	>200	172 ± 45	>200
7	>200	>200	>200	>200	>200	>200
8	>200	178 ± 14	>200	>200	163 ± 32	>200
9	>200	>200	>200	>200	147 ± 15	>200
10	42 ± 3	69 ± 12	54 ± 2	>200	29 ± 4	>200
Cp_2WCl_2	>100	>100	>100	>100	>100	>100

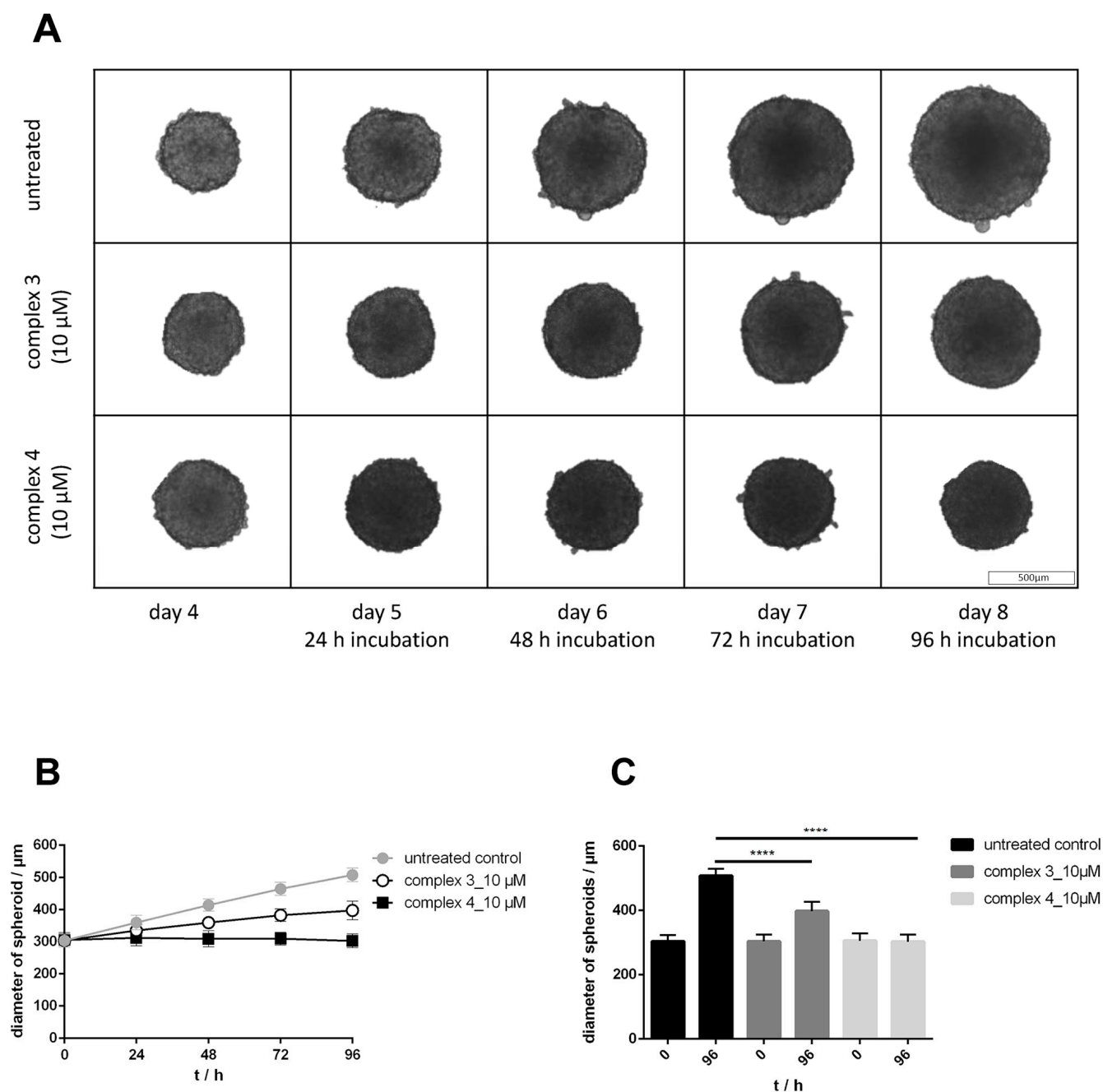


Figure 4. (A) Time-dependent growth of HCT116 spheroids upon treatment with complexes 3 and 4 (both 10 μ M) and untreated control, detected for 96 h (treatment started on day 4 after seeding). Images represent one captured spheroid out of three experiments. Scale bar = 500 μ m (B) Growth curves for spheroids of HCT116 cells treated with complexes 3 and 4 (both 10 μ M) and untreated control. Measurements started on day 4 (after seeding) with spheroid diameters of 304 ± 1 μ m and were continued every 24 h for additional 4 days. (C) Spheroids were initiated on day 0, treated with complexes 3 and 4 (both 10 μ M) on day 4, diameters of spheroids were obtained at the beginning of the treatment (0 h) and at the end (96 h). Unpaired *t*-test with Welch's correction ($n = 72$) was performed for statistical evaluation (**** $p < 0.0001$).

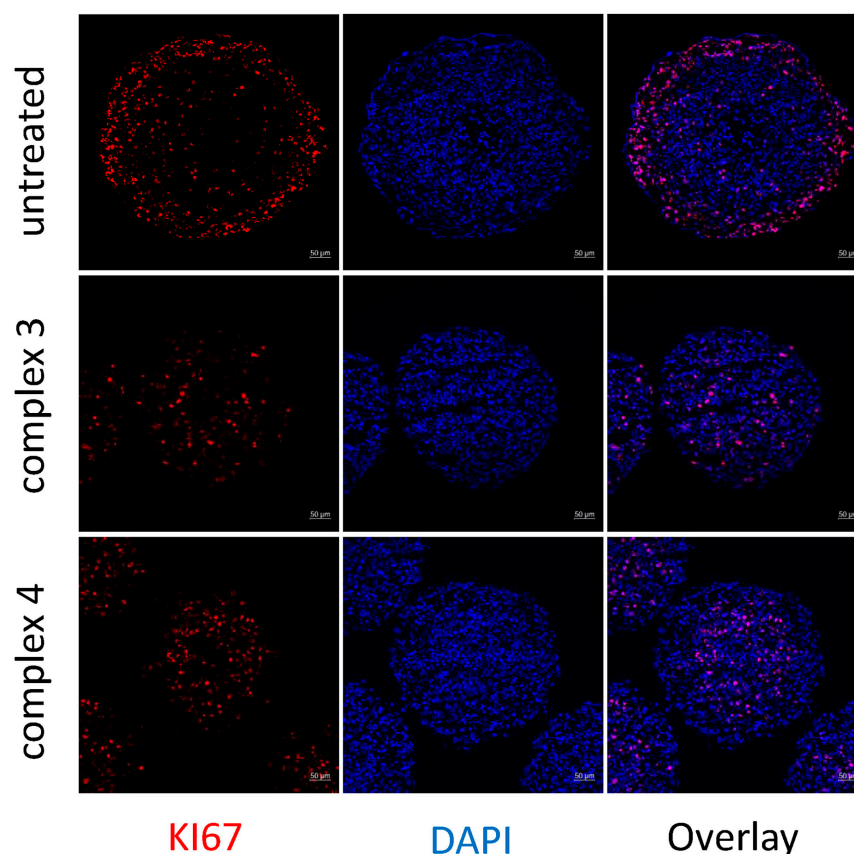


Figure 5. Confocal laser scanning fluorescence microscopy of paraformaldehyde (PFA)-fixed, 5 μm cryo-sectioned HCT116 spheroids, untreated or treated with complex 3 (20 μM) and complex 4 (15 μM) for 72 h. Detection of KI67 (proliferation marker, red) and DAPI-staining (nuclei, blue), and co-localized fluorescence (magenta). Scale bar = 50 μm .

3.7. W Distribution in HCT116 Tumor Spheroids

Multicellular HCT116 spheroids were treated with complexes 3 and 4 at sub cytotoxic concentration (10 μM) for analyzing the distribution of W (Figure 6) and qualifying a panel of other abundant elements by using high-resolution laser ablation (LA) in combination with inductively coupled plasma time-of-flight mass spectrometry (ICP-TOF-MS). Signal intensity of elements (isotopes) of biological relevance were measured after 48 and 72 h incubation with the two selected tungstenocenes. Flavone complex 3 yielded the highest tungsten accumulation in the inner layers of multicellular tumor spheroids (indicated by yellow color in Figure 6). These findings correlate well with proliferation marker (KI67) analysis (Figure 5), where a significantly lower level of KI67 signal was detected in the outer rim of the spheroid section, illustrating a clear decline in proliferative activity. The capacity of deep penetration of complex 3 indicates an increased chance of damaging non-proliferating tumor cells in the quiescent zone and in the spheroid core. Remarkably, thioflavone complex 4 displayed a tungsten enrichment predominantly in the proliferating zone and slighter signals in the quiescent zone or in the inner core. This suggests that the actively dividing cancer cells in the periphery of MTS accumulate tungsten to a high extent. Thus, the W distribution pattern of the two most cytotoxic tungstenocenes differs extremely, although these two complexes only differ in the presence of W–S or W–O bonds. These findings also suggest that the flavone ligand is more favorable for entering the spheroids and reaching the inner core, whereas it induces less cytotoxicity in all three tested cell lines than the thioflavone counterpart. In addition to tungsten ($^{182}\text{W}^+$), qualitative bioimaging was evaluated of $^{23}\text{Na}^+$, $^{24}\text{Mg}^+$, $^{31}\text{P}^+$ and $^{66}\text{Zn}^+$ after 48- and 72-h incubation at different concentrations, at 10 μM and additionally roughly at IC_{50} values (15 μM and 20 μM) for

each compound. A pronounced accumulation of $^{23}\text{Na}^+$, $^{24}\text{Mg}^+$ and $^{31}\text{P}^+$ could be observed in the outer rim of the selected spheroid sections, whereas the accumulation of $^{66}\text{Zn}^+$ is negligible (Figures S38–S40).

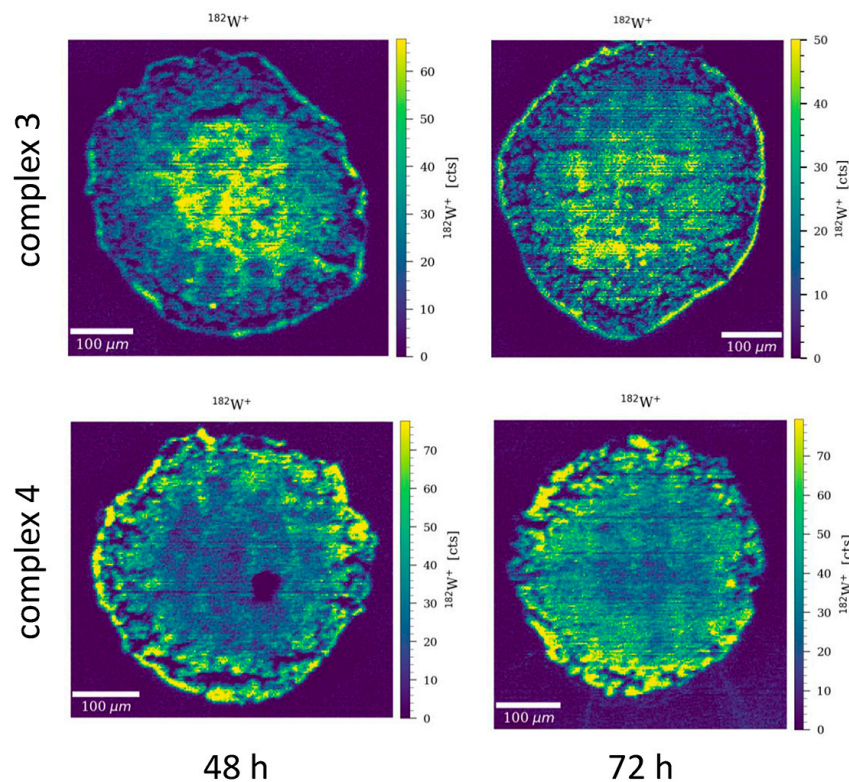


Figure 6. Qualitative images obtained by LA-ICP-TOF-MS for W distribution in HCT116 spheroids after treatment with complex 3 (10 μM) and complex 4 (10 μM) for 48 and 72 h. High-resolution laser ablation images were measured with a 5 μm square mask, double oversampling and a repetition rate of 200 Hz. (Scale bar = 100 μm).

3.8. Oxidative Stress Studies

Intracellular reactive oxygen species (ROS) levels were quantified in HCT116, HT29 and MCF-7 cell lines by using the fluorescent dye DCFH-DA. Fluorescence induction is proportional to the number of radicals produced by cells which were exposed to metallogenenes, and the development of the fluorescence signal was detected every 10 min for two hours. Due to the short incubation time, higher concentrations (2, 20, 100 and 200 μM) than the IC_{50} values determined after 96 h were applied. Pronounced elevation of ROS levels by complexes **1A**, **3** and **9**, which are (O,O-) chelate bearing tungstenocenes, was observed in all three cell lines (Figure 7, Figures S41 and S42). Complex **10** is the only tungstenocene with a (S,O-) chelate, which elicits relevant ROS generation. With these four complexes, clear concentration- and time-dependent effects could be observed. Unexpectedly, the behavior of compound **4** differed markedly, because measurements suggest concentration-dependent anti-oxidative properties. However, the intensely red colored complex **4** was found to quench the fluorescence of the product DCF (2',7'-dichlorofluorescein) in a cell-free assay. DCF (5 μM) and complex **4** (20 and 200 μM) were applied alone as well as in combination (DCF (5 μM) + complex **4** (20 μM) and DCF (5 μM) + complex **4** (200 μM)). In the combination with lower concentration of complex **4** 36.8% and with the higher concentration 77.2% lower fluorescence intensities were detected relative to DCF (5 μM) alone (Figure S43). The other tested complexes caused only minor or negligible alterations of ROS signals in a concentration- and time-dependent manner. These findings indicate that ROS production may play a role in the mode of action for at least some of the compounds. For a better understanding of the biological activity of tungstenocenes containing (O,O-),

(*S,O*–) and (*N,O*–) chelates, the relation between intracellular ROS levels and initiation of apoptosis and necrosis was examined.

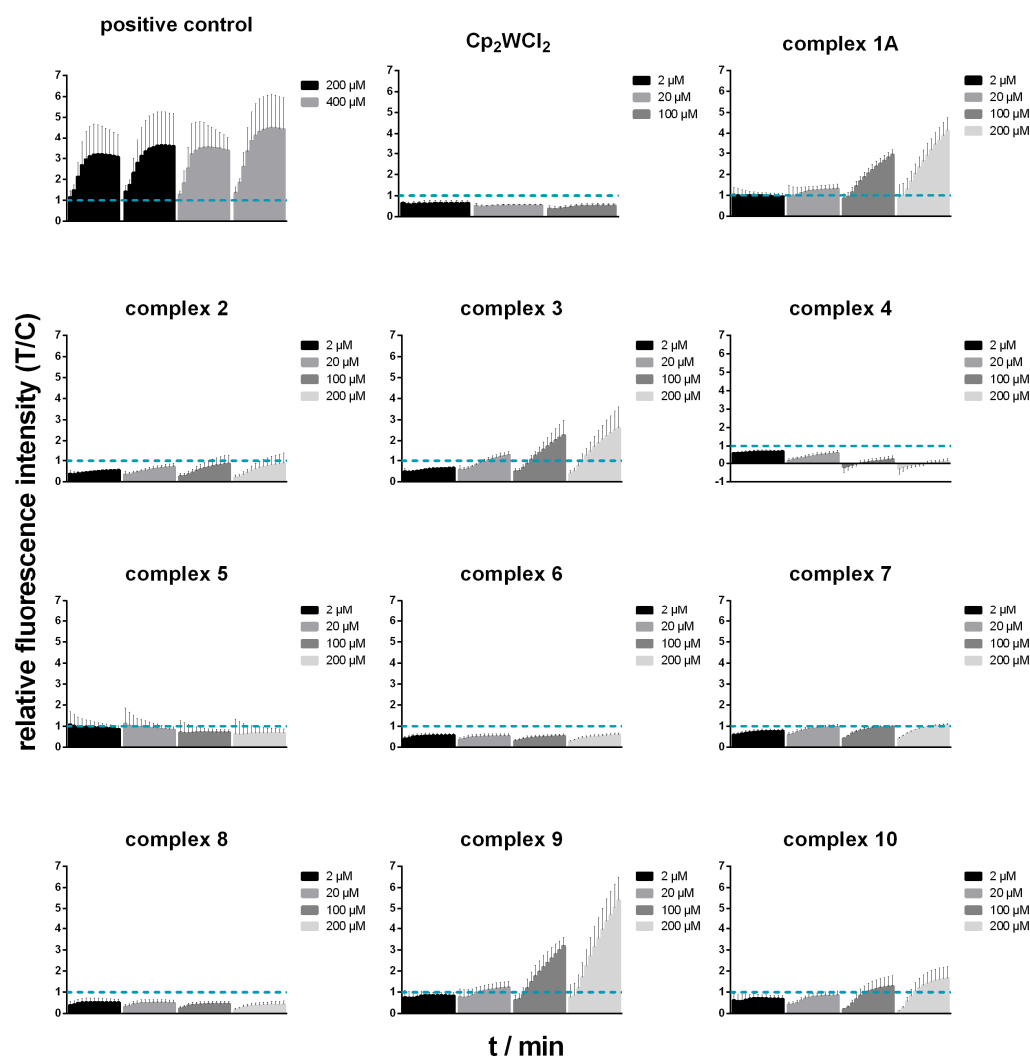


Figure 7. Time- and concentration-dependent intracellular reactive oxygen species (ROS) generation in HCT116 cells. Cells were treated with tungstenocenes (2, 20, 100 and 200 μM) and tert-butylperoxide (200 and 400 μM) as positive control. Fluorescence intensities of DCF indicating ROS levels (relative to the untreated control) were measured with a spectrophotometer. Data from three independent experiments with $n = 3$ wells per measurement are given as means $\pm$ standard deviations. The dashed, blue line indicates the ROS level of untreated controls to which the other values were normalized.

3.9. Apoptosis Studies

Translocation of phosphatidylserines (PS) is a classic apoptotic hallmark [57,58]. In viable cells, they are located on the inner leaflet of the plasma membrane, whereas they are translocated to outer leaflet in cells undergoing apoptosis. There, they can be detected with fluorescent-labeled annexin V, which specifically binds to PS. Besides annexin V, the DNA-intercalating agent propidium iodide (PI) is used to stain the nuclei of necrotic and late apoptotic cells. This annexin V-FITC/PI double staining enables a differentiation between viable cells, which are negative for both dyes (AV $^-$ /PI $^-$), early apoptotic cells, which are annexin V positive and PI negative (AV $^+$ /PI $^-$), late apoptotic cells, which are positive for both annexin V and PI (AV $^+$ /PI $^+$), and necrotic cells, which are positive for PI only (AV $^-$ /PI $^+$) [59,60].

In accordance with ROS and cytotoxicity studies (resazurin assay), 200 μM were applied of almost all tested tungstenocenes for 24 h; only complexes **3** and **4**, with the most promising cytotoxicity, were examined with a wider concentration range. Flavone complex **3** strongly induced apoptosis at the highest tested concentrations with up to 94% in HCT116 (200 μM), 83% in HT29 (200 μM) and 17.5% in MCF-7 (50 μM) cells (Table S26). Furthermore, complex **3** caused a tremendous concentration-dependent increase in early and late apoptotic phases in all three cell lines (Figure S44), exemplarily illustrated for HCT116 cells in Figure 8. The other potent compound was thioflavone complex **4**, which induced apoptosis even at 20 μM in up to 33% (HCT116), 36% (HT29) and 13% (MCF-7) of cells (Table S26 and Figure S45). In MCF-7 cells a distinct concentration dependency was shown for the detection of total apoptotic events induced by compound **4**; however, also the necrotic population increased. This specific phenomenon was obtained only in MCF-7 cells, whereas in the other two cell lines the necrotic fractions were rather minor (Figure S45). Surprisingly, thiomaltol complex **2** (200 μM), demonstrated strong apoptotic effect in HCT116 cells with up to 34% and in MCF-7 cells with up to 30%. Thioethylmaltol complex **10** (200 μM) also induced apoptosis in up to 52%, but only in MCF-7 cells (Table S26).

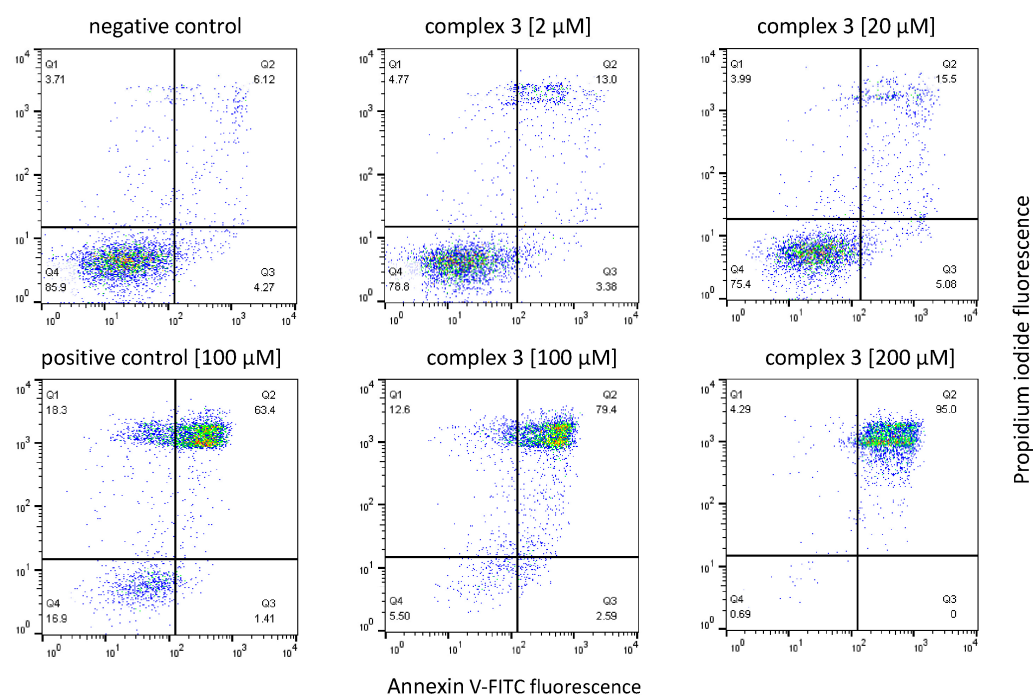


Figure 8. Flow-cytometric analysis (annexin V/PI assay) of apoptosis induction by complex **3** after 24 h in HCT116 cells. Scatter plots represent necrotic (Q1: AV−/PI+), late apoptotic (Q2: AV+/PI+), early apoptotic (Q3: AV+/PI−) and viable (Q4: AV−/PI−) cell fractions (percentages indicated correspondingly) of a representative experiment.

To sum up the results of annexin V/PI assay, the tungstenocenes bearing a (S,O−) chelate revealed a higher ability to induce early or late apoptosis than the complexes with (O,O−) or (N,O−) chelates.

3.10. Cell-Free DNA Interaction Assay

In order to elucidate if tungstenocenes interact with DNA, a cell-free dsDNA plasmid assay was performed. The intact dsDNA plasmid is mostly present in negatively supercoiled (sc) form, and upon drug interaction it may gradually convert to an open circular (oc) form, linear or interhelically cross-linked DNA, which results in altered electrophoretic mobility of the plasmid. This DNA damage may result from various forms of interaction such as cross-linking, intercalation or strand breakage. Platinum complexes such as cisplatin are capable of altering the secondary structure of the DNA mainly by formation of

cross-links [61,62]. To investigate a possible platinum-like behaviour of tungstenocenes, 50 μM of the complexes were incubated with pUC19 plasmid up to 6 h at 37 °C. A considerable interaction was observed by complexes 2–4, 8 and 10, suggesting single-strand breaks rather than cross-linking (Figure S46) in contrast to cisplatin [63]. Complex 8 showed the highest ability to induce single-strand breaks ($15 \pm 2\%$) untwisting the sc form to the oc form, even though this complex did not show pronounced cytotoxicity (Figure S47). From a comparison of the five most dsDNA damaging complexes (2–4, 8 and 10), it becomes obvious that all of the (*O,S*–) chelate bearing tungstenocenes interact with DNA. The only complex with an (*O,O*–) chelate showing the ability to induce DNA nicks is complex 3. The other tested tungstenocenes did not induce any or only negligible changes in the electrophoretic pattern. The minor effects on dsDNA observed and their poor correlation with cytotoxic potency argue against a crucial involvement of DNA interactions in the mode of action of the tested compounds.

4. Conclusions

Severe side effects, acquired tumor resistance and a limited number of responsive tumors are the limiting factors for platinum-based anticancer drug therapy in the clinic. Among the diverse metal compounds that might overcome these obstacles by potentially different modes of action and more selective anticancer activity, tungsten complexes have hardly received any attention thus far. Hence, we synthesized and characterized ten tungstenocene derivatives bearing different ligand scaffolds with various coordination motifs. Stability studies over 24 h in aqueous solution (1% DMSO in 10% PBS) via UV/Vis spectroscopy showed that some compounds precipitated over time; however, no hydrolysis or decomposition is indicated. The electrochemistry of all compounds was investigated by cyclic voltammetry. Although a direct comparison to the physiological system is not possible, due to measurements in MeCN, all complexes except for 6 show reversible W^{IV} to W^{V} peaks at such high potentials that decomposition due to redox processes in the physiological range should not be problematic.

Very auspicious in vitro anticancer activity was found in six human cell lines (A549, CH1/PA-1, HCT116, HT29, MCF-7, SW480), especially for complexes 3 and 4, exhibiting IC_{50} values mostly in the low micromolar range in monolayer cultures. An impact of the bidentate ligand donor system on the cytotoxicity was observed, with (*S,O*–) donors demonstrating improved IC_{50} values when compared to the (*O,O*–) donors. Furthermore, a certain selectivity of compounds 2, 3, 4 and 10 for malignant cells over non-malignant fibroblasts was observed. The cytotoxicity of complexes 3 and 4 is hardly affected in 3D cell culture (IC_{50} ratios 3D/2D: 2.3 and 2.5, respectively). Growth curves of HCT116, HT29 and MCF-7 multicellular tumor spheroids under treatment with complexes 3 and 4 (both 10 μM) indicate that both complexes were able to inhibit or completely stop growth and induce changes in the morphology of spheroids within the first 24 h of a 96-h treatment. To support these results, proliferating cells in cryo-sectioned HCT116 spheroids were detected via immunofluorescence imaging of KI67. Notable decreases of KI67⁺ cells were observed upon treatment with complexes 3 and 4 after 72 h incubation. A 2.4-fold reduction in number of KI67⁺ cells by complex 3 (20 μM) and 2-fold reduction by complex 4 (15 μM) was observed relative to the untreated control, ($p < 0.05$, respectively). Both compounds inhibited the proliferation of cells in the periphery of the spheroid with differing distribution patterns of KI67⁺ cells. This prompted us to investigate the W distribution in cryo-sectioned HCT116 spheroids via LA-ICP-TOF-MS. The signal intensity maps of W differed fundamentally, suggesting that the flavone-bearing complex 3 is more capable of entering deeper parts of the spheroids, whereas the thio-analogue 4 showed a higher accumulation in the outer rim of the spheroids.

To dig deeper into the mode of action of the novel tungstenocenes, the potential to form reactive oxygen species and apoptosis induction were studied in monolayer cultures of three cell lines (HCT116, HT29, MCF-7). The measured intracellular ROS levels spanned a wide range from slightly antioxidant up to highly oxidant effects, depending on the

cell line as well. In all three cell lines an increase of ROS upon treatment with complexes **1A**, **3**, **9** and **10** (except complex **3** in MCF-7 cells) was detected. Complex **10** is the only tungstenocene with a (*S,O*−) chelate which was able to induce ROS development. Both complexes **3** and **4** induced apoptosis (and necrosis) in a concentration-dependent manner in all three cell lines. Furthermore, the capacity of altering DNA secondary structure of the ten tungstenocenes was investigated in a cell-free assay. Complexes **2–4**, **8** and **10** interacted with plasmid dsDNA by causing single-strand breaks to a certain extent, but no other forms of DNA damage were indicated. Over all assays, the (*S,O*−) chelate bearing complexes tend to be more active than their (*O,O*−) and (*N,O*−) counterparts, but the current study also suggests a somewhat different mode of action of these complexes, although they only differ by the presence of a W–S or W–O bond.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pharmaceutics15071875/s1>, Synthesis of ligands, materials, NMR-spectroscopy (Figures S1–S11), mass spectrometry data (Table S1), experimental parameters for X-ray analysis (Table S2), sample and crystal data of complexes, data collection and structure refinement of compound (Tables S3–S22), cyclic voltammetry data (Figures S12–S17), UV/Vis spectroscopy data (Figures S18–S27), wavelength of the peak maxima and molar extinction coefficients (Table S23), concentration-effect curves of MTT assay (Figures S28 and S29), resazurin assay (Table S24, Figures S30–S32), time-dependent growth of spheroids and growth curves (Figures S33–S36), statistical data of immunofluorescence staining (Table S25, Figure S37), laser ablation (Figures S38–S40), reactive oxygen species detection (Figures S41 and S42), fluorescence-quenching assay (Figure S43), apoptosis induction (Figures S44 and S45, Table S26), dsDNA plasmid assay (Figures S46 and S47). Reference [42] is cited in the Supporting Information.

Author Contributions: Conceptualization, K.C. and M.S.M.; methodology, K.C., D.W. and M.S.M.; formal analysis, K.C. and M.S.M.; investigation, K.C., I.B., V.F., A.B., A.S., A.P.-R. and M.H.; resources, G.K., M.A.J., W.K., M.S.M. and B.K.K.; data curation, K.C., A.S., A.P.-R., M.H., M.A.J. and M.S.M.; writing—original draft preparation, K.C. and M.S.M.; writing—review and editing, K.C., V.F., M.A.J., W.K. and M.S.M.; visualization, K.C., I.B., V.F., A.B. and A.P.-R.; supervision, G.K., M.A.J., W.K., M.S.M. and B.K.K.; project administration, M.S.M.; funding acquisition, G.K., M.A.J., W.K., M.S.M. and B.K.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding. Open Access Funding by University of Vienna.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: CCDC 2074974–2074983 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.com.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; Fax: +44-1223-336033.

Acknowledgments: Authors express their gratitude to Anton Legin and Endre Kiss who provided their expertise and helped with the data evaluation of confocal images. Open Access Funding by the University of Vienna.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gasser, G.; Ott, I.; Metzler-Nolte, N. Organometallic anticancer compounds. *J. Med. Chem.* **2011**, *54*, 3–25. [CrossRef]
2. Jaouen, G.; Top, S.; Vessières, A. Organometallics Targeted to Specific Biological Sites: The Development of New Therapies. In *Bioorganometallics: Biomolecules, Labeling, Medicine*; Wiley-VCH Verlag GmbH & Co., KGaA: Weinheim, Germany, 2005.
3. Schirrmacher, V. *Quo Vadis Cancer Therapy?* LAP Lambert Academic Publishing: London, UK, 2017.
4. Keith, L.S.; Moffett, D.B.; Rosemond, Z.A.; Wohlers, D.W. ATSDR evaluation of health effects of tungsten and relevance to public health. *Toxicol. Ind. Health* **2007**, *23*, 347–387. [CrossRef]
5. Vyskočil, A.; Viau, C. Assessment of molybdenum toxicity in humans. *J. Appl. Toxicol.* **1999**, *19*, 185–192. [CrossRef]
6. Köpf, H.; Köpf-Maier, P. Titanocene Dichloride—The First Metallocene with Cancerostatic Activity. *Angew. Chem. Int. Ed. Engl.* **1979**, *18*, 477–478. [CrossRef]

7. Köpf-Maier, P.; Leitner, M.; Voigtländer, R.; Köpf, H. Molybdocene Dichloride as an Antitumor Agent. *Z. für Nat. CA J. Biosci.* **1979**, *34*, 1174–1176.
8. Köpf-Maier, P.; Leitner, M.; Köpf, H. Tumor Inhibition by Metallocenes: Antitumor Activity of Niobocene and Tungstocene Dichlorides. *J. Inorg. Nucl. Chem.* **1980**, *42*, 1789–1791. [[CrossRef](#)]
9. Köpf-Maier, P.; Köpf, H. Vanadocen-dichlorid—Ein weiteres Antitumor-Agens aus der Metallocenreihe/Vanadocene Dichloride—Another Antitumor Agent from the Metallocene Series. *Z. Nat. B* **1979**, *34*, 805–807. [[CrossRef](#)]
10. Ghosh, P.; D’Cruz, O.J.; Narla, R.K.; Uckun, F.M. Apoptosis-Inducing Vanadocene Compounds against Human Testicular Cancer. *Clin. Cancer Res.* **2000**, *6*, 1536–1545.
11. Harding, M.M.; Mokdsi, G. Antitumour Metallocenes: Structure-Activity Studies and Interactions with Biomolecules. *Curr. Med. Chem.* **2000**, *7*, 1289–1303. [[CrossRef](#)]
12. Köpf-Maier, P. Complexes of Metals Other than Platinum as Antitumour Agents. *Eur. J. Clin. Pharmacol.* **1994**, *47*, 1–16. [[CrossRef](#)]
13. Strohfeldt, K.; Tacke, M. Bioorganometallic fulvene-derived titanocene anti-cancer drugs. *Chem. Soc. Rev.* **2008**, *37*, 1174–1187. [[CrossRef](#)]
14. Waern, J.B.; Harding, M.M. Bioorganometallic chemistry of molybdocene dichloride. *J. Organomet. Chem.* **2004**, *689*, 4655–4668. [[CrossRef](#)]
15. Gao, L.M.; Vera, J.L.; Matta, J.; Meléndez, E. Synthesis and cytotoxicity studies of steroid-functionalized titanocenes as potential anticancer drugs: Sex steroids as potential vectors for titanocenes. *J. Biol. Inorg. Chem.* **2010**, *15*, 851–859. [[CrossRef](#)]
16. Dominguez-Garcia, M.; Ortega-Zuniga, C.; Meléndez, E. New tungstenocenes containing 3-hydroxy-4-pyrone ligands: Antiproliferative activity on HT-29 and MCF-7 cell lines and binding to human serum albumin studied by fluorescence spectroscopy and molecular modeling methods. *J. Biol. Inorg. Chem.* **2013**, *18*, 195–209. [[CrossRef](#)]
17. Gomez-Ruiz, S.; Maksimovic-Ivanic, D.; Mijatovic, S.; Kaluderovic, G.N. On the discovery, biological effects, and use of Cisplatin and metallocenes in anticancer chemotherapy. *Bioinorg. Chem. Appl.* **2012**, *2012*, 140284. [[CrossRef](#)]
18. Meléndez, E. Metallocenes as Target Specific Drugs for Cancer Treatment. *Inorg. Chim. Acta* **2012**, *393*, 36–52. [[CrossRef](#)]
19. Kuo, L.Y.; Kanatzidis, M.G.; Sabat, M.; Tipton, A.L.; Marks, T.J. Metallocene Antitumor Agents. Solution and Solid-State Molybdenocene Coordination Chemistry of DNA Constituents. *J. Am. Chem. Soc.* **1991**, *113*, 9027–9045. [[CrossRef](#)]
20. Balzarek, C.; Weakley, T.J.R.; Kuo, L.Y.; Tyler, D.R. Investigation of the Monomer–Dimer Equilibria of Molybdocenes in Water. *Organometallics* **2000**, *19*, 2927–2931. [[CrossRef](#)]
21. Kandioller, W.; Reikersdorfer, M.; Theiner, S.; Roller, A.; Hejl, M.; Jakupec, M.A.; Malarek, M.S.; Keppler, B.K. The Impact of Leaving Group Variation on the Anticancer Activity of Molybdenocenes. *Organometallics* **2018**, *37*, 3909–3916. [[CrossRef](#)]
22. Feliciano, I.; Matta, J.; Meléndez, E. Water-soluble molybdenocene complexes with both proliferative and antiproliferative effects on cancer cell lines and their binding interactions with human serum albumin. *J. Biol. Inorg. Chem.* **2009**, *14*, 1109–1117. [[CrossRef](#)]
23. Meléndez, E. Bioorganometallic Chemistry of Molybdenocene Dichloride and Its Derivatives. *J. Organomet. Chem.* **2012**, *706–707*, 4–12. [[CrossRef](#)]
24. Fuchs, V.; Cseh, K.; Hejl, M.; Vician, P.; Neuditschko, B.; Meier-Menches, S.M.; Janker, L.; Bileck, A.; Gajic, N.; Kronberger, J.; et al. Highly Cytotoxic Molybdenocenes with Strong Metabolic Effects Inhibit Tumour Growth in Mice. *Chem. Eur. J.* **2022**, *29*, e202202648. [[CrossRef](#)]
25. Kandioller, W.; Kurzwernhart, A.; Hanif, M.; Meier, S.M.; Henke, H.; Keppler, B.K.; Hartinger, C.G. Pyrone derivatives and metals: From natural products to metal-based drugs. *J. Organomet. Chem.* **2011**, *696*, 999–1010. [[CrossRef](#)]
26. Kandioller, W.; Hartinger, C.G.; Nazarov, A.A.; Bartel, C.; Skocic, M.; Jakupec, M.A.; Arion, V.B.; Keppler, B.K. Maltol-derived ruthenium-cymene complexes with tumor inhibiting properties: The impact of ligand-metal bond stability on anticancer activity in vitro. *Chemistry* **2009**, *15*, 12283–12291. [[CrossRef](#)]
27. Kandioller, W.; Hartinger, C.G.; Nazarov, A.A.; Kuznetsov, M.L.; John, R.O.; Bartel, C.; Jakupec, M.A.; Arion, V.B.; Keppler, B.K. From Pyrone to Thiopyrone Ligands—Rendering Maltol-Derived Ruthenium(II)–Arene Complexes That Are Anticancer Active in Vitro. *Organometallics* **2009**, *28*, 4249–4251. [[CrossRef](#)]
28. Reddy, V.D.; Dayal, D.; Szalda, D.J.; Cosenza, S.C.; Ramana Reddy, M.V. Syntheses, structures, and anticancer activity of novel organometallic ruthenium–maltol complexes. *J. Organomet. Chem.* **2012**, *700*, 180–187. [[CrossRef](#)]
29. Bhattacharya, S.; Seth, N.; Gupta, V.D.; Nöth, H.; Polborn, K.; Thomann, M.; Schwenk, H. Synthesis and Structure of Organotin(IV) Complexes of Maltol. *Chem. Ber.* **1994**, *127*, 1895–1900. [[CrossRef](#)]
30. Lamboy, J.L.; Pasquale, A.; Rheingold, A.L.; Meléndez, E. Synthesis, Solution and Solid State Structure of Titanium-Maltol Complex. *Inorg. Chim. Acta* **2007**, *360*, 2115–2120. [[CrossRef](#)]
31. Berasaluce, I.; Cseh, K.; Roller, A.; Hejl, M.; Heffeter, P.; Berger, W.; Jakupec, M.A.; Kandioller, W.; Malarek, M.S.; Keppler, B.K. The First Anticancer Tris(pyrazolyl)borate Molybdenum(IV) Complexes: Tested In Vitro and In Vivo—A Comparison of O,O-, S,O-, and N,N-Chelate Effects. *Chem. Eur. J.* **2020**, *26*, 2211–2221. [[CrossRef](#)]
32. Middleton, E.; Kandaswami, C.; Theoharides, T.C. The Effects of Plant Flavonoids on Mammalian Cells: Implications for Inflammation, Heart Disease, and Cancer. *Pharmacol. Rev.* **2000**, *52*, 673–751.
33. Grant, R.S.; Coggan, S.E.; Smythe, G.A. The Physiological Action of Picolinic Acid in the Human Brain. *Int. J. Tryptophan Res. IJTR* **2009**, *2*, 71–79. [[CrossRef](#)] [[PubMed](#)]

34. Yasumoto, E.; Nakano, K.; Nakayachi, T.; Morshed, S.R.M.; Hashimoto, K.; Kikuchi, H.; Nishikawa, H.; Kawase, M.; Sakagami, H. Cytotoxic Activity of Deferiprone, Maltol and Related Hydroxyketones against Human Tumor Cell Lines. *Anticancer Res.* **2004**, *24*, 755–762. [\[PubMed\]](#)
35. Williams, D.B.; Lawton, M. Drying of organic solvents: Quantitative evaluation of the efficiency of several desiccants. *J. Org. Chem.* **2010**, *75*, 8351–8354. [\[CrossRef\]](#)
36. Chaves, S.; Gil, M.; Canario, S.; Jelic, R.; Romao, M.J.; Trincão, J.; Herdtweck, E.; Sousa, J.; Diniz, C.; Fresco, P.; et al. Biologically relevant O,S-donor compounds. Synthesis, molybdenum complexation and xanthine oxidase inhibition. *Dalton Trans.* **2008**, *13*, 1773–1782. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Kurzwernhart, A.; Kandiolle, W.; Bachler, S.; Bartel, C.; Martić, S.; Buczkowska, M.; Mühlgassner, G.; Jakupec, M.A.; Kraatz, H.-B.; Bednarski, P.J.; et al. Structure–Activity Relationships of Targeted Ru^{II}(η^6 -p-Cymene) Anticancer Complexes with Flavonol-Derived Ligands. *J. Med. Chem.* **2012**, *55*, 10512–10522. [\[CrossRef\]](#)
38. Enyedy, E.A.; Sija, E.; Jakusch, T.; Hartinger, C.G.; Kandiolle, W.; Keppler, B.K.; Kiss, T. Solution equilibria of anticancer ruthenium(II)-(eta(6)-p-cymene)-hydroxy(thio)pyr(id)one complexes: Impact of sulfur vs. oxygen donor systems on the speciation and bioactivity. *J. Inorg. Biochem.* **2013**, *127*, 161–168. [\[CrossRef\]](#)
39. Agrawal, A.; Johnson, S.L.; Jacobsen, J.A.; Miller, M.T.; Chen, L.H.; Pellicchia, M.; Cohen, S.M. Chelator fragment libraries for targeting metalloproteinases. *ChemMedChem* **2010**, *5*, 195–199. [\[CrossRef\]](#)
40. Pavlishchuk, V.V.; Addison, A.W. Conversion Constants for Redox Potentials Measured versus Different Reference Electrodes in Acetonitrile Solutions at 25 °C. *Inorg. Chim. Acta* **2000**, *298*, 97–102. [\[CrossRef\]](#)
41. Cseh, K.; Geisler, H.; Stanojkovska, K.; Westermayr, J.; Brunmayr, P.; Wenisch, D.; Gajic, N.; Hejl, M.; Schaiër, M.; Koellensperger, G.; et al. Arene Variation of Highly Cytotoxic Tridentate Naphthoquinone-Based Ruthenium(II) Complexes and In-Depth In Vitro Studies. *Pharmaceutics* **2022**, *14*, 2466. [\[CrossRef\]](#)
42. Scaffidi-Domianello, Y.Y.; Legin, A.; Jakupec, M.A.; Arion, V.B.; Kukushkin, V.Y.; Galanski, M.S.; Keppler, B.K. Synthesis, characterization, and cytotoxic activity of novel potentially pH-sensitive nonclassical platinum(II) complexes featuring 1,3-dihydroxyacetone oxime ligands. *Inorg. Chem.* **2011**, *50*, 10673–10681. [\[CrossRef\]](#)
43. Socrates, G. *Infrared and Raman Characteristic Group Frequencies: Tables and Charts*, 3rd ed.; The University of West London: Middlesex, UK, 2002; Volume 124, p. 1830. [\[CrossRef\]](#)
44. Spek, A.L. Structure Validation in Chemical Crystallography. *Acta Crystallogr. D* **2009**, *65*, 148–155. [\[CrossRef\]](#)
45. Berger, W.; Elbling, L.; Micksche, M. Expression of the major vault protein LRP in human non-small-cell lung cancer cells: Activation by short-term exposure to antineoplastic drugs. *Int. J. Cancer* **2000**, *88*, 293–300. [\[CrossRef\]](#)
46. Kubanik, M.; Tu, J.K.Y.; Söhnle, T.; Hejl, M.; Jakupec, M.A.; Kandiolle, W.; Keppler, B.K.; Hartinger, C.G. Expanding on the Structural Diversity of Flavone-Derived Ruthenium(II)(η^6 -arene) Anticancer Agents. *Metallo drugs* **2015**, *1*, 24–35. [\[CrossRef\]](#)
47. Khater, M.; Ravishankar, D.; Greco, F.; Osborn, H.M. Metal complexes of flavonoids: Their synthesis, characterization and enhanced antioxidant and anticancer activities. *Future Med. Chem* **2019**, *11*, 2845–2867. [\[CrossRef\]](#) [\[PubMed\]](#)
48. Duval, K.; Grover, H.; Han, L.; Mou, Y.; Pegoraro, A.F.; Fredberg, J.; Chen, Z. Modeling Physiological Events in 2D vs. 3D Cell Culture. *Physiology* **2017**, *32*, 266–277. [\[CrossRef\]](#) [\[PubMed\]](#)
49. Fontoura, J.C.; Viezzer, C.; Dos Santos, F.G.; Ligabue, R.A.; Weinlich, R.; Puga, R.D.; Antonow, D.; Severino, P.; Bonorino, C. Comparison of 2D and 3D cell culture models for cell growth, gene expression and drug resistance. *Mater. Sci. Eng. C* **2020**, *107*, 110264. [\[CrossRef\]](#)
50. Nirmalanandhan, V.S.; Duren, A.; Hendricks, P.; Vielhauer, G.; Sittampalam, G.S. Activity of anticancer agents in a three-dimensional cell culture model. *Drug Dev. Technol.* **2010**, *8*, 581–590. [\[CrossRef\]](#)
51. Ravi, M.; Paramesh, V.; Kaviya, S.R.; Anuradha, E.; Paul Solomon, F.D. 3D Cell Culture Systems Advantages and Applications. *J. Cell. Physiol.* **2014**, *230*, 16–26. [\[CrossRef\]](#)
52. Wang, H.; Brown, P.C.; Chow, E.C.Y.; Ewart, L.S.; Ferguson, S.S.; Fitzpatrick, S.; Freedman, B.S.; Guo, G.L.; Hedrich, W.; Heyward, S.; et al. 3D cell culture models: Drug pharmacokinetics, safety assessment, and regulatory consideration. *Clin. Transl. Sci.* **2021**, *14*, 1659–1680. [\[CrossRef\]](#)
53. Bonnier, F.; Keating, M.E.; Wróbel, T.P.; Majzner, K.; Baranska, M.; Garcia-Munoz, A.; Blanco, A.; Byrne, H.J. Cell viability assessment using the Alamar blue assay: A comparison of 2D and 3D cell culture models. *Toxicol. In Vitro* **2015**, *29*, 124–131. [\[CrossRef\]](#)
54. Uxa, S.; Castillo-Binder, P.; Kohler, R.; Stangner, K.; Müller, G.A.; Engeland, K. Ki-67 gene expression. *Cell Death Differ.* **2021**, *28*, 3357–3370. [\[CrossRef\]](#)
55. Sun, X.; Kaufman, P.D. Ki-67: More than a proliferation marker. *Chromosoma* **2018**, *127*, 175–186. [\[CrossRef\]](#)
56. Li, L.T.; Jiang, G.; Chen, Q.; Zheng, J.N. Ki67 is a promising molecular target in the diagnosis of cancer (Review). *Mol. Med. Rep* **2015**, *11*, 1566–1572. [\[CrossRef\]](#)
57. Sharma, B.; Kanwar, S.S. Phosphatidylserine: A cancer cell targeting biomarker. *Semin. Cancer Biol.* **2018**, *52*, 17–25. [\[CrossRef\]](#)
58. Nagata, S. Apoptosis and Clearance of Apoptotic Cells. *Annu. Rev. Immunol.* **2018**, *36*, 489–517. [\[CrossRef\]](#)
59. Kupcho, K.; Shultz, J.; Hurst, R.; Hartnett, J.; Zhou, W.; Machleidt, T.; Grailer, J.; Worzella, T.; Riss, T.; Lazar, D.; et al. A real-time, bioluminescent annexin V assay for the assessment of apoptosis. *Apoptosis* **2019**, *24*, 184–197. [\[CrossRef\]](#)
60. Eray, M.; Mättö, M.; Kaartinen, M.; Andersson, L.C.; Pelkonen, J. Flow Cytometric Analysis of Apoptotic Subpopulations with a Combination of Annexin V-FITC, Propidium Iodide, and SYTO 17. *Cytometry* **2001**, *43*, 134–142. [\[CrossRef\]](#)

61. Göschl, S.; Schreiber-Brynzak, E.; Pichler, V.; Cseh, K.; Heffeter, P.; Jungwirth, U.; Jakupec, M.A.; Berger, W.; Keppler, B.K. Comparative studies of oxaliplatin-based platinum(IV) complexes in different in vitro and in vivo tumor models. *Metallomics* **2017**, *9*, 309–322. [[CrossRef](#)] [[PubMed](#)]
62. Valiahdi, S.M.; Heffeter, P.; Jakupec, M.A.; Marculescu, R.; Berger, W.; Rappersberger, K.; Keppler, B.K. The gallium complex KP46 exerts strong activity against primary explanted melanoma cells and induces apoptosis in melanoma cell lines. *Melanoma Res.* **2009**, *19*, 283–293. [[CrossRef](#)] [[PubMed](#)]
63. Geisler, H.; Westermayr, J.; Cseh, K.; Wenisch, D.; Fuchs, V.; Harringer, S.; Plutzar, S.; Gajic, N.; Hejl, M.; Jakupec, M.A.; et al. Tridentate 3-Substituted Naphthoquinone Ruthenium Arene Complexes: Synthesis, Characterization, Aqueous Behavior, and Theoretical and Biological Studies. *Inorg. Chem.* **2021**, *60*, 9805–9819. [[CrossRef](#)] [[PubMed](#)]

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SUPPORTING INFORMATION

Anticancer tungstenocenes with a diverse set of (O,O–) (O,S–) and (O,N–) chelates – a detailed biological study using an improved evaluation *via* 3D spheroid models

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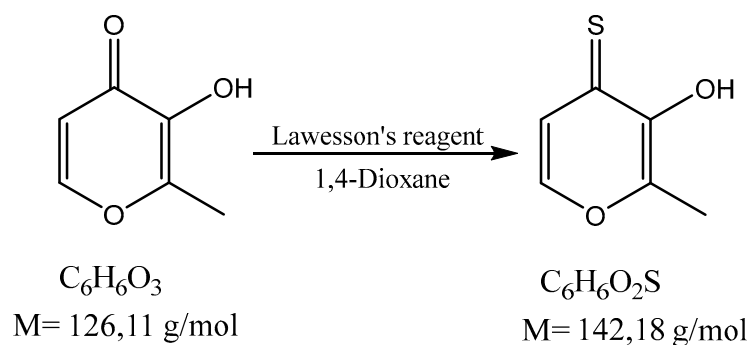
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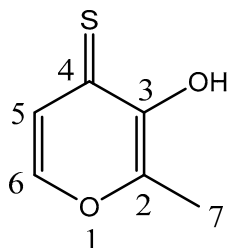
3-Hydroxy-2-methyl-4H-pyran-4-thione (L2)



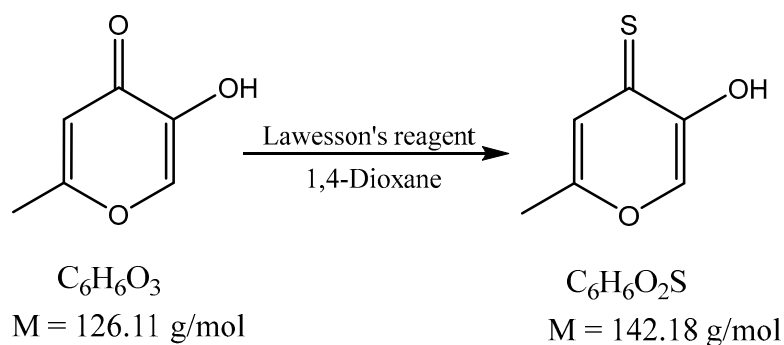
Maltol (1.00 g, 8.2 mmol) and Lawesson's reagent (1.70 g, 4.2 mmol) were dissolved in 1,4-dioxane (20 mL) and refluxed for 4 hours. The solvent was removed and after column chromatography (n-hexane/ethyl acetate = 10:1) the yellow crystalline product was obtained and dried *in vacuo*.

Yield: 0.368 mg (32%), yellow crystals

NMR-Spectroscopy: ^1H -NMR (d_6 -DMSO): $\delta = 2.40$ (s, 3H, CH_3), 7.35 (d, 1H, $^3J(\text{H}, \text{H}) = 5 \text{ Hz}$, H5), 8.0 (d, 1H, $^3J(\text{H}, \text{H}) = 5 \text{ Hz}$, H6), 8.28 (s, 1H, OH).



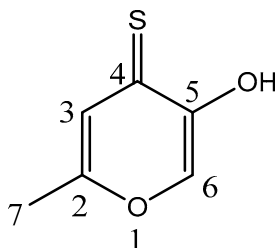
5-Hydroxy-2-methyl-4H-pyran-4-thione (L8)



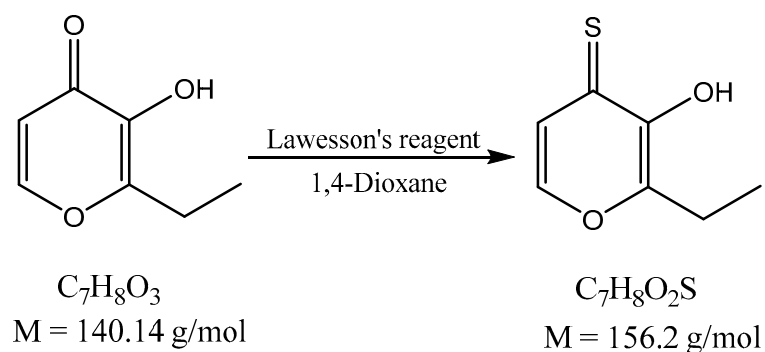
Allomaltol (1.00 g, 8.2 mmol) and Lawesson's reagent (1.70 g, 4.2 mmol) were dissolved in 1,4-dioxane (20 mL) and refluxed for 4 hours. The solvent was removed and after column chromatography (n-hexane/ethyl acetate = 10:1) the yellow crystalline product was obtained and dried *in vacuo*.

Yield: 0.210 mg (17%), yellow crystals

NMR-Spectroscopy: ¹H-NMR (d₆-DMSO): δ = 2.33 (s, 3H, CH₃), 7.35 (s, 1H, H3), 8.27 (s, 1H, H6), 8.42 (s, 1H, OH).



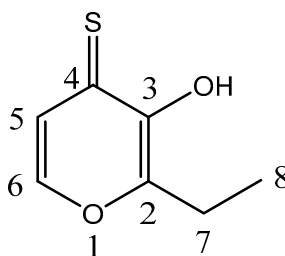
2-Ethyl-3-hydroxy-4H-pyran-4-thione (L10)



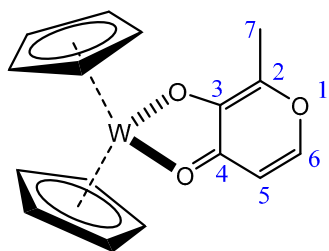
Ethylmaltol (2.00 g, 14.3 mmol) and Lawesson's reagent (1.92 g, 4.8 mmol) were dissolved in 1,4-dioxane (40 mL) and refluxed for 4 hours. The solvent was removed and after column chromatography (n-hexane/ethyl acetate = 10:1) the yellow product was obtained and dried *in vacuo*.

Yield: 1.304g (59%), yellow-orange oil

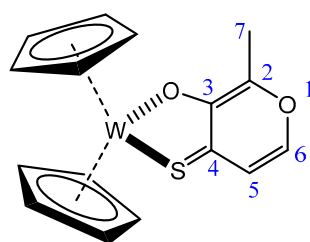
NMR-Spectroscopy: ^1H -NMR (d_6 -DMSO): $\delta = 1.22$ (t, 3H, $^3J(\text{H}, \text{H}) = 7.6 \text{ Hz}$, CH_3), 2.78 (q, 2H, $^3J(\text{H}, \text{H}) = 7.6 \text{ Hz}$, CH_2), 7.36 (d, 1H, $^3J(\text{H}, \text{H}) = 5 \text{ Hz}$, H5), 8.13 (d, 1H, $^3J(\text{H}, \text{H}) = 4.9 \text{ Hz}$, H6), 8.29 (s, 1H, OH).



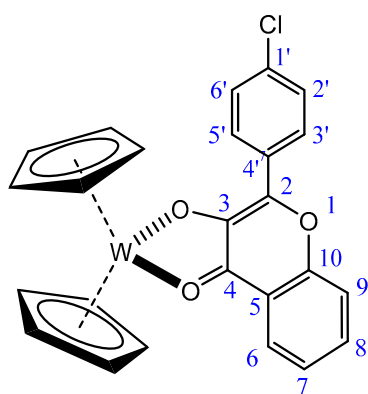
NMR-spectroscopy



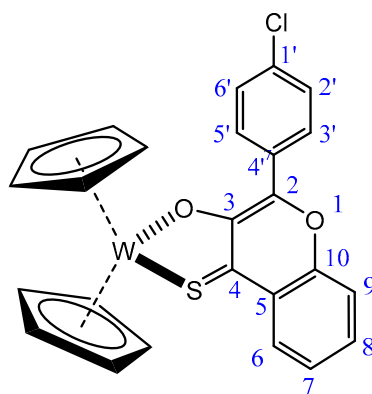
1



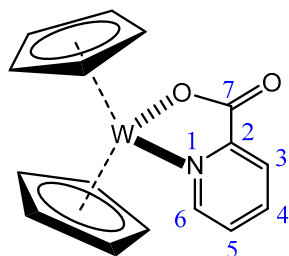
2



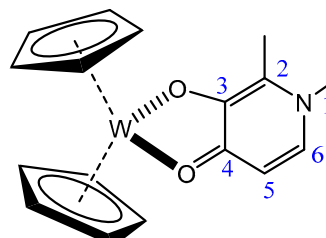
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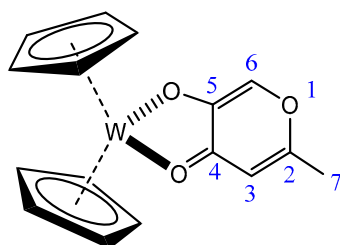
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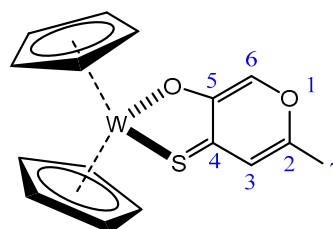
5



6



7



8

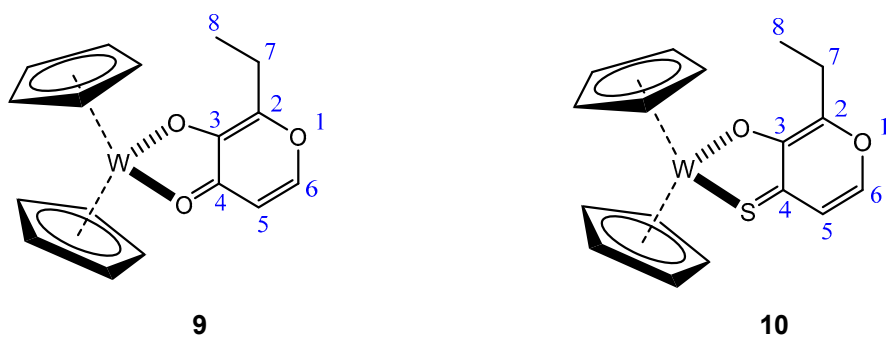


Figure S1: Complex atom numbering for NMR peak assignments of compounds **1–10**.

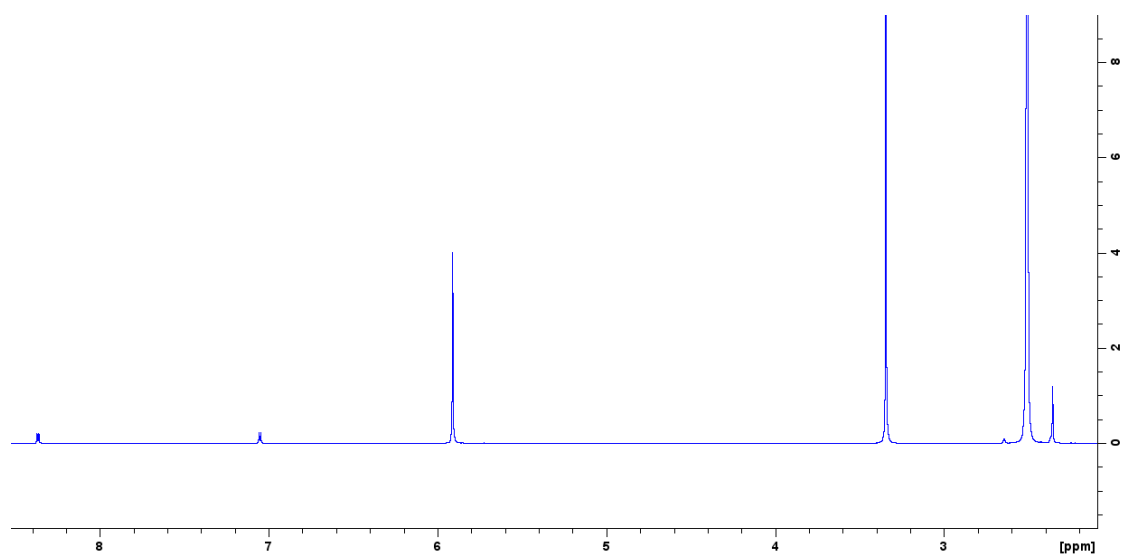


Figure S2: ^1H NMR of compound **1** measured in DMSO-d_6 .

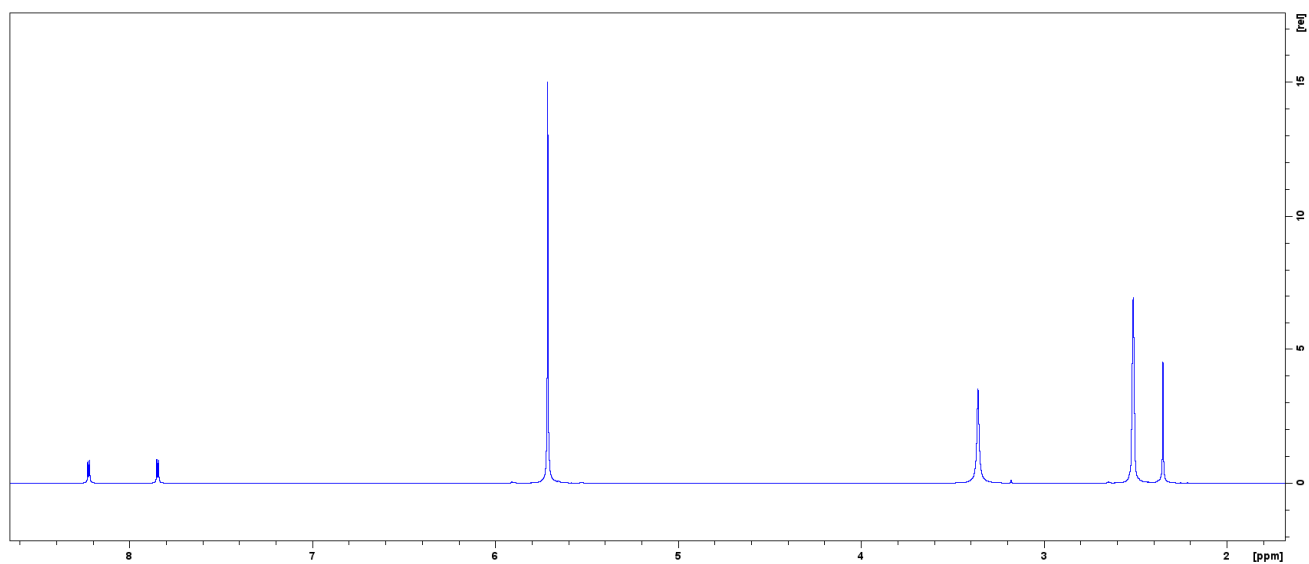


Figure S3: ^1H NMR of compound **2** measured in DMSO-d_6 .

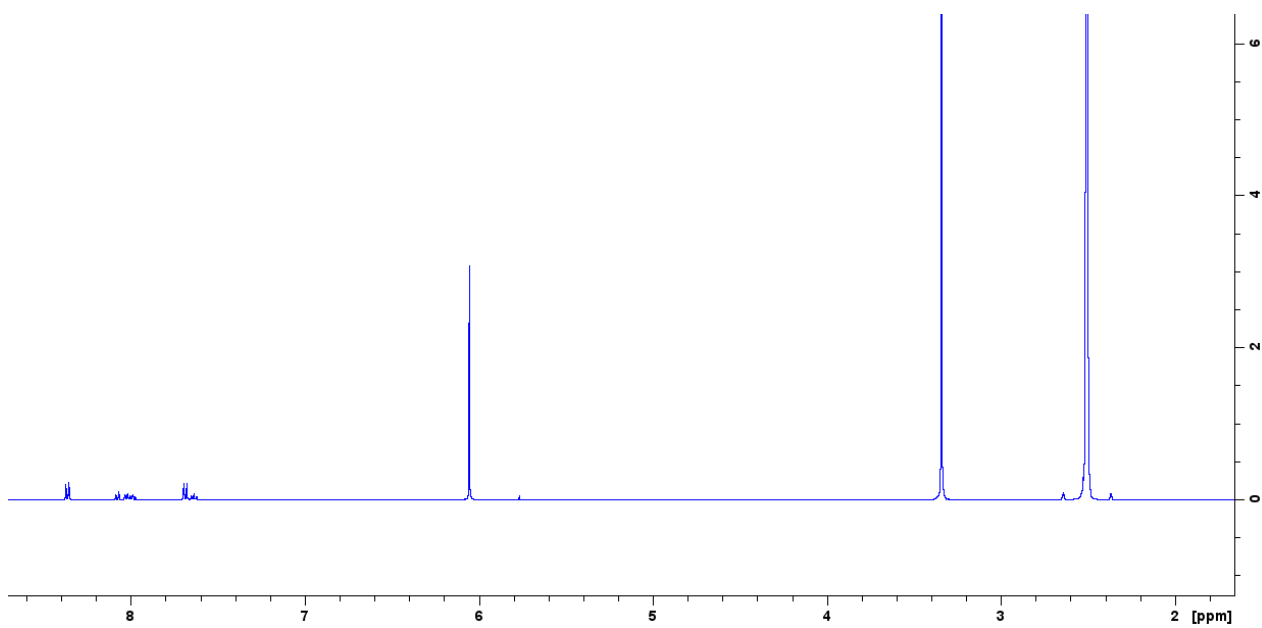


Figure S4: ^1H NMR of compound **3** measured in DMSO-d_6 .

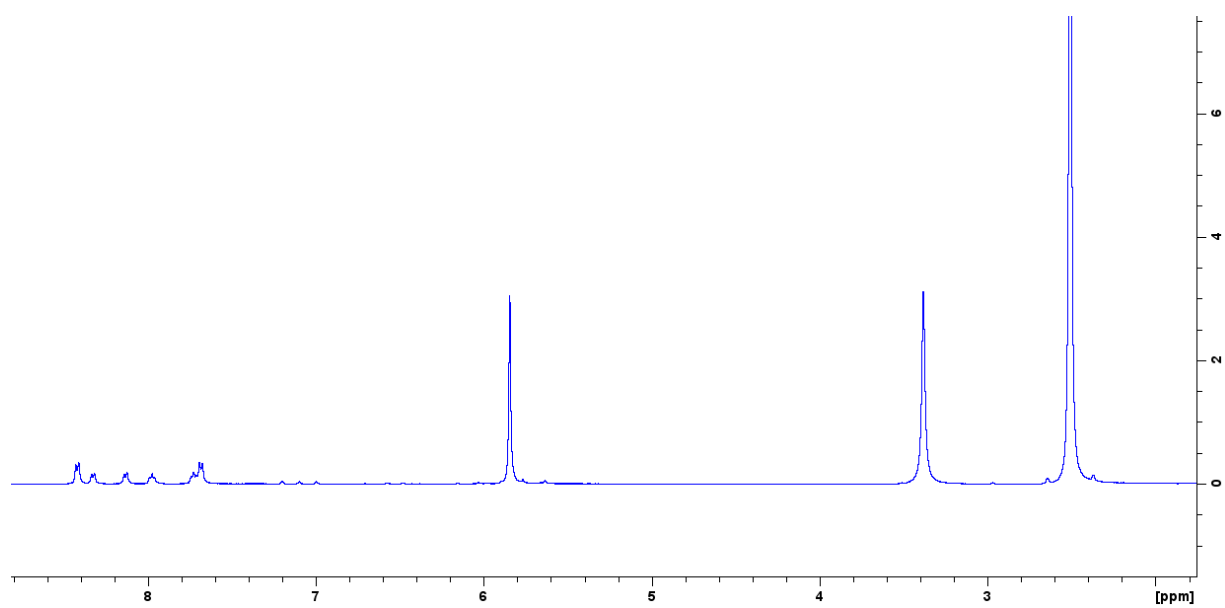


Figure S5: ^1H NMR of compound **4** measured in DMSO-d_6 .

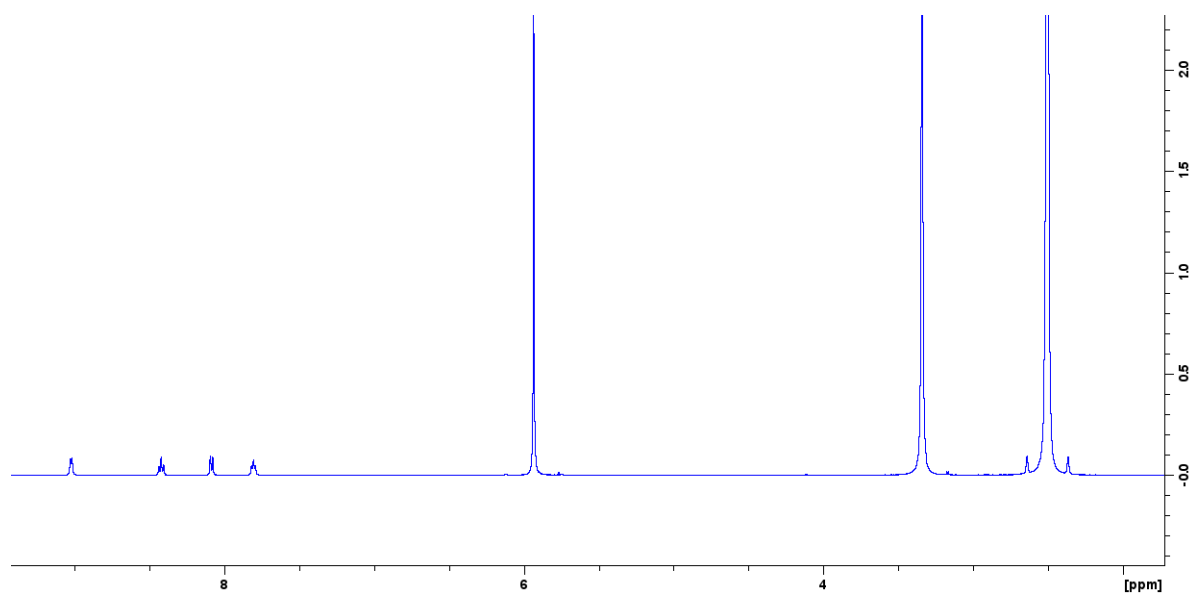


Figure S6: ^1H NMR of compound **5** measured in DMSO-d_6 .

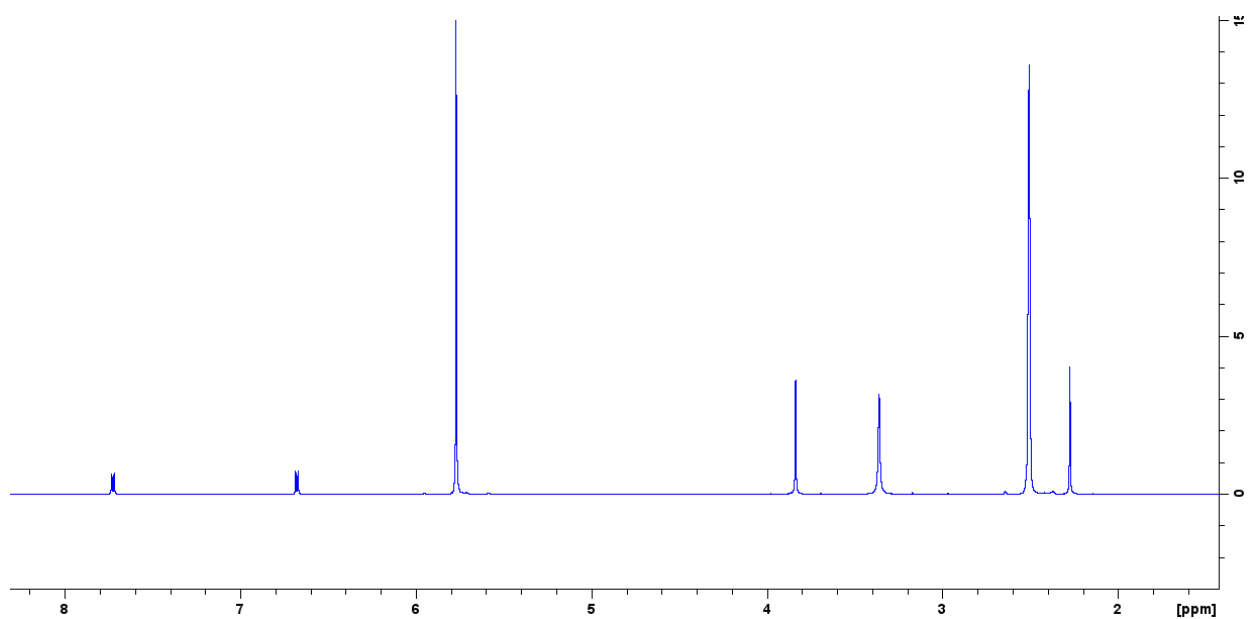


Figure S7: ^1H NMR of compound **6** measured in DMSO-d_6 .

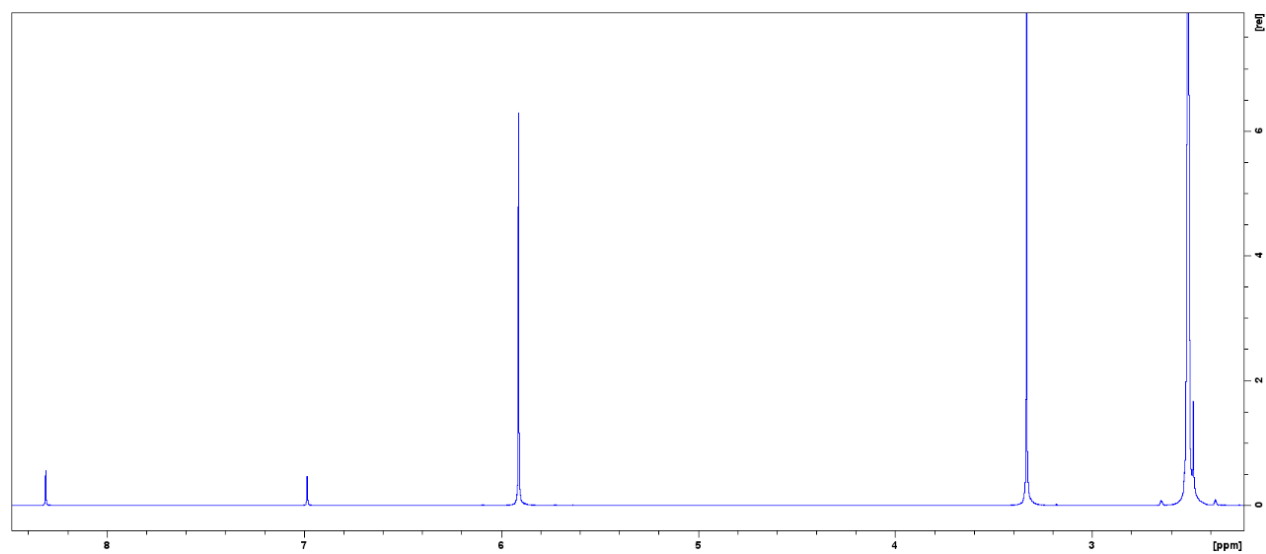


Figure S8: ^1H NMR of compound **7** measured in DMSO-d_6 .

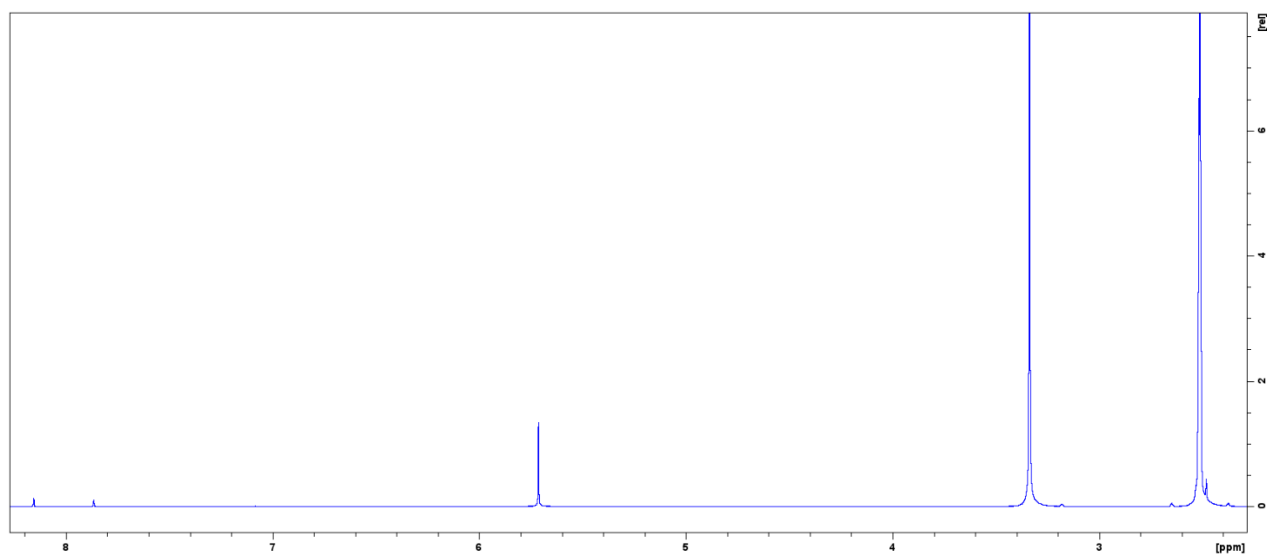


Figure S9: ^1H NMR of compound **8** measured in DMSO-d_6 .

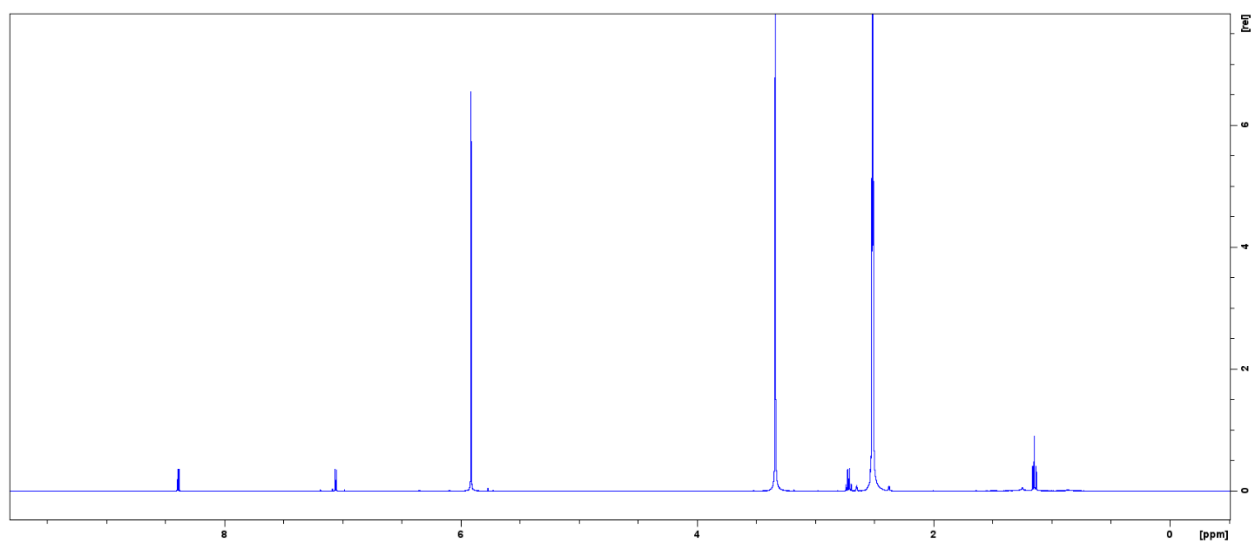


Figure S10: ^1H NMR of compound **9** measured in DMSO-d_6 .

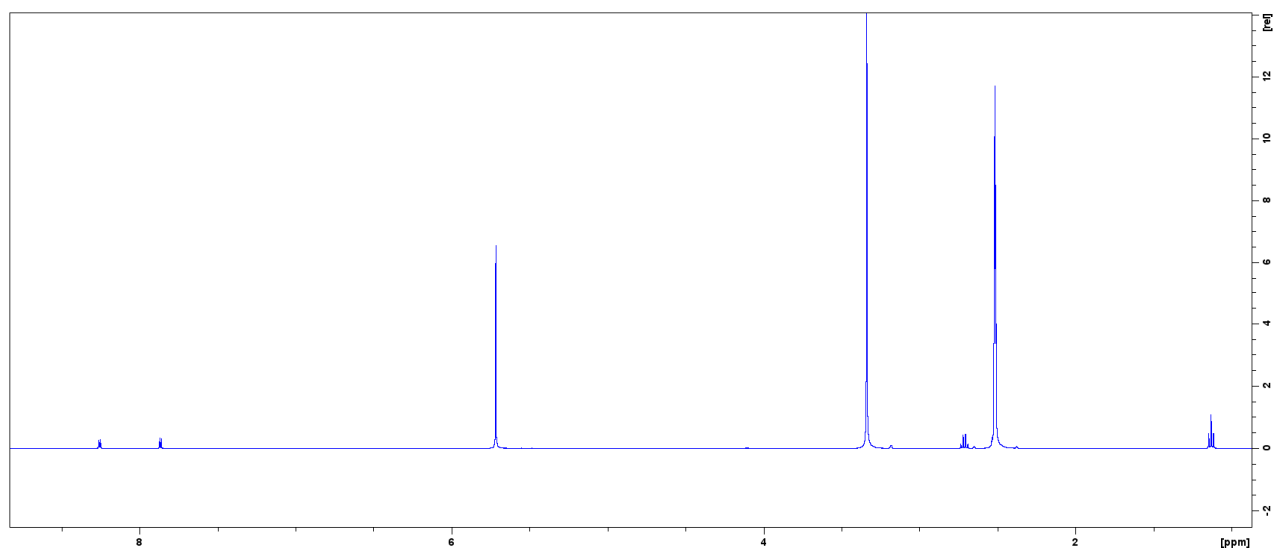


Figure S11: ^1H NMR of compound **10** measured in DMSO-d_6 .

Mass Spectrometry

Table S1: ESI-MS experimental and theoretical m/z values of compounds **1–10**.

Detected ion	m/z	$[\text{Cp}_2\text{W}(\text{L})]^+_{\text{calc}}$
$[\text{Cp}_2\text{W}(\text{L1})]^+$	439.05	439.05
$[\text{Cp}_2\text{W}(\text{L2})]^+$	455.17	455.03
$[\text{Cp}_2\text{W}(\text{L3})]^+$	585.05	585.05
$[\text{Cp}_2\text{W}(\text{L4})]^+$	601.02	601.10
$[\text{Cp}_2\text{W}(\text{L5})]^+$	436.05	436.05
$[\text{Cp}_2\text{W}(\text{L6})]^+$	452.08	452.07
$[\text{Cp}_2\text{W}(\text{L7})]^+$	439.02	439.05
$[\text{Cp}_2\text{W}(\text{L8})]^+$	454.98	455.03
$[\text{Cp}_2\text{W}(\text{L9})]^+$	452.97	453.07
$[\text{Cp}_2\text{W}(\text{L10})]^+$	468.93	469.05

X-ray diffraction analysis

Table S2: Experimental parameters.

Sample	Machine	Source	Temp.	Detector Distance	Time/ Frame	#Frames	Frame width	CCDC
	Bruker		[K]	[mm]	[s]		[°]	
1 KP2576	D8	Mo	100.0	40	6	180	1.000	2074976
2 Alba112	D8	Mo	100.0	34	10	473	0.500	2074974
3 Ikbe318a	X8	Mo	100.0	35	60	1100	0.500	2074975
4 Ikbe313a	X8	Mo	100.0	35	60	1100	0.500	2074977
5 Ikbe335a	D8	Cu	100.0	34	10	5316	0.500	2074979
6 Ikbe334a	X8	Mo	100.0	35	10	5615	0.500	2074980
7 Alba119	D8	Mo	100.0	35	6	758	0.500	2074978
8 Alba115	D8	Mo	100.0	35	50	2168	0.500	2074981
9 Alba139	D8	Mo	100.0	35	60	239	0.500	2074982
10 Alba147	D8	Mo	100.0	35	7	5150	0.500	2074983

Bis(η^5 -cyclopentadienyl)[2-methyl-3-(oxo- κ O)-4-(1H)-pyran-4-ato- κ O]tungsten(IV) chloride, **1A**

Table S3: Sample and crystal data of compound **1A**.

Chemical formula	C ₁₆ H ₁₅ ClO ₃ W	Crystal system	orthorhombic	
Formula weight [g/mol]	474.58	Space group	Cmca	
Temperature [K]	100	Z	8	
Measurement method	$\backslash$ f and $\backslash$ w scans	Volume [Å³]	3638.3(7)	
Radiation (Wavelength [Å])	MoK α (λ = 0.71073)	Unit cell dimensions [Å] and [°]	12.8287(10)	90
Crystal size / [mm³]	0.2 × 0.1 × 0.09		24.661(3)	90
Crystal habit	clear red needle		11.5002(12)	90
Density (calculated) / [g/cm³]	1.733	Absorption coefficient / [mm⁻¹]	6.502	
Abs. correction Tmin	0.6395	Abs. correction Tmax	0.7452	
Abs. correction type	multiscan	F(000) [e⁻]	1808	

Table S4: Data collection and structure refinement of compound **1A**.

Index ranges	-15 ≤ h ≤ 14, -28 ≤ k ≤ 29, -13 ≤ l ≤ 13	Theta range for data collection [°]	4.844 to 50.724	
Reflections number	11264	Data / restraints / parameters	1750/24/127	
Refinement method	Least squares	Final R indices	all data	R1 = 0.0331, wR2 = 0.0497
Function minimized	$\sum w(F_o^2 - F_c^2)^2$		I > 2σ(I)	R1 = 0.0245, wR2 = 0.0468
Goodness-of-fit on F²	1.062	Weighting scheme	w=1/[σ ² (F _o ²)+(0.0116P) ² +4.3718P]	
Largest diff. peak and hole [e Å⁻³]	0.57/-0.66		where P=(F _o ² +2F _c ²)/3	

Bis(η^5 -cyclopentadienyl)[2-methyl-3-(oxo- κ O)-4-(1H)-pyran-4-thionato- κ S]tungsten(IV) hexafluorophosphate, **2**.

Table S5: Sample and crystal data of compound **2**.

Chemical formula	C ₁₆ H ₁₅ O ₂ SWPF ₆	Crystal system	orthorhombic	
Formula weight [g/mol]	600.16	Space group	Pnma	
Temperature [K]	100	Z	4	
Measurement method	$\backslash\Phi$ and $\backslash\omega$ scans	Volume [Å³]	1745.34(7)	
Radiation (Wavelength [Å])	MoK α (λ = 0.71073)	Uni cell dimensions [Å] and [°]	16.7909(4)	90
Crystal size / [mm³]	0.1 × 0.07 × 0.03		8.3122(2)	90
Crystal habit	clear red block		12.5052(3)	90
Density calculated / [g/cm³]	2.284	Absorption coefficient / [mm⁻¹]	6.901	
Abs. correction type	multiscan	F(000) [e⁻]	1144.0	

Table S6: Data collection and structure refinement of compound **2**.

Index ranges	-23 ≤ h ≤ 23, -10 ≤ k ≤ 11, -17 ≤ l ≤ 17	Theta range for data collection [°]	4.852 to 60.138	
Reflections number	14654	Data / restraints / parameters	2718/0/143	
Refinement method	Least squares	Final R indices	[all data]	R ₁ = 0.0185, wR ₂ = 0.0355
Function minimized	$\sum w(F_o^2 - F_c^2)^2$		>=2σ (I)	R ₁ = 0.0156, wR ₂ = 0.0347
Goodness-of-fit on F²	1.065	Weighting scheme	w=1/[σ ² (F _o ²)+(0.0182P) ² + 0.0507P]	
Largest diff. peak and hole [e Å⁻³]	0.46/-1.22		where P=(F _o ² +2F _c ²)/3	

Bis(η^5 -cyclopentadienyl)[2-(4-chlorophenyl)-3-(oxo- κ O)-4(H)-chromen-4-oato- κ O]tungsten(IV) hexafluorophosphate, **3**.

Table S7: Sample and crystal data of compound 3.

Chemical formula	C ₂₈ H ₂₄ ClF ₆ O ₄ PW	Crystal system	monoclinic	
Formula weight [g/mol]	788.74	Space group	$P2_1/n$	
Temperature [K]	100.0	Z	4	
Measurement method	$\backslash\Phi$ and $\backslash\omega$ scans	Volume [Å³]	2687.6(7)	
Radiation (Wavelength [Å])	MoK α (λ = 0.71073)	Unit cell dimensions [Å] and [°]	11.5665(17))	90
Crystal size / [mm³]	0.1 × 0.09 × 0.02		15.593(2)	99.365(7)
Crystal habit	clear brown plate		15.103(2)	90
Density (calculated) / [g/cm³]	1.949	Absorption coefficient / [mm⁻¹]	4.534	
Abs. correction Tmin	0.5993	Abs. correction Tmax	0.7452	
Abs. correction type	multiscan	F(000) [e⁻]	1536.0	

Table S8: Data collection and structure refinement of compound 3.

Index ranges	-13 ≤ h ≤ 13, -18 ≤ k ≤ 18, -17 ≤ l ≤ 18	Theta range for data collection [°]	4.422 to 50.792	
Reflections number	33217	Data / restraints / parameters	4860/24/372	
Refinement method	Least squares	Final R indices	all data	R1 = 0.1453, wR2 = 0.1280
Function minimized	$\sum w(F_o^2 - F_c^2)^2$		$I > 2\sigma(I)$	R1 = 0.0592, wR2 = 0.1023
Goodness-of-fit on F^2	0.966	Weighting scheme	$w = 1/[\sigma^2(F_o^2) + (0.0533P)^2]$	
Largest diff. peak and hole [e Å⁻³]	1.35/-1.80		where $P = (F_o^2 + 2F_c^2)/3$	

Bis(η^5 -cyclopentadienyl)[2-(4-chlorophenyl)-3-(oxo- κ O)-4(H)-chromen-4-thioato- κ S]tungsten(IV) hexafluorophosphate, **4**.

Table S9: Sample and crystal data of compound 4.

Chemical formula	C ₂₈ H ₂₄ ClF ₆ O ₃ PSW	Crystal system	monoclinic	
Formula weight [g/mol]	804.80	Space group	$P2_1/n$	
Temperature [K]	100.0	Z	4	
Measurement method	$\backslash\Phi$ and $\backslash\omega$ scans	Volume [Å³]	2749.9(2)	
Radiation (Wavelength [Å])	MoK α (λ = 0.71073)	Unit cell dimensions [Å] and [°]	11.4548(5)	90
Crystal size / [mm³]	0.2 × 0.1 × 0.08		15.8196(7)	98.874(2)
Crystal habit	clear brown block		15.3591(7)	90
Density (calculated) / [g/cm³]	1.944	Absorption coefficient / [mm⁻¹]	4.504	
Abs. correction Tmin	0.5497	Abs. correction Tmax	0.7460	
Abs. correction type	multiscan	F(000) [e⁻]	1568.0	

Table S10: Data collection and structure refinement of compound 4.

Index ranges	-16 ≤ h ≤ 16, -22 ≤ k ≤ 22, -21 ≤ l ≤ 21	Theta range for data collection [°]	4.426 to 60.558	
Reflections number	191709	Data / restraints / parameters	8120/1/391	
Refinement method	Least squares	Final R indices	all data	R1 = 0.0251, wR2 = 0.0499
Function minimized	$\sum w(F_o^2 - F_c^2)^2$		$ I > 2\sigma(I)$	R1 = 0.0200, wR2 = 0.0464
Goodness-of-fit on F^2	1.070	Weighting scheme	$w = 1/[\sigma^2(F_o^2) + (0.0186P)^2 + 3.9570P]$	
Largest diff. peak and hole [e Å⁻³]	1.15/-0.78		where $P = (F_o^2 + 2F_c^2)/3$	

Bis(η^5 -cyclopentadienyl)[2-(carboxylato- κ O)-pyridine- κ S]tungsten(IV) hexafluorophosphate, **5**.

Table S11: Sample and crystal data of compound 5.

Chemical formula	C ₁₆ H ₁₄ F ₆ NO ₂ PW	Crystal system	orthorhombic	
Formula weight [g/mol]	581.10	Space group	<i>Pca</i> 2 ₁	
Temperature [K]	100.0	Z	4	
Measurement method	$\backslash \Phi$ and $\backslash \omega$ scans	Volume [Å³]	1685.40(15)	
Radiation (Wavelength [Å])	MoK α (λ = 0.71073)	Unit cell dimensions [Å] and [°]	12.3684(6)	90
Crystal size / [mm³]	0.388 × 0.267 × 0.026		9.9666(5)	90
Crystal habit	clear brown plate		13.6723(7)	90
Density (calculated) / [g/cm³]	2.290	Absorption coefficient / [mm⁻¹]	14.334	
Abs. correction Tmin	0.3492	Abs. correction Tmax	0.7536	
Abs. correction type	multiscan	F(000) [e⁻]	1104.0	

Table S12: Data collection and structure refinement of compound 5.

Index ranges	-14 ≤ h ≤ 14, -11 ≤ k ≤ 11, -16 ≤ l ≤ 14	Theta range for data collection [°]	6.464 to 130.124	
Reflections number	33349	Data / restraints / parameters	2841/1/245	
Refinement method	Least squares	Final R indices	all data	R1 = 0.0381, wR2 = 0.1051
Function minimized	$\sum w(F_o^2 - F_c^2)^2$		$I > 2\sigma(I)$	R1 = 0.0371, wR2 = 0.0997
Goodness-of-fit on F^2	1.107	Weighting scheme	$w = 1/[\sigma^2(F_o^2) + (0.0748P)^2 + 1.5724P]$	
Largest diff. peak and hole [e Å⁻³]	3.51/-1.43		where $P = (F_o^2 + 2F_c^2)/3$	

Bis(η^5 -cyclopentadienyl)[1,2-dimethyl-3-(oxo- κ O)-4-(1H)-pyridonato- κ O]tungsten(IV) hexafluorophosphate, **6**.

Table S13: Sample and crystal data of compound 6.

Chemical formula	C ₁₇ H ₁₈ F ₆ NO ₂ PW	Crystal system	orthorhombic	
Formula weight [g/mol]	597.14	Space group	<i>Pna</i> 2 ₁	
Temperature [K]	100.0	Z	4	
Measurement method	$\backslash \Phi$ and $\backslash \omega$ scans	Volume [Å³]	1782.06(14)	
Radiation (Wavelength [Å])	MoK α (λ = 0.71073)	Unit cell dimensions [Å] and [°]	20.8991(10)	90
Crystal size / [mm³]	0.15 × 0.13 × 0.04		7.9947(4)	90
Crystal habit	clear brown plate		10.6658(4)	90
Density (calculated) / [g/cm³]	2.226	Absorption coefficient / [mm⁻¹]	6.646	
Abs. correction Tmin	0.5250	Abs. correction Tmax	0.7460	
Abs. correction type	multiscan	F(000) [e⁻]	1144.0	

Table S14: Data collection and structure refinement of compound 6.

Index ranges	-29 ≤ h ≤ 29, -11 ≤ k ≤ 11, -15 ≤ l ≤ 14	Theta range for data collection [°]	1.948 to 60.322	
Reflections number	115981	Data / restraints / parameters	5108/1/280	
Refinement method	Least squares	Final R indices	all data	R1 = 0.0139, wR2 = 0.0311
Function minimized	$\sum w(F_o^2 - F_c^2)^2$		$I > 2\sigma(I)$	R1 = 0.0131, wR2 = 0.0307
Goodness-of-fit on F²	1.045	Weighting scheme	$w = 1/[\sigma^2(F_o^2) + (0.0142P)^2 + 0.4852P]$	
Largest diff. peak and hole [e Å⁻³]	1.36/-0.69		where $P = (F_o^2 + 2F_c^2)/3$	

Bis(η^5 -cyclopentadienyl)[2-methyl-5-(oxo- κ O)-4-(1H)-pyran-4-ato- κ O] hexafluorophosphate, **7**.

tungsten(IV)

Table S15: Sample and crystal data of compound **7**.

Chemical formula	C ₁₆ H ₁₅ F ₆ O ₃ PW	Crystal system	orthorhombic	
Formula weight [g/mol]	584.10	Space group	Pmn2 ₁	
Temperature [K]	100	Z	2	
Measurement method	\Phi and \omega scans	Volume [Å³]	854.39(7)	
Radiation (Wavelength [Å])	MoK α (λ = 0.71073)	Unit cell dimensions [Å] and [°]	8.5280(4)	90
Crystal size / [mm³]	0.14 × 0.14 × 0.04		6.8174(3)	90
Crystal habit	Clear brown block		14.6957(6)	90
Density calculated / [g/cm³]	2.270	Absorption coefficient / [mm⁻¹]	6.931	
Abs. correction type	multiscan	F(000) [e⁻]	556.0	

Table S16: Data collection and structure refinement of compound **7**.

Index ranges	-10 ≤ h ≤ 10, -8 ≤ k ≤ 8, -17 ≤ l ≤ 17	Theta range for data collection [°]	5.522 to 50.692	
Reflections number	6904	Data / restraints / parameters	1666/1/143	
Refinement method	Least squares	Final R indices	[all data]	R ₁ = 0.0283, wR ₂ = 0.0647
Function minimized	$\sum w(F_o^2 - F_c^2)^2$		I ≥ 2σ (I)	R ₁ = 0.0269, wR ₂ = 0.0642
Goodness-of-fit on F²	1.059	Weighting scheme		w=1/[σ ² (F _o ² +(0.0383P) ² +1.1113P]
Largest diff. peak and hole [e Å⁻³]	2.08/-0.65			where P=(F _o ² +2F _c ²)/3

Bis(η^5 -cyclopentadienyl)[2-methyl-5-(oxo- κ O)-pyran-4-(1H)-thionato- κ S]
hexafluorophosphate, **8**.

tungsten(IV)

Table S17: Sample and crystal data of compound **8**.

Chemical formula	C ₁₆ H ₁₅ F ₆ O ₂ PSW	Crystal system	monoclinic	
Formula weight [g/mol]	600.16	Space group	P2 ₁ /c	
Temperature [K]	100	Z	4	
Measurement method	\Phi and \omega scans	Volume [Å³]	1730.5(6)	
Radiation (Wavelength [Å])	MoK α (λ =0.71073)	Unit cell dimensions [Å] and [°]	7.6463(16)	90
Crystal size / [mm³]	0.3 × 0.2 × 0.01		20.383(4)	93.003(7)
Crystal habit	clear brown block		11.118(2)	90
Density calculated / [g/cm³]	2.304	Absorption coefficient / [mm⁻¹]	6.960	
Abs. correction type	multiscan	F(000) [e⁻]	1144.0	

Table S18: Data collection and structure refinement of compound **8**.

Index ranges	-8 ≤ h ≤ 9, -24 ≤ k ≤ 24, -13 ≤ l ≤ 13	Theta range for data collection [°]	5.334 to 50.698	
Reflections number	18772	Data / restraints / parameters	3111/60/245	
Refinement method	Least squares	Final R indices	[all data]	R ₁ = 0.0859, wR ₂ = 0.1671
Function minimized	$\sum w(F_o^2 - F_c^2)^2$		>=2σ (I)	R ₁ = 0.0671, wR ₂ = 0.1546
Goodness-of-fit on F²	1.164	Weighting scheme	w=1/[σ ² +(F _o ² +(0.0175P) ² +93.7428P]	
Largest diff. peak and hole [e Å⁻³]	2.13/-2.36		where P=(F _o ² +2F _c ²)/3	

Bis(η^5 -cyclopentadienyl)[2-ethyl-3-(oxo- κ O)-4-(1*H*)-pyran-4-ato- κ O]
hexafluorophosphate, **9**.

tungsten(IV)

Table S19: Sample and crystal data of compound **9**.

Chemical formula	C ₁₇ H ₁₇ F ₆ O ₃ PW	Crystal system	orthorhombic	
Formula weight [g/mol]	598.12	Space group	Pnma	
Temperature [K]	100	Z	4	
Measurement method	$\backslash \Phi$ and $\backslash \omega$ scans	Volume [Å³]	1782.58(12)	
Radiation (Wavelength [Å])	MoK α (λ = 0.71073)	Unit cell dimensions [Å] and [°]	13.7943(4)	90
Crystal size / [mm³]	0.12 × 0.11 × 0.03		8.3019(3)	90
Crystal habit	clear brown block		15.5658(7)	90
Density calculated / [g/cm³]	2.229	Absorption coefficient / [mm⁻¹]	6.647	
Abs. correction type	multiscan	F(000) [e⁻]	1144.0	

Table S20: Data collection and structure refinement of compound **9**.

Index ranges	-10 ≤ h ≤ 16, -9 ≤ k ≤ 7, -11 ≤ l ≤ 18	Theta range for data collection [°]	5.562 to 50.688	
Reflections number	4136	Data / restraints / parameters	1732/0/149	
Refinement method	Least squares	Final R indices	[all data]	R ₁ = 0.0339, wR ₂ = 0.0577
Function minimized	$\sum w(F_o^2 - F_c^2)^2$		>=2σ (I)	R ₁ = 0.0252, wR ₂ = 0.0548
Goodness-of-fit on F²	1.048	Weighting scheme	w=1/[σ ² +(F _o ² +(0.0228P) ² +5.4016P]	
Largest diff. peak and hole [e Å⁻³]	1.10/-1.11		where P=(F _o ² +2F _c ²)/3	

Bis(η^5 -cyclopentadienyl)[2-ethyl-3-(oxo- κ O)-4-(1H)-pyran-4-ato- κ S]
hexafluorophosphate, **10**.

tungsten(IV)

Table S21: Sample and crystal data of compound **10**.

Chemical formula	C ₁₇ H ₁₇ F ₆ O ₂ PSW	Crystal system	triclinic	
Formula weight [g/mol]	614.19	Space group	P-1	
Temperature [K]	100	Z	2	
Measurement method	$\backslash\Phi$ and $\backslash\omega$ scans	Volume [Å³]	905.88(6)	
Radiation (Wavelength [Å])	MoK α (λ = 0.71073)	Unit cell dimensions [Å] and [°]	7.6662(3)	82.659(2)
Crystal size / [mm³]	0.14 × 0.1 × 0.04		10.7376(4)	79.687(2)
Crystal habit	clear brown block		11.3297(4)	83.341(2)
Density calculated / [g/cm³]	2.252	Absorption coefficient / [mm⁻¹]	6.650	
Abs. correction type	multiscan	F(000) [e⁻]	588.0	

Table S22: Data collection and structure refinement of compound **10**.

Index ranges	-10 ≤ h ≤ 10, -15 ≤ 5, -15 ≤ l ≤ 15	Theta range for data collection [°]	5.018 to 60.416	
Reflections number	50145	Data / restraints / parameters	5295/0/254	
Refinement method	Least squares	Final R indices	[all data]	R ₁ = 0.0181, wR ₂ = 0.0364
Function minimized	$\sum w(F_o^2 - F_c^2)^2$		>=2σ (I)	R ₁ = 0.0165, wR ₂ = 0.0357
Goodness-of-fit on	1.031	Weighting scheme	w=1/[σ ² +(F _o ² +(0.0132P) ² +1.1500P]	
Largest diff. peak and hole [e Å⁻³]	0.91/-0.88		where P=(F _o ² +2F _c ²)/3	

Cyclic Voltammetry

Due to low water-solubility of the compounds **1–10** MeCN was utilized in order to reach the desired concentration of 2 mM. All complexes exhibit a reversible one-electron process W^{IV} to W^V .

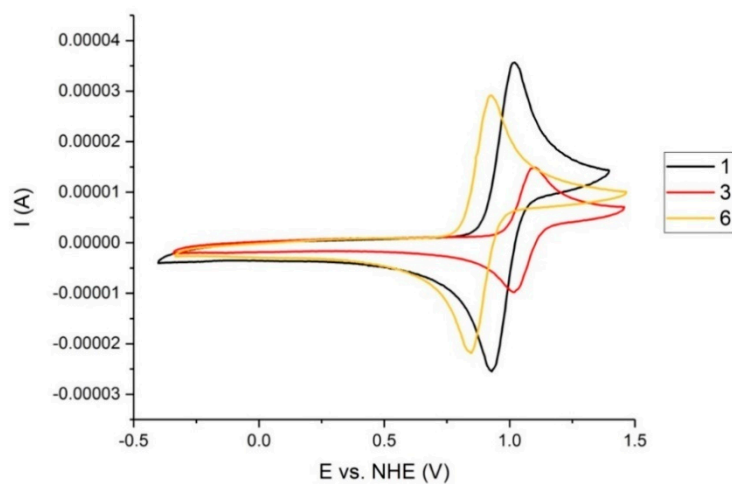


Figure S12: Cyclic Voltammogram of the (O,O-) chelates, compounds **1**, **3** and **6** in MeCN referenced to the NHE.

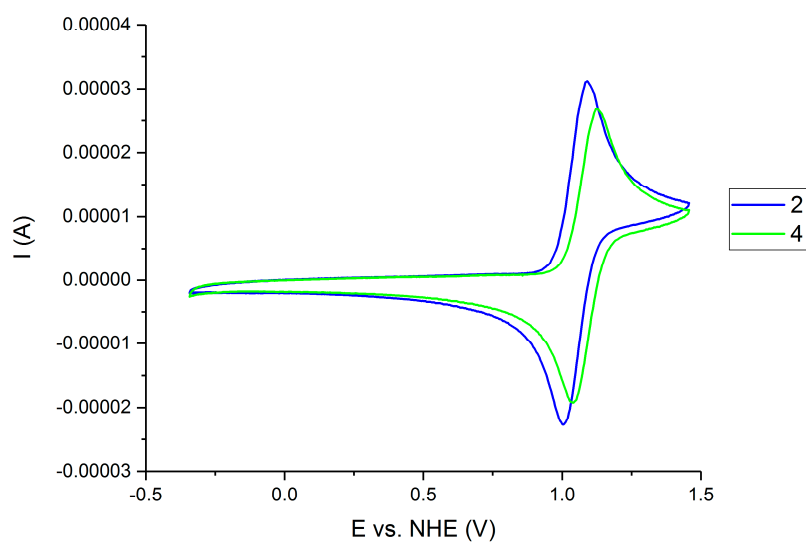


Figure S13: Cyclic Voltammogram of the (S,O-) chelates, compounds **2** and **4** in MeCN referenced to the NHE.

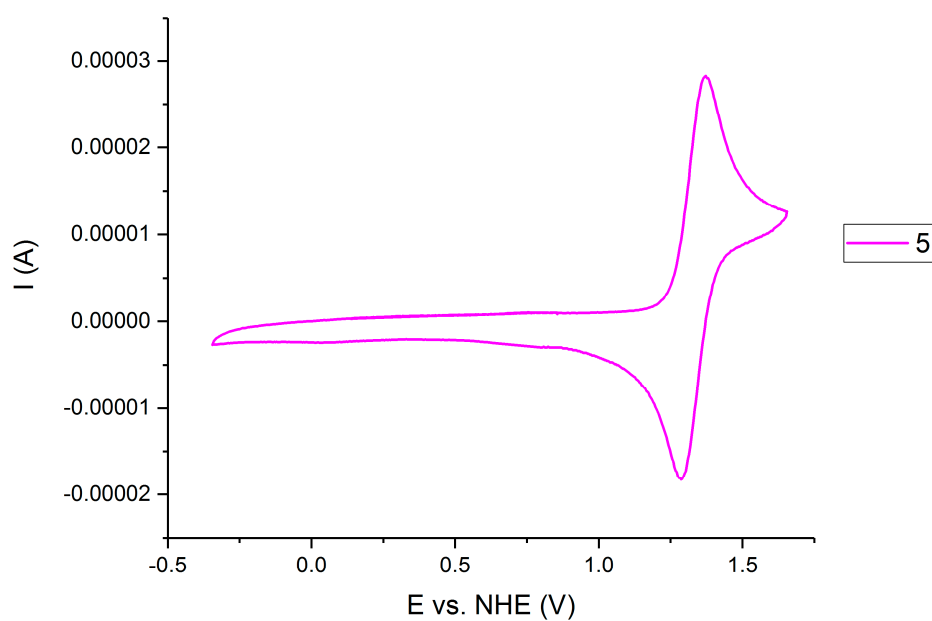


Figure S14: Cyclic Voltammogram of the (*N,O*)-chelate compound **5** in MeCN referenced to the NHE.

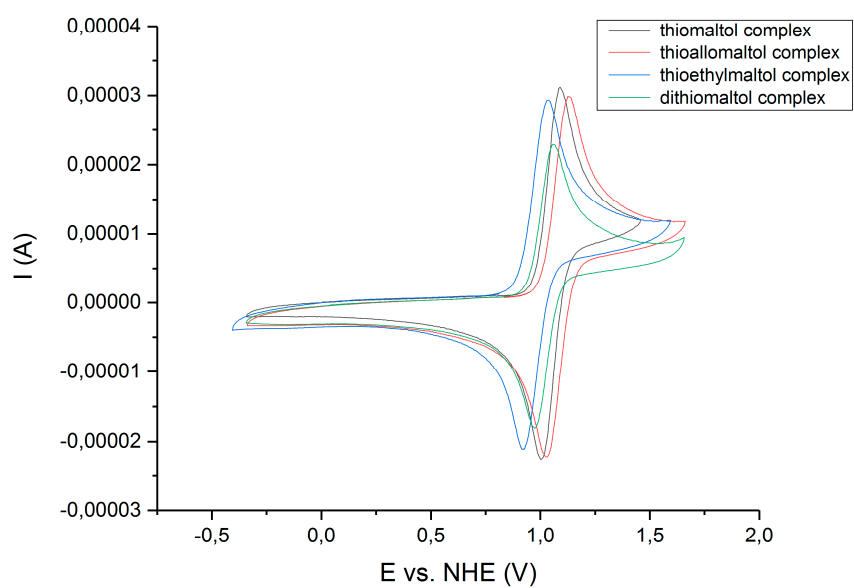


Figure S15: Cyclic Voltammogram of the (*S,O*-) chelates compounds **2**, **8**, **10** and a dithiomaltolato derivative in MeCN referenced to the NHE.

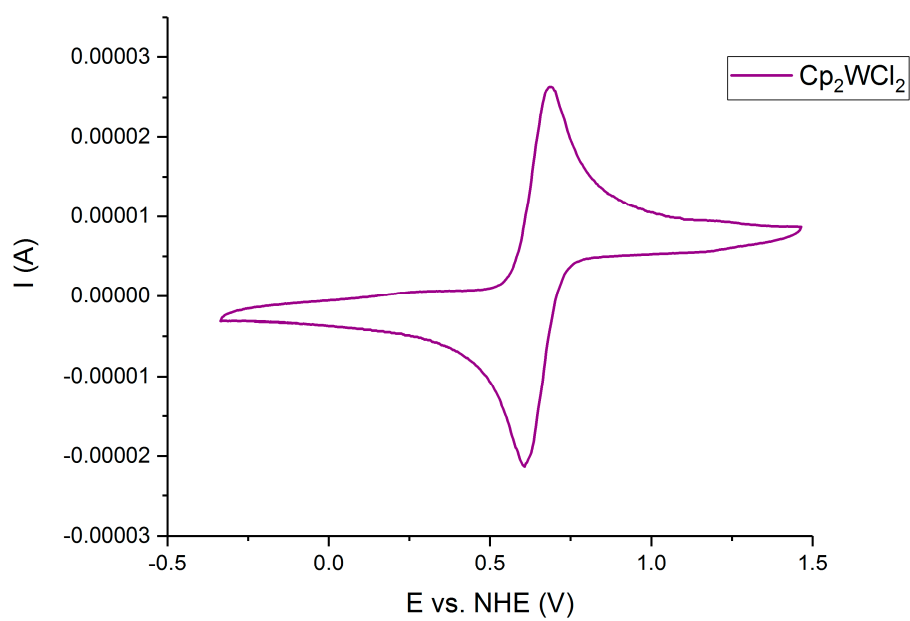


Figure S16: Cyclic Voltammogram of Cp_2WCl_2 in MeCN referenced to the NHE.

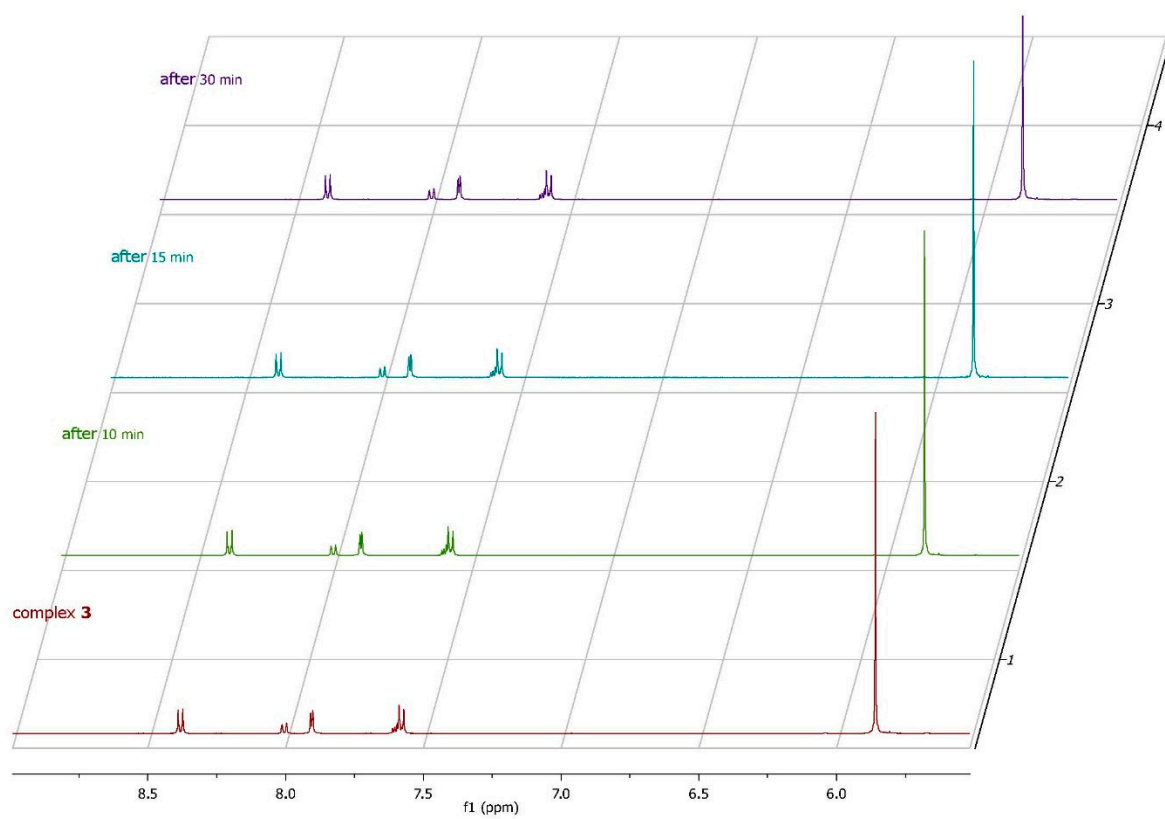


Figure S17: ^1H NMR in MeCN of complex **3** to gauge the stability over the timespan of a cyclic voltammetry experiment.

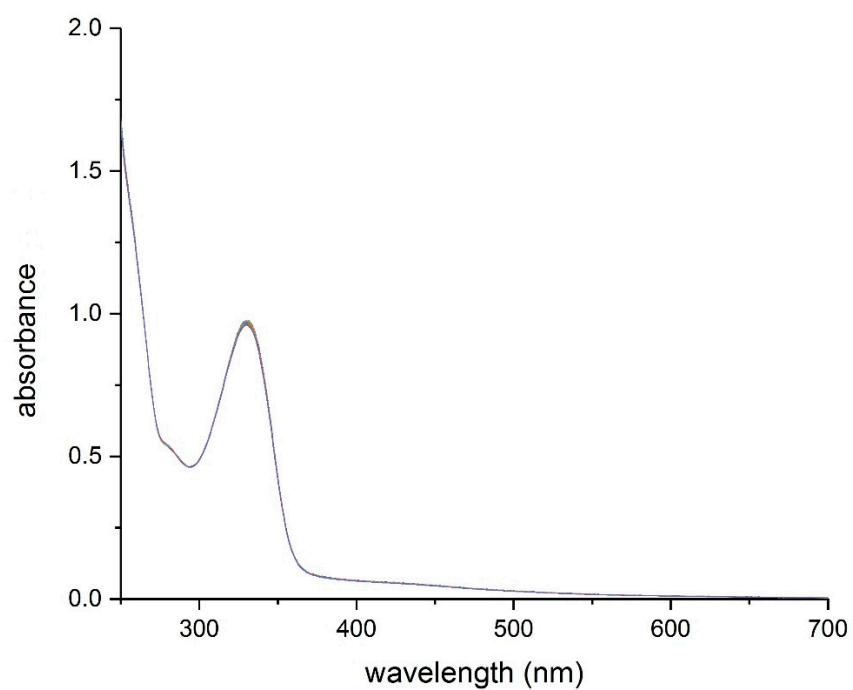


Figure S18: UV/Vis spectra of complex 1 (0.039 mM) over 24 h in PBS (1% DMSO).

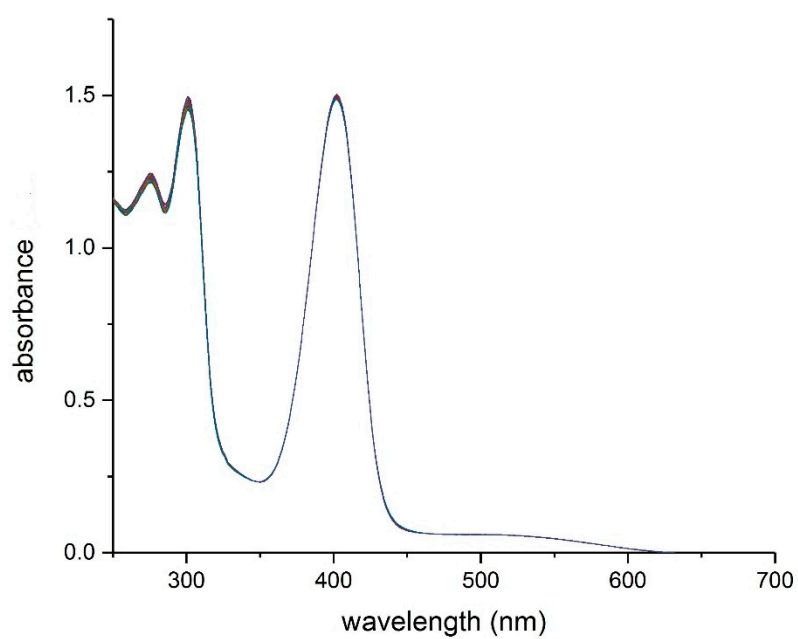


Figure S19: UV/Vis spectra of complex **2** (0.042 mM) over 24 h in PBS (1% DMSO).

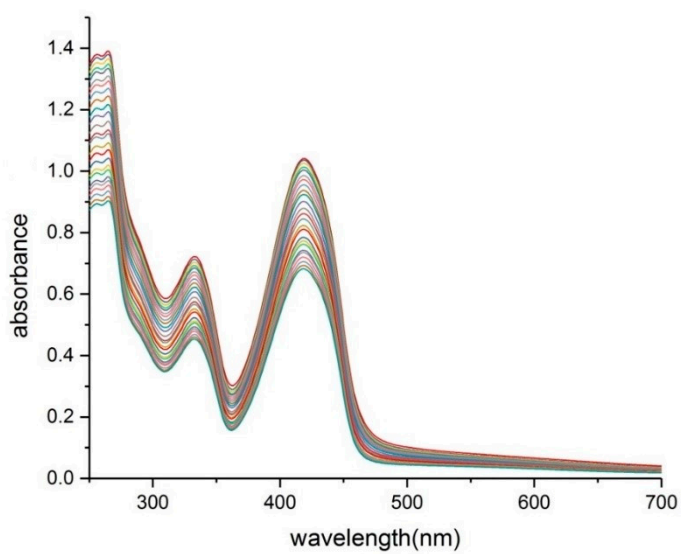


Figure S20: UV/Vis spectra of complex **3** (0.041 mM) over 24 h in PBS (1% DMSO). Precipitation over the time can be observed.

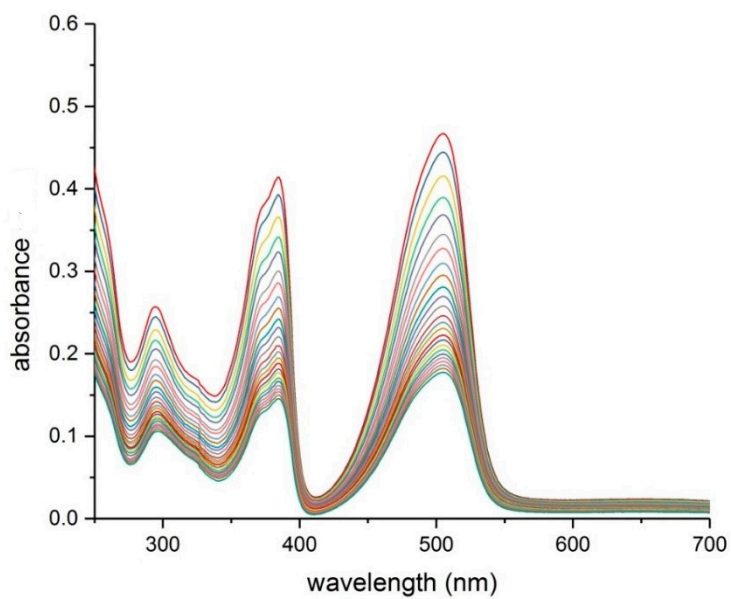


Figure S21: UV/Vis spectra of complex **4** (0.013 mM) over 24 h in PBS (1% DMSO). Precipitation over the time can be observed.

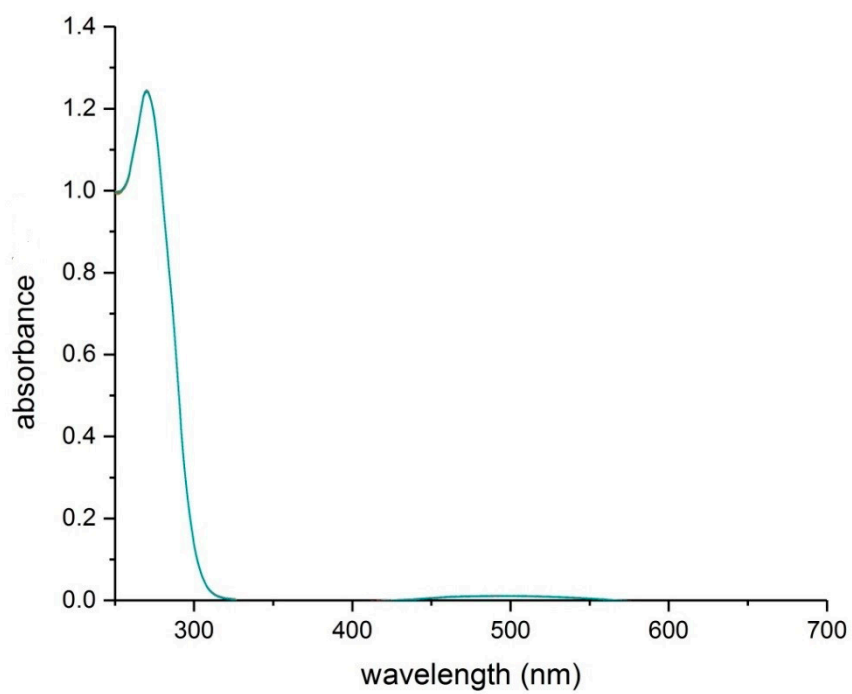


Figure S22: UV/Vis spectra of complex 5 (0.031 mM) over 24 h in PBS (1% DMSO).

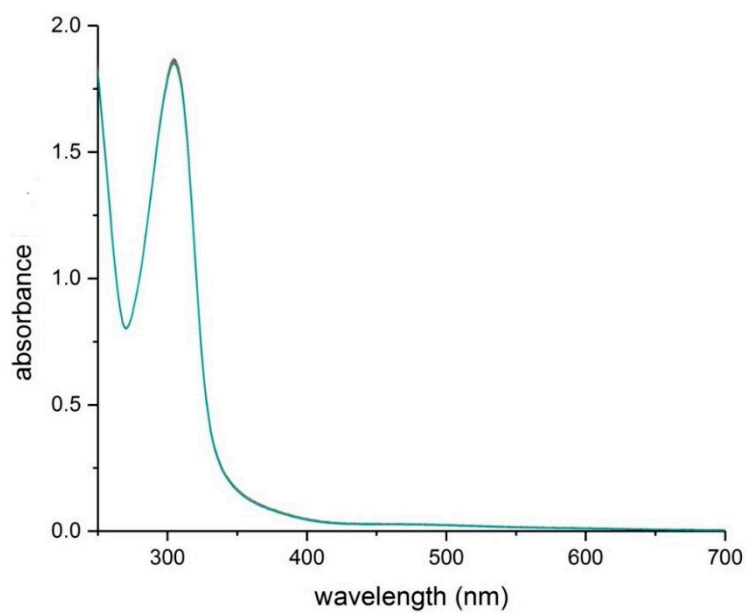


Figure S23: UV/Vis spectra of complex 6 (0.080 mM) over 24 h in PBS (1% DMSO).

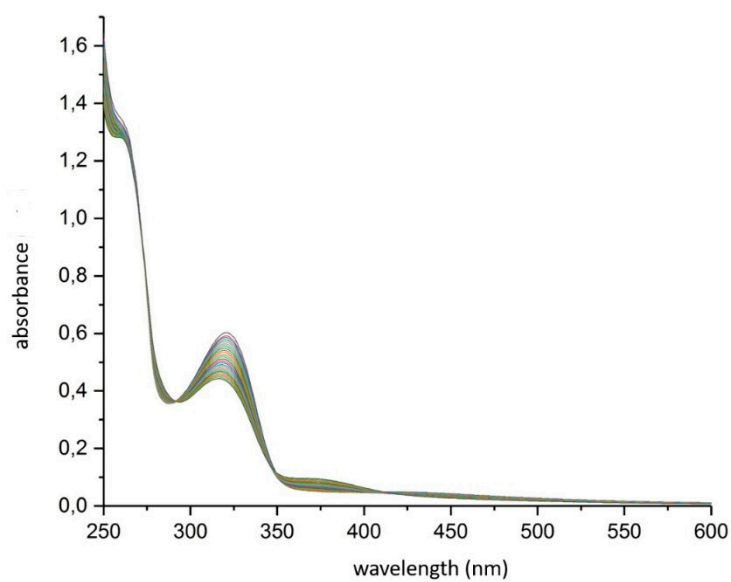


Figure S24: UV/Vis spectra of complex **7** (0.062 mM) over 24 h in PBS (1% DMSO).

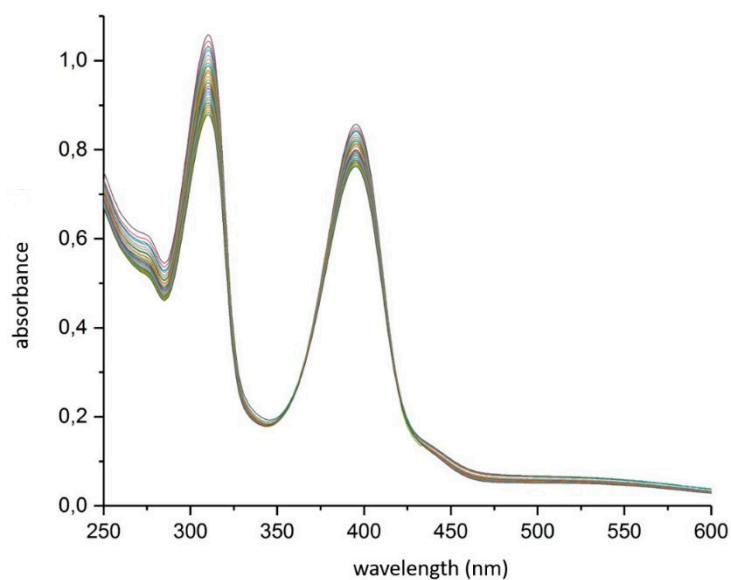


Figure S25: UV/Vis spectra of complex **8** (0.034 mM) over 24 h in PBS (1% DMSO).

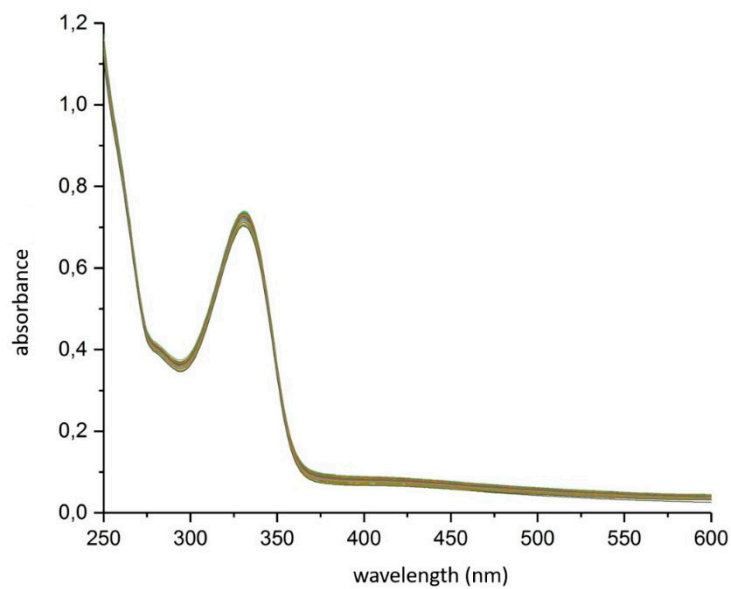


Figure S26: UV/Vis spectra of complex **9** (0.043 mM) over 24 h in PBS (1% DMSO).

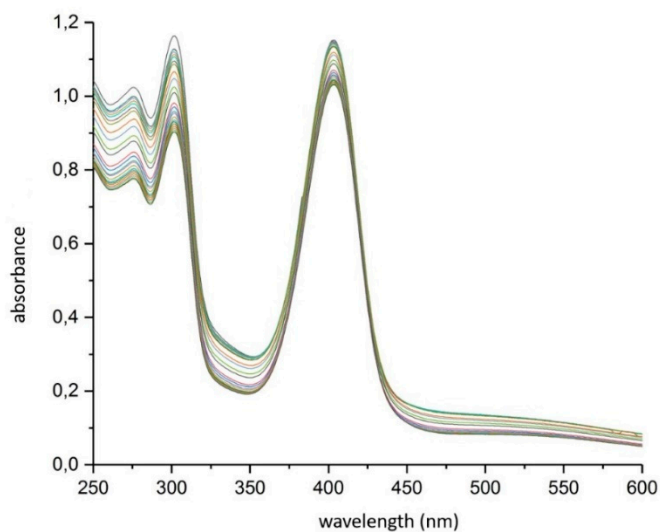


Figure S27: UV/Vis spectra of complex 10 (0.052 mM) over 24 h in PBS (1% DMSO).

Table S23: Wavelength of peak maxima and molar extinction coefficients (ϵ) of compounds 1–10 in 10% PBS (1% DMSO)

Compound	λ_{max} [nm] (ϵ [$\text{M}^{-1} \text{cm}^{-1}$])
1	330 (39049)
2	402 (120301), 301 (119818), 276 (99628)
3	256 (110446), 265 (111269), 333 (57762), 419 (83286)
4	505 (93412), 384 (82852), 295 (51368), 242 (100662)
5	305 (74304)
6	270 (49723)
7	320 (60272)
8	395 (85700), 310 (105823)
9	331 (73797)
10	403 (115229), 302 (116416), 276 (102373)

MTT assay

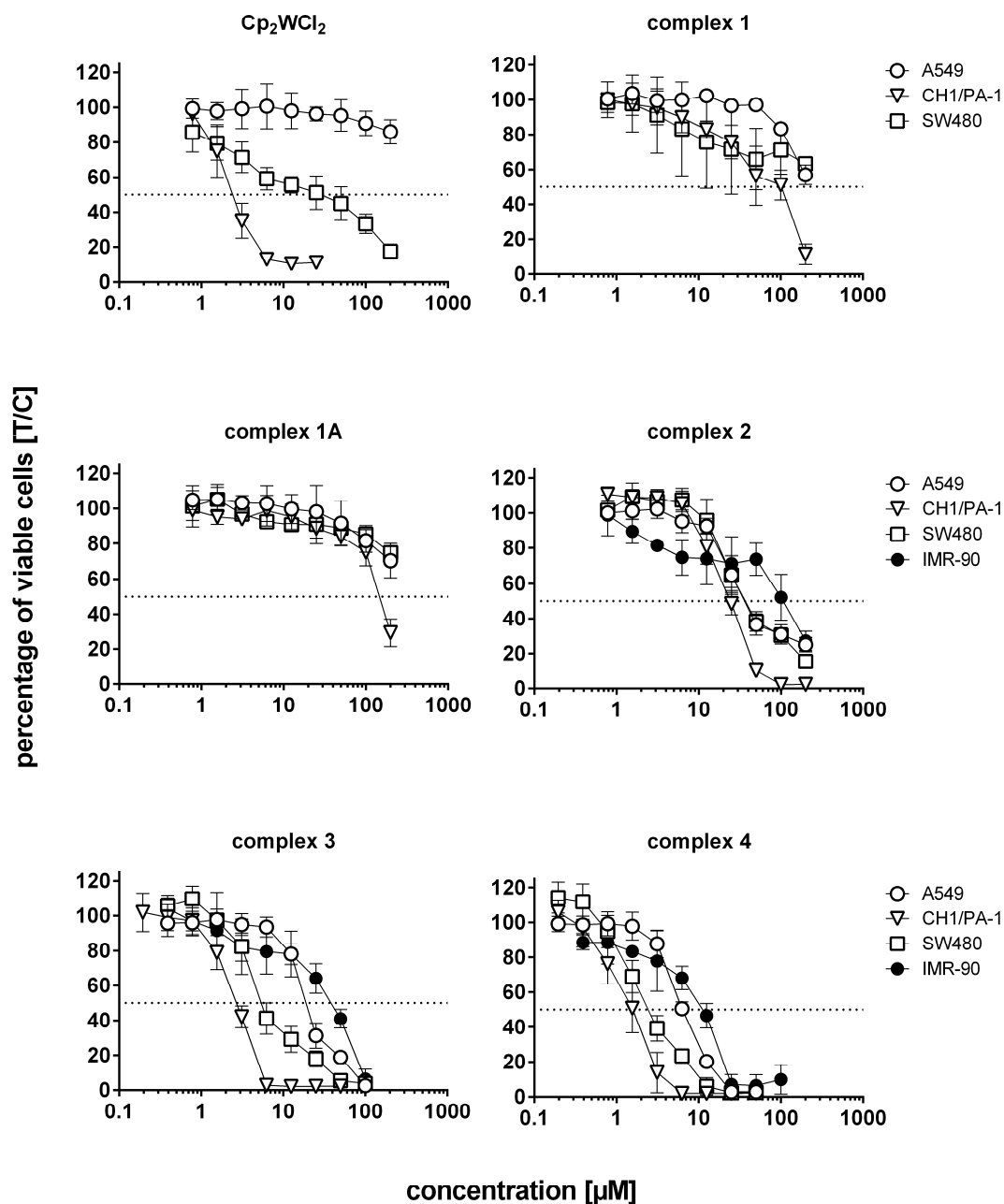


Figure S28: Concentration-effect curves of Cp₂WCl₂ and five other tungstenocenes (**1A**, **1–4**) in A549, CH1/PA-1 and SW480 cancer cells as well as (partially) in IMR-90 fibroblasts, obtained by the colorimetric MTT assay after 96 h incubation. Data are means ± standard deviations from at least three experiments (except for compounds that did not yield an IC₅₀ value; these were tested only twice). The dashed line refers to the 50% inhibitory level relative to the untreated controls.

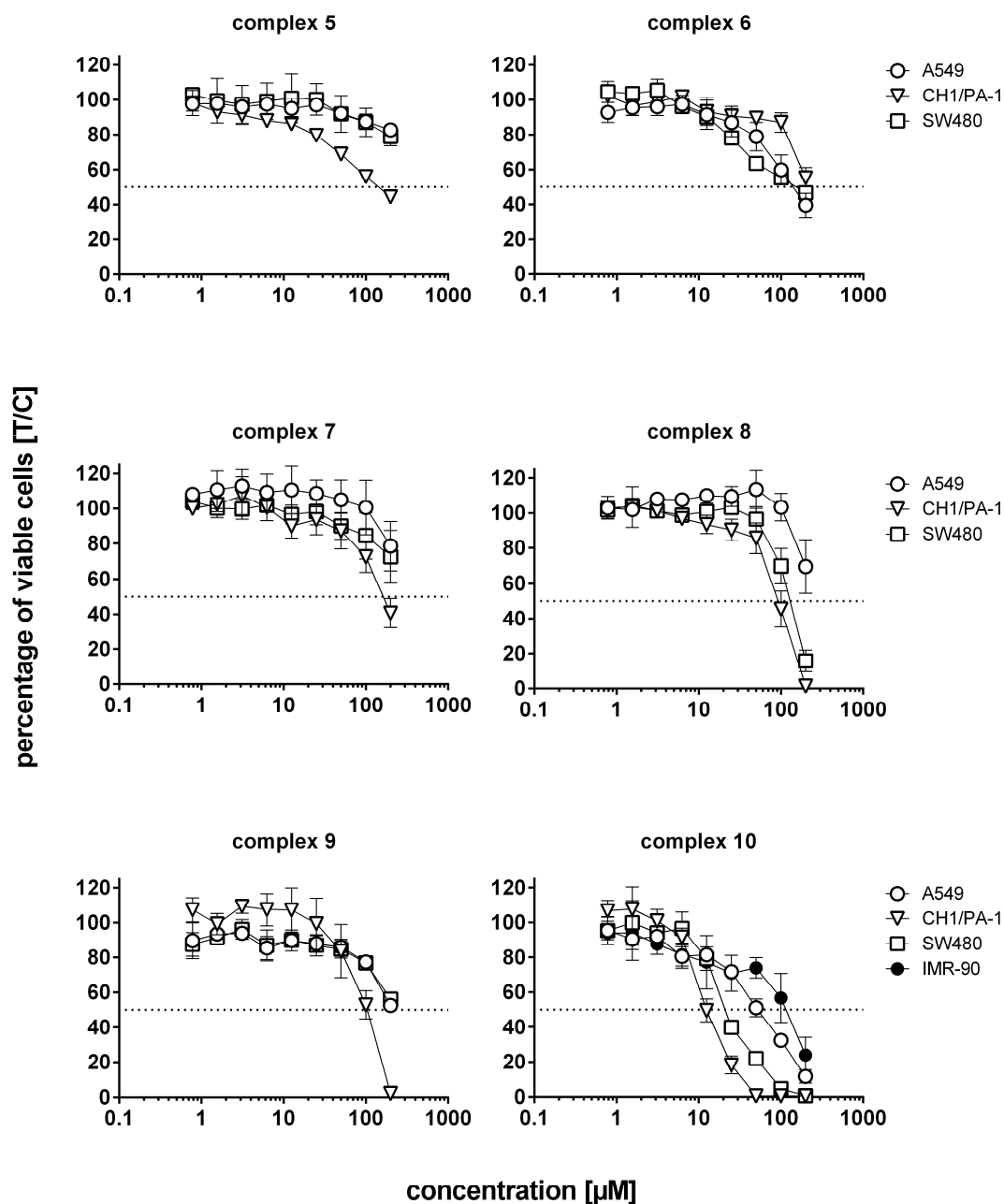


Figure S29: Concentration-effect curves of six tungstenocenes (**5–10**) in A549, CH1/PA-1, and SW480 cancer cells as well as (partially) in IMR-90 fibroblasts, obtained by the colorimetric MTT assay after 96 h incubation. Data are means $\pm$ standard deviations from at least three experiments (except for compounds that did not yield an IC₅₀ value; these were tested only twice). The dashed line refers to the 50% inhibitory level relative to the untreated controls.

Resazurin assay

Table S24: IC₅₀ values and calculated ratios of IC₅₀ values obtained in 2D and 3D cultures (determined by the resazurin assay) for complexes **2–4** and **10** in three cell lines (HCT116, HT29 and MCF-7).

compound	IC ₅₀ [μM]								
	HCT116			HT29			MCF-7		
	2D	3D	ratio 3D/2D	2D	3D	ratio 3D/2D	2D	3D	ratio 3D/2D
2	98 ± 12	158 ± 28	1.6	53 ± 9	> 200	> 3.8	66 ± 12	> 200	> 3.0
3	9.2 ± 1.4	21 ± 4	2.3	17 ± 4	86 ± 2	5.1	6.5 ± 1.8	44 ± 1	6.8
4	6.0 ± 0.9	15 ± 4	2.5	5.5 ± 1.0	31 ± 6	5.6	2.7 ± 0.7	19 ± 2	7.0
10	42 ± 3	69 ± 12	1.6	54 ± 2	> 200	> 3.7	29 ± 4	> 200	> 6.9

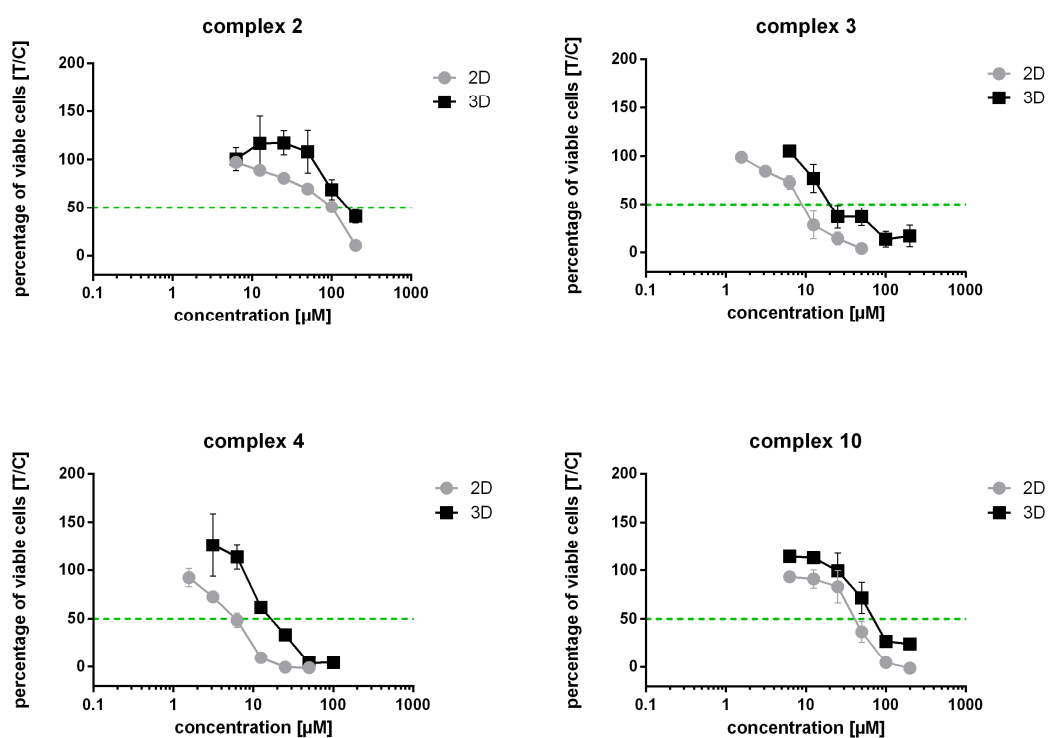


Figure S30: Concentration-effect curves of complexes **2–4** and **10** obtained by the resazurin assay (96 hours exposure) in monolayer (2D, grey lines) and multicellular spheroid (3D, black lines) cultures of HCT116 cells. The dashed, green line refers to the 50% inhibitory level relative to untreated controls.

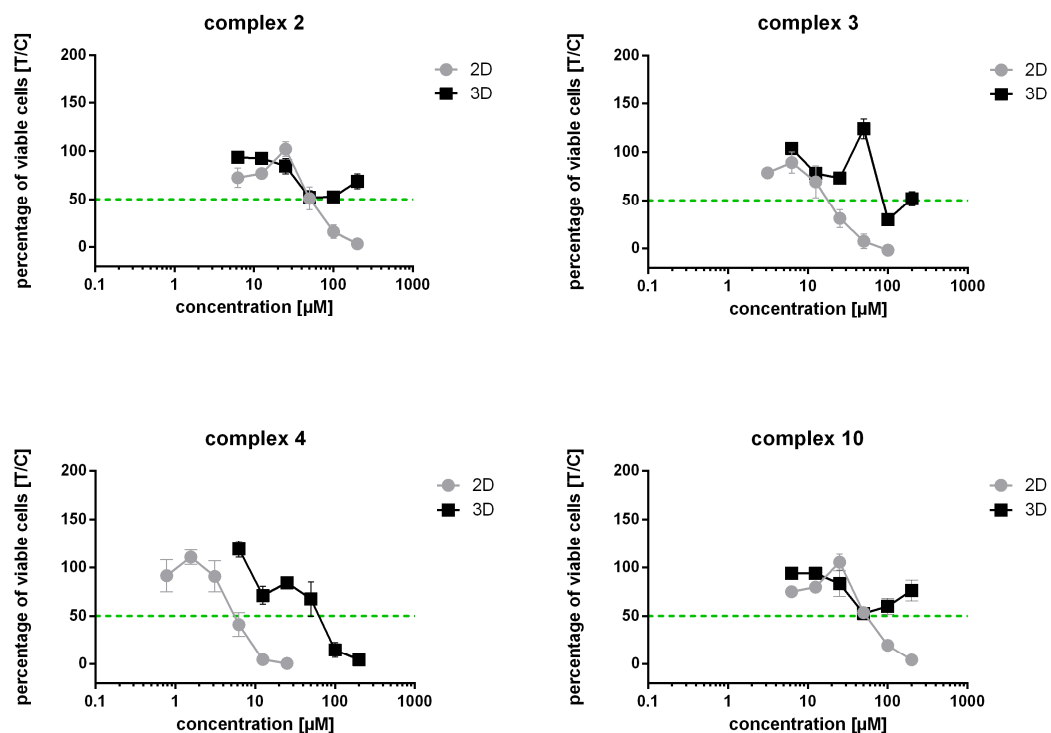


Figure S31: Concentration-effect curves of complexes **2–4** and **10** obtained by the resazurin assay (96 hours exposure) in monolayer (2D, grey lines) and multicellular spheroid (3D, black lines) cultures of HT29 cells. The dashed, green line refers to the 50% inhibitory level relative to untreated controls.

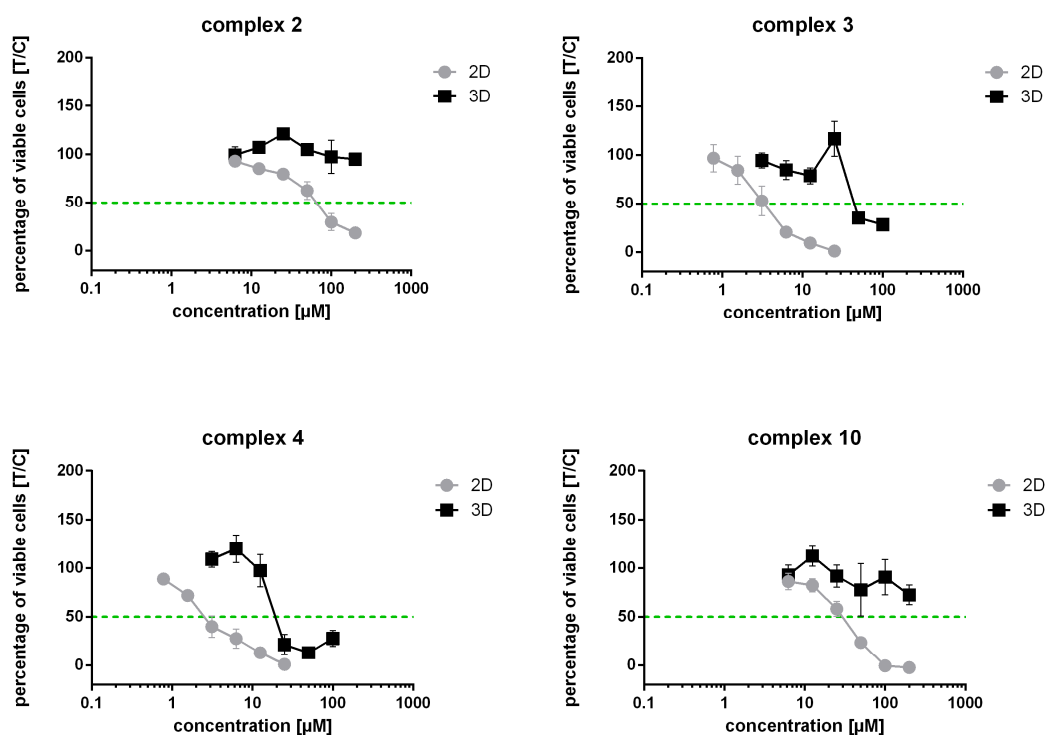


Figure S32: Concentration-effect curves of complexes **2–4** and **10** obtained by the resazurin assay (96 hours exposure) in monolayer (2D, grey lines) and multicellular spheroid (3D, black lines) cultures of MCF-7 cells. The dashed, green line refers to the 50% inhibitory level relative to untreated controls.

Growth curve

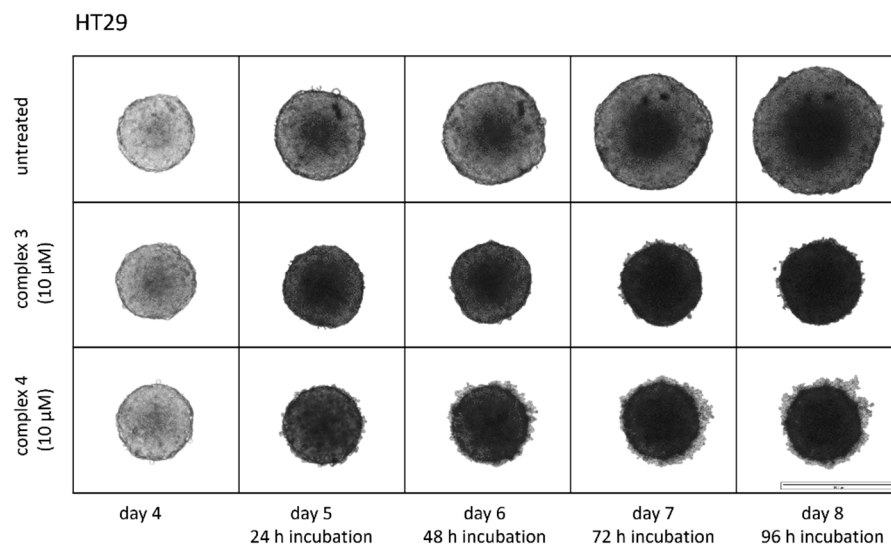


Figure S33: Time-dependent growth of HT29 spheroids upon treatment with complexes **3** and **4** (both 10 μM) and untreated control, detected for 96 hours (treatment started on day 4 after seeding). Images represent one captured spheroid out of three experiments. Scale bar = 500 μm

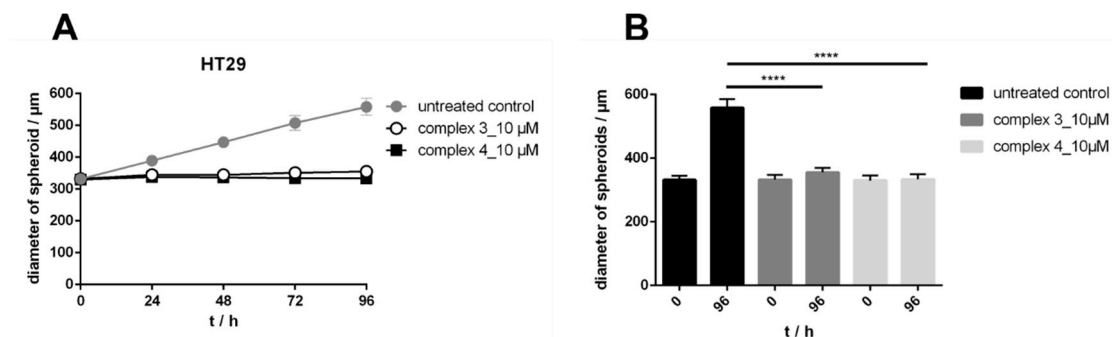


Figure S34: (A) Growth curves for spheroids of HT29 cells treated with complexes **3** and **4** (both 10 μM) and untreated control. Measurements started on day 4 (after seeding) with spheroid diameters of $304 \pm 1 \mu\text{m}$ and were continued every 24 hours for additional 4 days. **(B)** Spheroids were initiated on day 0, treated with complexes **3** and **4** (both 10 μM) on day 4, diameter of spheroids were obtained at the beginning of the treatment (0 h) and at the end (96 h). Unpaired t -test with Welch's correction ($n=72$) was performed for statistical evaluation (**** $p < 0.0001$).

MCF-7

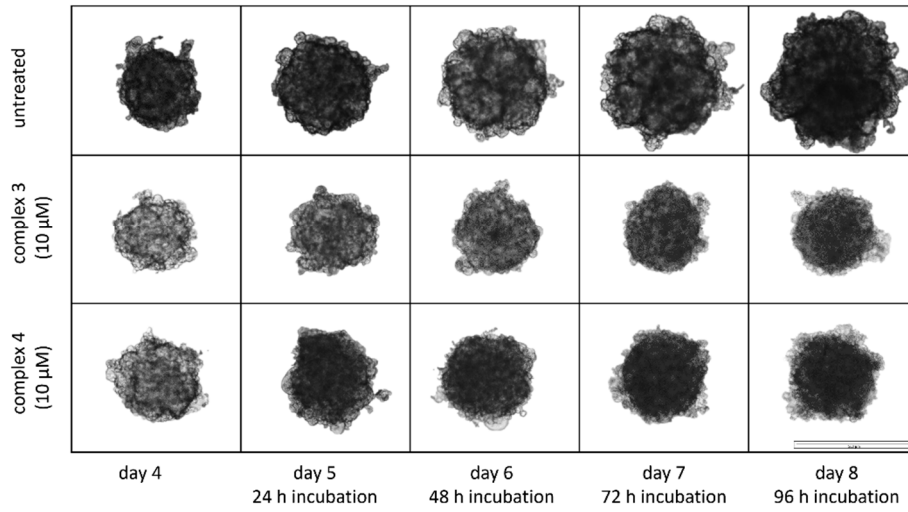


Figure S35: Time-dependent growth of MCF-7 spheroids upon treatment with complexes **3** and **4** (both 10 μM) and untreated control, detected for 96 hours (treatment started on day 4 after seeding). Images represent one captured spheroid out of three experiments. Scale bar = 500 μm

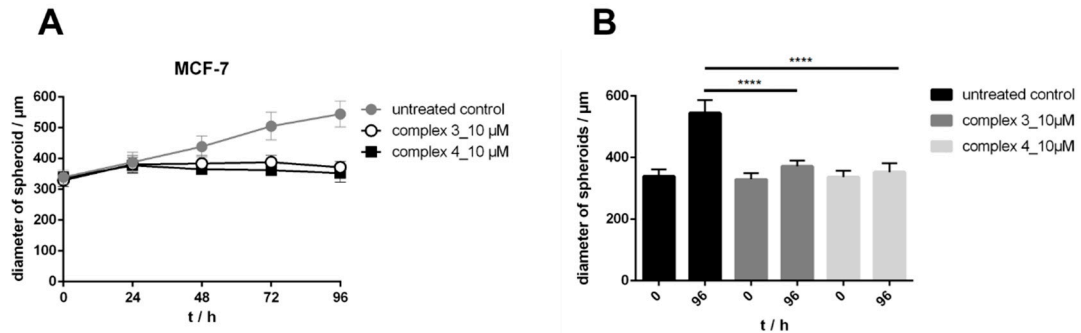


Figure S36: (A) Growth curves for spheroids of MCF-7 cells treated with complexes **3** and **4** (both 10 μM) and untreated control. Measurements started on day 4 (after seeding) with spheroid diameters of $304 \pm 1 \mu\text{m}$ and were continued every 24 hours for additional 4 days. (B) Spheroids were initiated on day 0, treated with complexes **3** and **4** (both 10 μM) on day 4, diameter of spheroids were obtained at the beginning of the treatment (0 h) and at the end (96 h). Unpaired t -test with Welch's correction ($n=72$) was performed for statistical evaluation (**** $p < 0.0001$).

Table S25: Statistical analysis of KI67⁺ cells in cryo-sectioned HCT116 spheroids of untreated control and treated samples with complex **3** (20 μ M) and complex **4** (15 μ M) after 72 hours exposure.

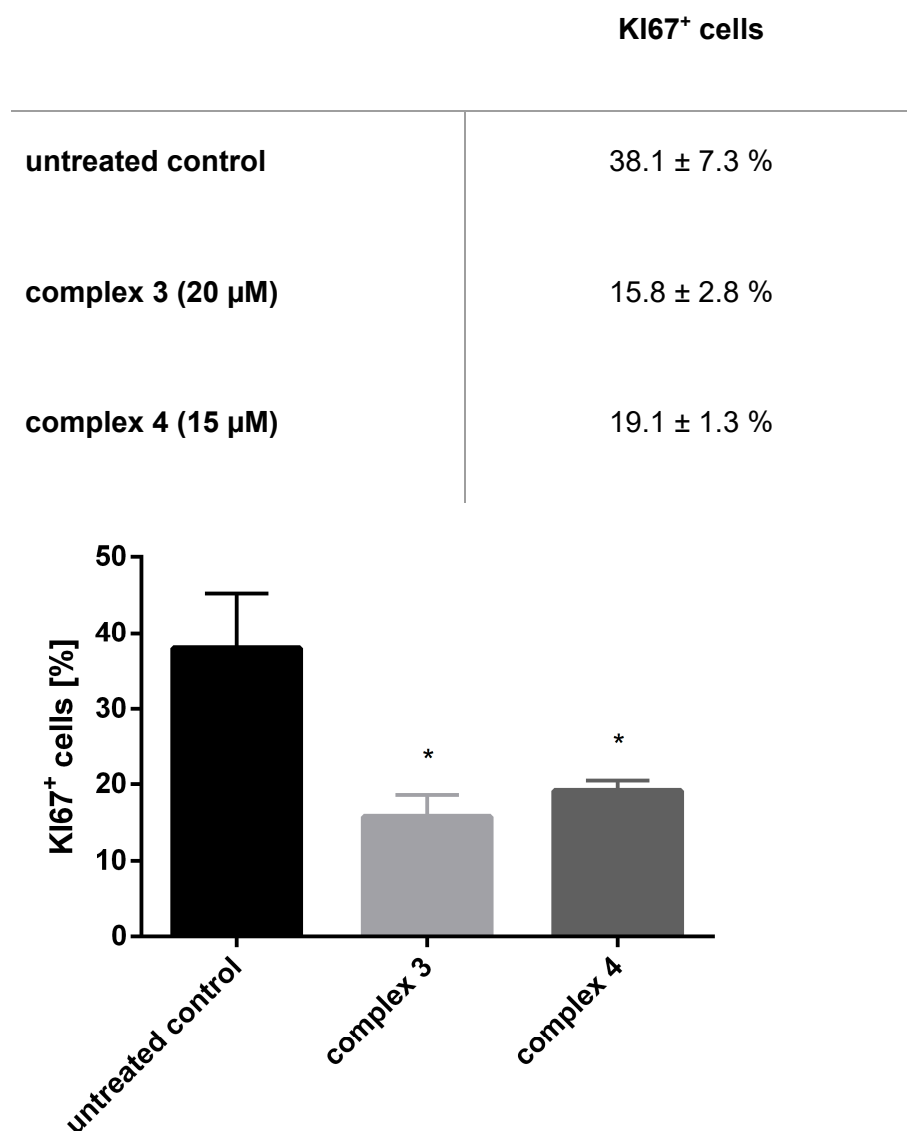


Figure S37: Quantitative analysis of KI67⁺ cells in cryo-sectioned HCT116 spheroids, untreated or treated with complex **3** (20 μ M) and complex **4** (15 μ M) for 72 hours. Unpaired *t*-test with Welch's correction (*n*=3 for untreated control, *n*=4 for treated samples) was performed for statistical evaluation (* *p* < 0.05). The asterisks mark the difference relative to the untreated control, the difference between the means of complex **3** and complex **4** treated samples was found to be not significant.

Laser ablation

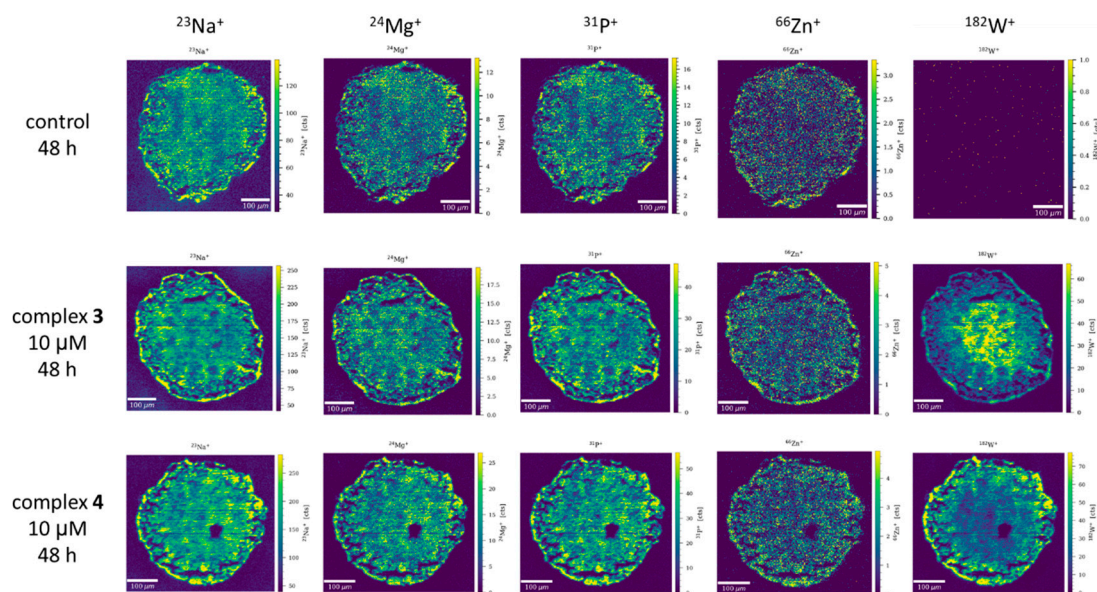


Figure S38: Signal intensity maps of $^{23}\text{Na}^+$, $^{24}\text{Mg}^+$, $^{31}\text{P}^+$, $^{66}\text{Zn}^+$ and $^{182}\text{W}^+$ obtained by LA-ICP-TOF-MS in selected HCT116 tumor spheroid sections after treatment with 10 μM of complexes **3** and **4** for 48 hours. High-resolution laser ablation images were measured with a 5 μm square mask, double oversampling and a repetition rate of 200 Hz.

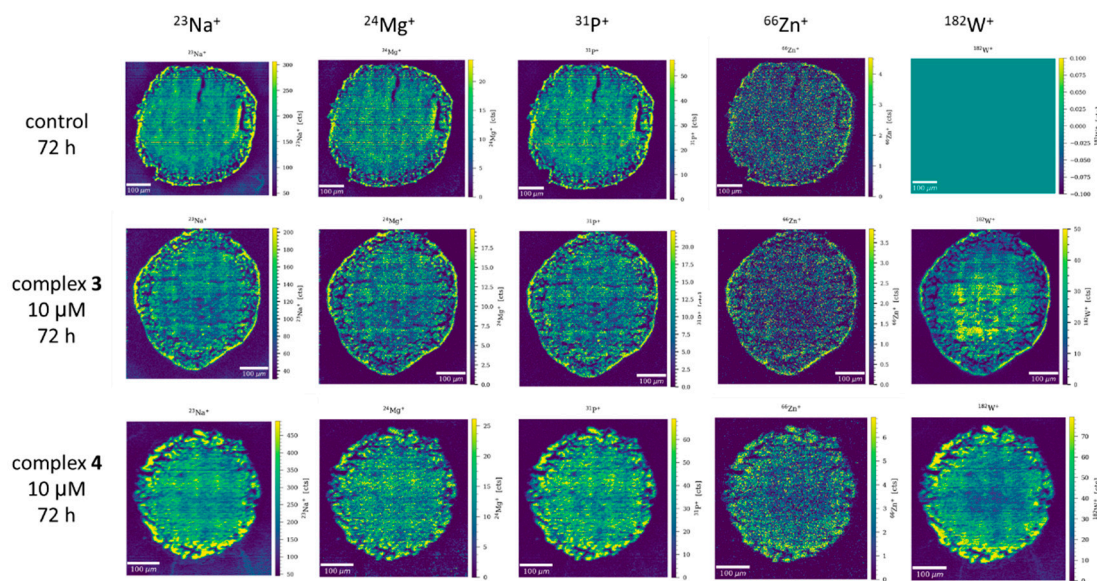


Figure S39: Signal intensity maps of $^{23}\text{Na}^+$, $^{24}\text{Mg}^+$, $^{31}\text{P}^+$, $^{66}\text{Zn}^+$ and $^{182}\text{W}^+$ obtained by LA-ICP-TOF-MS in selected HCT116 tumor spheroid sections after treatment with 10 μM of complexes **3** and **4** for 72 hours. High-resolution laser ablation images were measured with a 5 μm square mask, double oversampling and a repetition rate of 200 Hz.

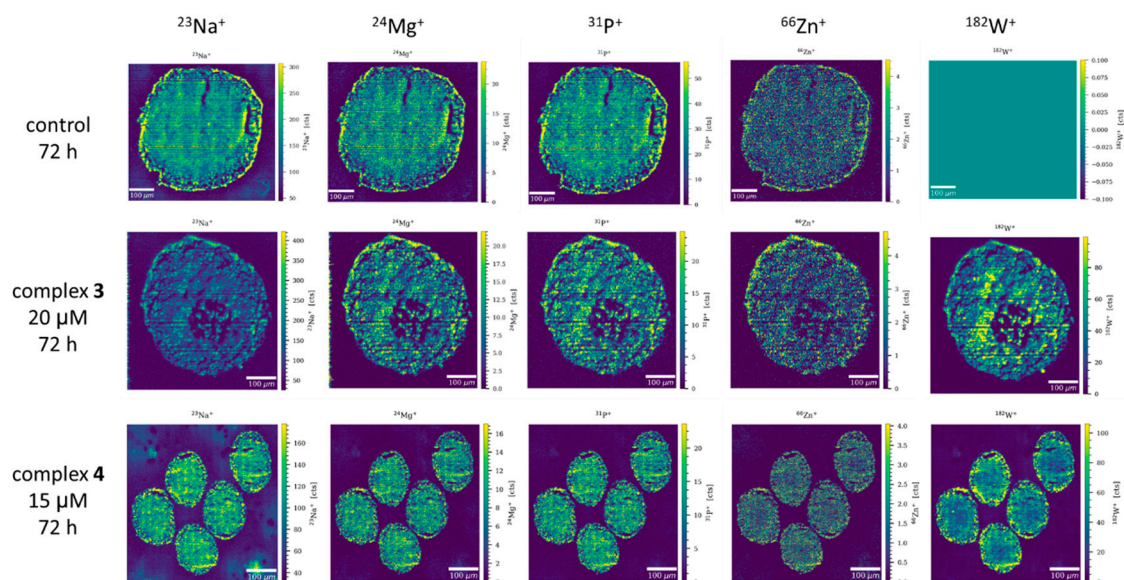


Figure S40: Signal intensity maps of $^{23}\text{Na}^+$, $^{24}\text{Mg}^+$, $^{31}\text{P}^+$, $^{66}\text{Zn}^+$ and $^{182}\text{W}^+$ obtained by LA-ICP-TOF-MS in selected HCT116 tumor spheroid sections after treatment with 20 μM of complex **3** and 15 μM of complex **4** for 72 hours. High-resolution laser ablation images were measured with a 5 μm square mask, double oversampling and a repetition rate of 200 Hz.

Reactive oxygen species (ROS) detection

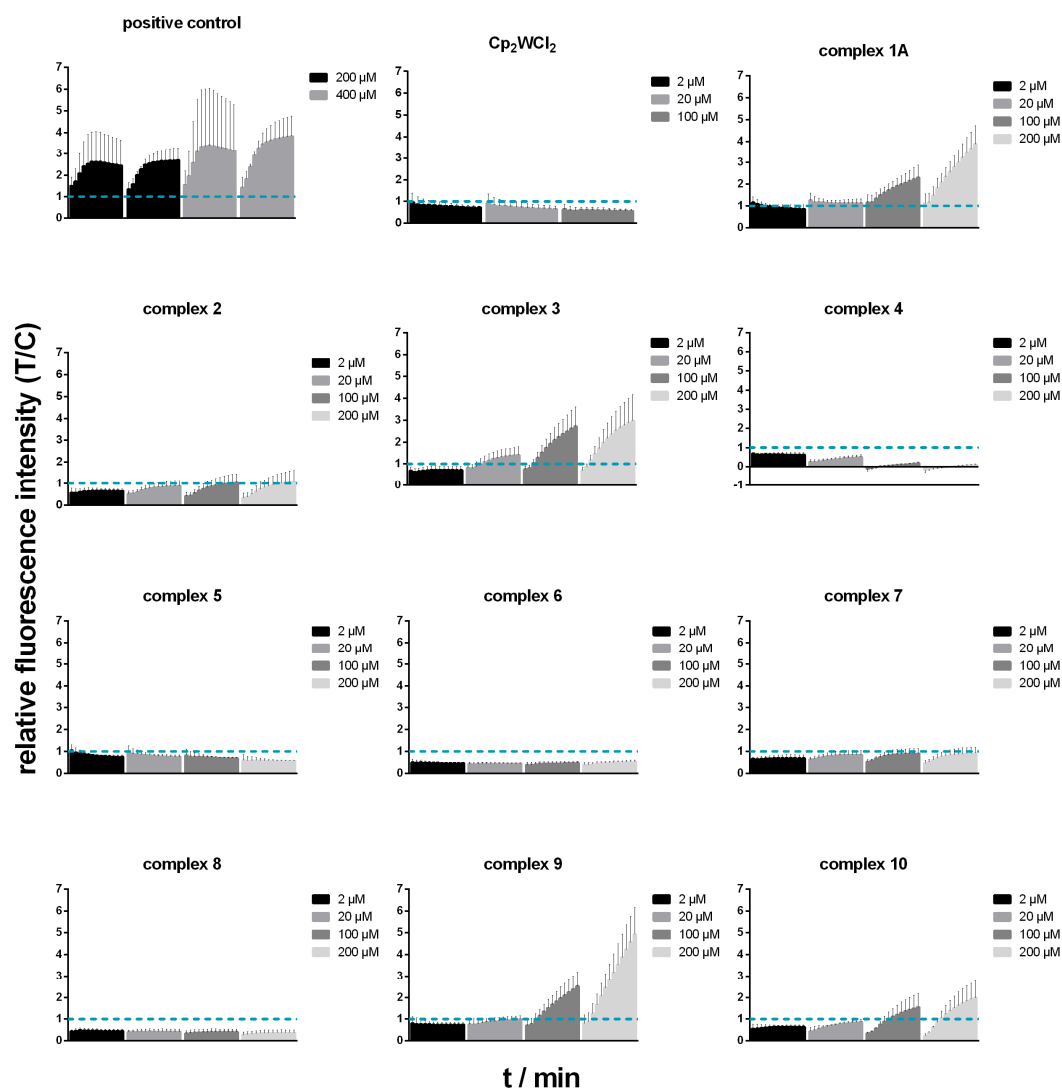


Figure S41: Time- and concentration-dependent intracellular reactive oxygen species (ROS) generation in HT29 cells. Cells were treated with tungstenocenes (2, 20, 100 and 200 μM) and tert-butyl-peroxide (200 and 400 μM) as positive control. Fluorescence intensities of DCF indicating ROS levels (relative to the untreated control) were measured with a spectrophotometer. Data from three independent experiments with $n=3$ wells per measurement are given as means $\pm$ standard deviations. The dashed, blue line indicates the ROS level of untreated controls to which the other values were normalized.

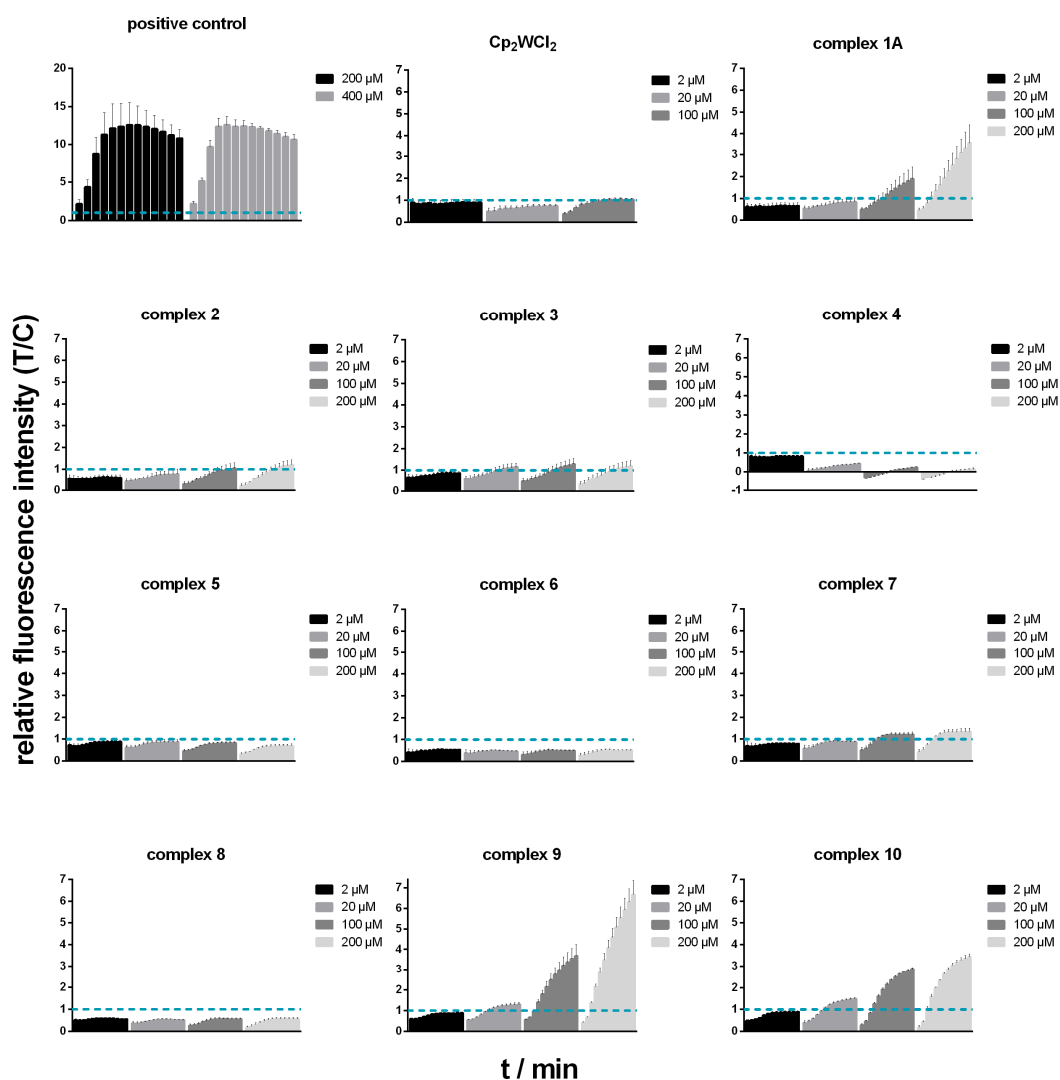


Figure S42: Time- and concentration-dependent intracellular reactive oxygen species (ROS) generation in MCF-7 cells. Cells were treated with tungstenocenes (2, 20, 100 and 200 μM) and tert-butyl-peroxide (200 and 400 μM) as positive control. Fluorescence intensities of DCF indicating ROS levels (relative to the untreated control) were measured with a spectrophotometer. Data from three independent experiments with $n=3$ wells per measurement are given as means $\pm$ standard deviations. The dashed, blue line indicates the ROS level of untreated controls to which the other values were normalized.

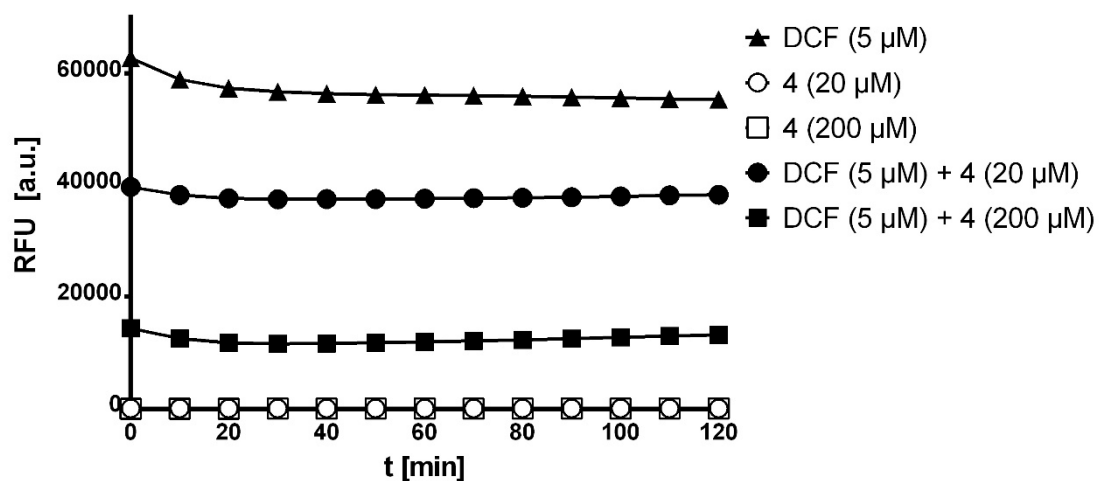
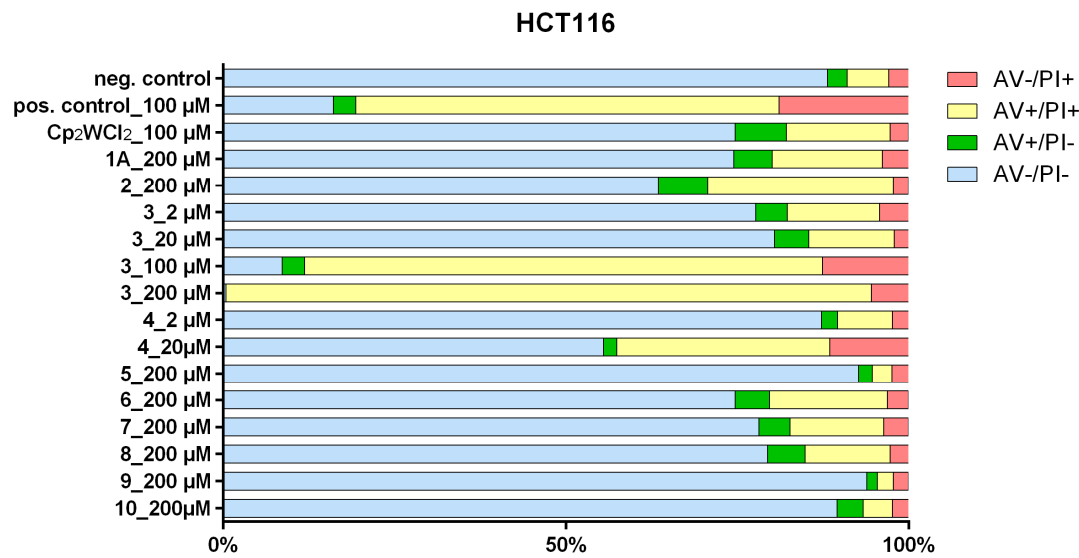
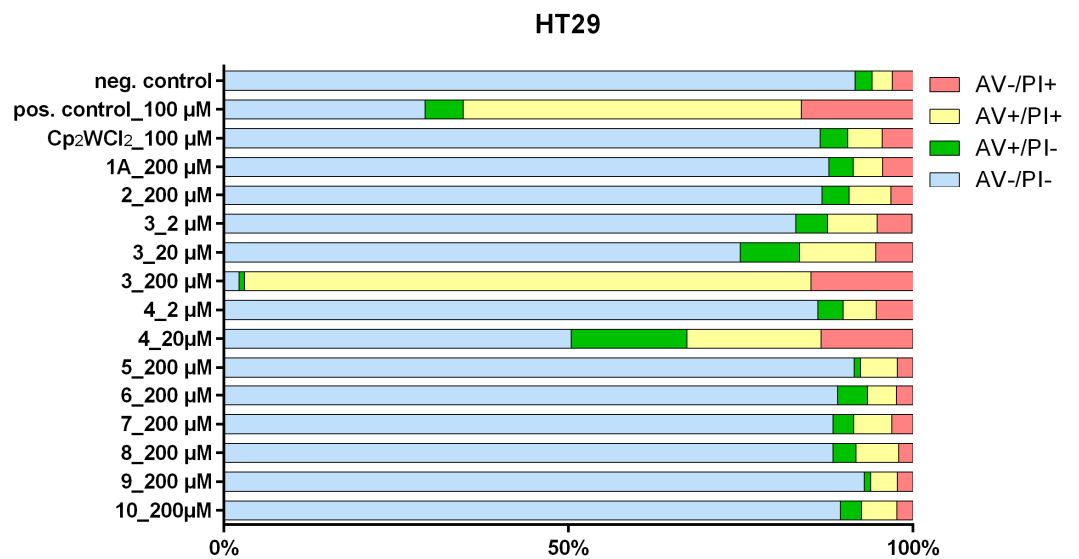


Figure S43: Quenching of DCF (2',7'-dichlorofluorescein) fluorescence by complex **4** in a cell-free assay. DCF (5 μ M) and complex **4** (20 and 200 μ M, the highest concentrations used in ROS assays) were applied alone as well as in combination. Fluorescence intensity was measured over 120 min every 10 min in triplicates (standard deviations are too small to be shown on this scale).



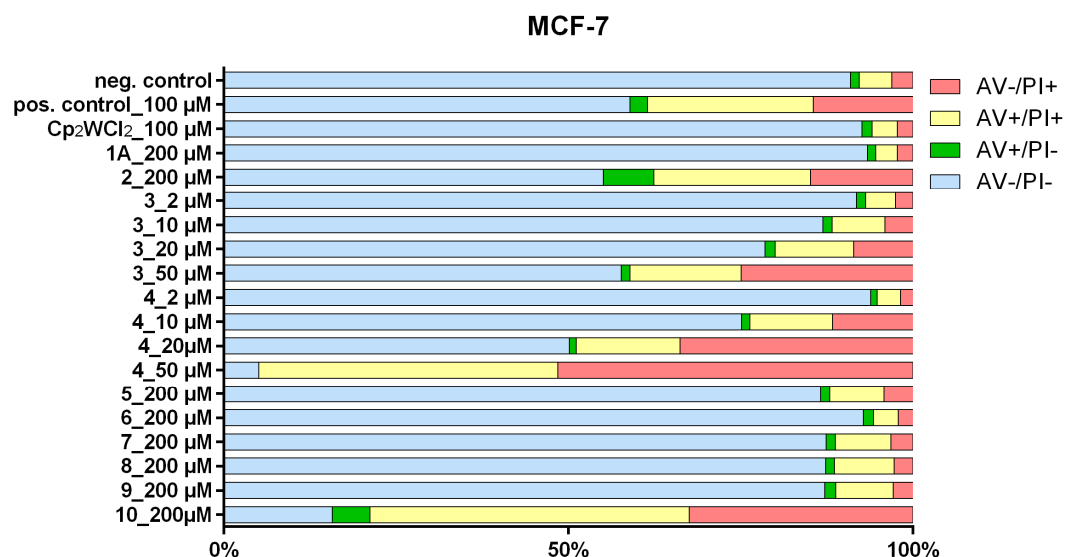


Figure S44: Apoptosis induction by Cp₂WCl₂ and ten other tungstenocenes (**1A–10**) after 24 h incubation in HCT116, HT29 and MCF-7 cancer cells. Cells were double-stained with annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry. Percentages of necrotic (AV-/PI+), late apoptotic (AV+/PI+), early apoptotic (AV+/PI-) and viable (AV-/PI-) cells are given as averages of at least three independent experiments. Positive control was treated with KP1998 (compound **7** in reference ⁶²)

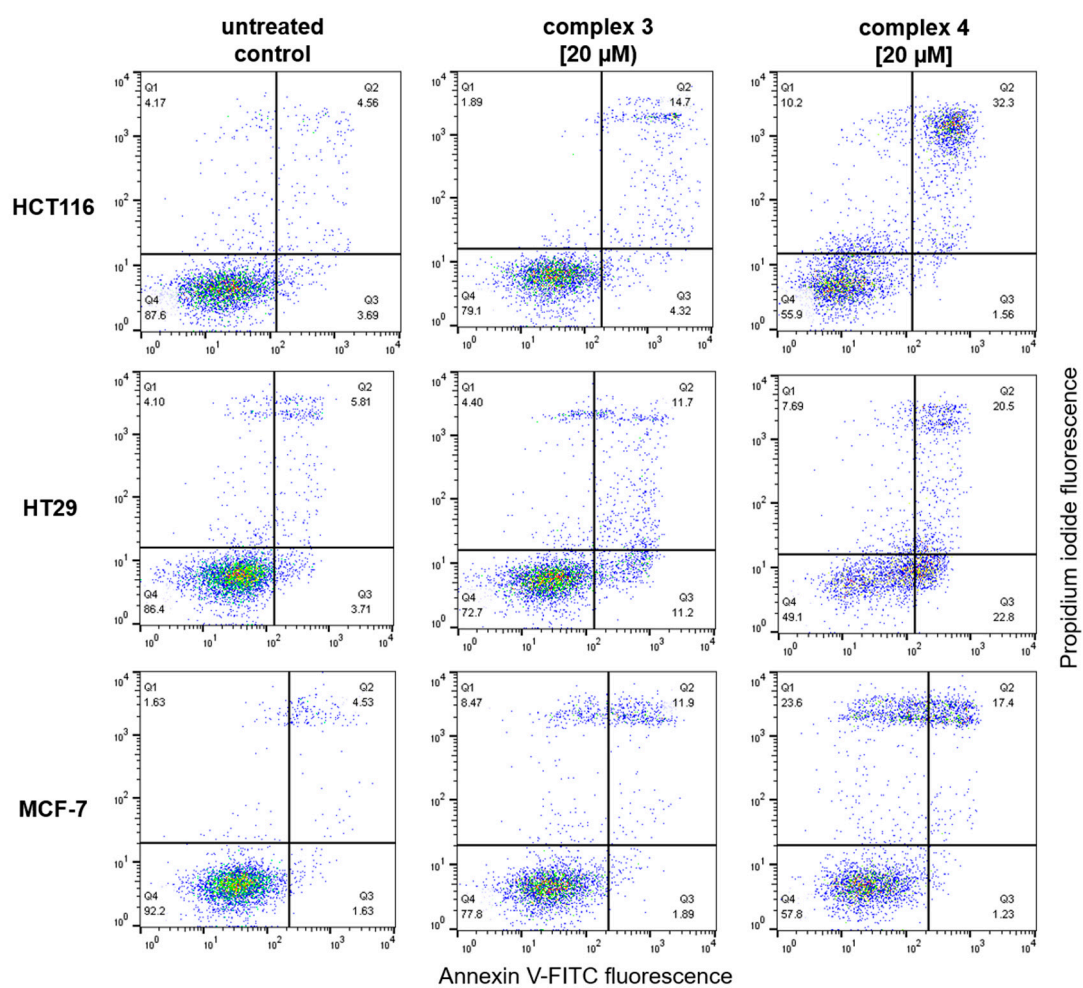


Figure S45: Dot plots of viable (Q4), early apoptotic (Q3), late apoptotic (Q2) and necrotic (Q1) HCT116, HT29 and MCF-7 cells treated with two most cytotoxic complexes **3** and **4** at 20 μM. The dot plots represent the annexin V-FITC (x-axis) and PI (y-axis) staining detected by flow cytometry and represent a single experiment out of at least three repetitions.

Table S26: Apoptosis induction by Cp_2WCl_2 and tungstenocenes containing (O,O–), (S,O–) and (N,O–)chelate in three different cell lines after 24 h exposure to the indicated concentrations. For each setting, the total apoptotic subpopulation is the sum of early and late apoptotic events (Σ apoptotic cells [%]). Results are means $\pm$ standard derivations from at least three independent experiments. Positive control was treated with KP1998 (compound 7 in reference ⁶³)

cell line	compound	concentration	viable cells [%] AV- / PI-	early apoptotic cells [%] AV+ / PI-	late apoptotic cells [%] AV+ / PI+	necrotic cells [%] AV- / PI+	Σ apoptotic cells [%]
HCT116	neg. ctrl		88.2 $\pm$ 8.6	2.8 $\pm$ 2.7	6.1 $\pm$ 5.5	2.9 $\pm$ 1.8	8.9
	pos. ctrl	100 μM	16.1 $\pm$ 1.9	3.3 $\pm$ 1.6	61.7 $\pm$ 2.7	19.0 $\pm$ 4.3	65
	Cp_2WCl_2	100 μM	74.7 $\pm$ 4.9	7.5 $\pm$ 3.0	15.1 $\pm$ 2.1	2.7 $\pm$ 0.5	22.6
	1A	200 μM	74.5 $\pm$ 7.0	5.6 $\pm$ 2.1	16.1 $\pm$ 4.6	3.8 $\pm$ 2.1	21.7
	2	200 μM	63.5 $\pm$ 18.6	7.2 $\pm$ 1.9	27.1 $\pm$ 17.6	2.2 $\pm$ 0.2	34.3
	3	2 μM	77.7 $\pm$ 1.6	4.6 $\pm$ 1.7	13.5 $\pm$ 0.6	4.3 $\pm$ 0.7	18.1
		20 μM	80.4 $\pm$ 2.6	5.0 $\pm$ 0.9	12.1 $\pm$ 2.9	2.5 $\pm$ 0.6	17.1
		100 μM	8.6 $\pm$ 4.3	3.3 $\pm$ 1.1	75.5 $\pm$ 5.6	12.8 $\pm$ 0.2	78.8
		200 μM	0.4 $\pm$ 0.3	0.0 $\pm$ 0.0	94.1 $\pm$ 4.8	5.5 $\pm$ 4.9	94.1
	4	2 μM	87.3 $\pm$ 7.8	2.3 $\pm$ 0.0	8.0 $\pm$ 6.2	2.4 $\pm$ 1.5	10.3
		20 μM	55.5 $\pm$ 4.8	1.9 $\pm$ 0.4	31.1 $\pm$ 4.7	11.5 $\pm$ 1.2	33
	5	200 μM	92.7 $\pm$ 1.3	2.0 $\pm$ 1.1	2.9 $\pm$ 0.7	2.4 $\pm$ 1.2	4.9
	6	200 μM	74.7 $\pm$ 14.1	5.0 $\pm$ 1.3	17.2 $\pm$ 12.2	3.1 $\pm$ 0.7	22.2
	7	200 μM	78.2 $\pm$ 9.3	4.5 $\pm$ 1.8	13.7 $\pm$ 8.2	3.6 $\pm$ 0.9	18.2
	8	200 μM	79.4 $\pm$ 3.5	5.5 $\pm$ 2.0	12.4 $\pm$ 2.4	2.8 $\pm$ 1.3	17.9
	9	200 μM	93.9 $\pm$ 0.5	1.5 $\pm$ 0.8	2.3 $\pm$ 0.3	2.3 $\pm$ 0.4	3.8
	10	200 μM	89.6 $\pm$ 7.5	3.8 $\pm$ 3.2	4.3 $\pm$ 3.6	2.4 $\pm$ 0.7	8.0
HT29	neg. ctrl		91.6 $\pm$ 5.3	2.5 $\pm$ 2.3	2.9 $\pm$ 2.2	3.1 $\pm$ 1.2	4.5
	pos. ctrl	100 μM	29.2 $\pm$ 9.4	5.5 $\pm$ 1.9	49.1 $\pm$ 7.8	16.1 $\pm$ 9.7	54.6
	Cp_2WCl_2	100 μM	86.5 $\pm$ 0.3	4.0 $\pm$ 2.2	5.0 $\pm$ 0.3	4.6 $\pm$ 2.5	9.0
	1A	200 μM	87.8 $\pm$ 4.0	3.5 $\pm$ 2.5	4.3 $\pm$ 0.9	4.4 $\pm$ 4.8	7.8
	2	200 μM	86.8 $\pm$ 2.4	3.9 $\pm$ 1.8	6.1 $\pm$ 1.0	3.3 $\pm$ 2.4	10
	3	2 μM	83.0 $\pm$ 3.8	4.6 $\pm$ 4.1	7.2 $\pm$ 3.8	5.1 $\pm$ 4.2	11.8
		20 μM	74.9 $\pm$ 3.3	8.6 $\pm$ 2.5	11.1 $\pm$ 1.0	5.4 $\pm$ 1.2	19.7
		200 μM	2.2 $\pm$ 1.3	0.8 $\pm$ 0.5	82.2 $\pm$ 4.1	14.9 $\pm$ 2.8	83.0
	4	2 μM	86.2 $\pm$ 3.1	3.7 $\pm$ 3.2	4.8 $\pm$ 1.3	5.4 $\pm$ 5.0	8.5
		20 μM	50.4 $\pm$ 4.1	16.8 $\pm$ 5.2	19.5 $\pm$ 2.0	13.3 $\pm$ 5.1	36.3
	5	200 μM	91.5 $\pm$ 8.9	0.9 $\pm$ 0.7	5.3 $\pm$ 6.9	2.3 $\pm$ 1.5	6.2
	6	200 μM	89.1 $\pm$ 0.4	4.3 $\pm$ 1.8	4.2 $\pm$ 0.6	2.4 $\pm$ 0.7	8.5
	7	200 μM	88.4 $\pm$ 3.8	3.0 $\pm$ 1.6	5.5 $\pm$ 1.9	3.1 $\pm$ 2.3	8.5
	8	200 μM	88.4 $\pm$ 3.6	3.3 $\pm$ 2.1	6.2 $\pm$ 4.1	2.1 $\pm$ 0.9	9.5
	9	200 μM	92.9 $\pm$ 5.8	0.9 $\pm$ 0.6	3.9 $\pm$ 4.5	2.3 $\pm$ 1.3	4.8
	10	200 μM	89.5 $\pm$ 8.5	3.1 $\pm$ 2.8	5.1 $\pm$ 5.2	2.3 $\pm$ 0.9	8.2

MCF-7	neg. ctrl		90.9 ± 5.1	1.3 ± 0.7	4.7 ± 3.1	3.1 ± 1.7	3.8
	pos. ctrl	100 µM	59.0 ± 1.9	2.5 ± 1.1	24.1 ± 2.6	14.5 ± 1.8	26.6
	Cp ₂ WCl ₂	100 µM	92.6 ± 2.1	1.5 ± 0.7	3.7 ± 1.2	2.3 ± 0.3	5.1
	1A	200 µM	94.3 ± 2.3	1.1 ± 0.6	3.2 ± 1.3	2.3 ± 0.5	4.3
	2	200 µM	55.1 ± 10.1	7.4 ± 2.7	22.7 ± 4.5	14.9 ± 8.3	30.1
	3	2 µM	91.8 ± 1.9	1.4 ± 0.5	4.3 ± 1.5	2.5 ± 0.6	5.6
		10 µM	86.9 ± 5.9	1.4 ± 0.3	7.7 ± 4.4	4.1 ± 1.8	9.1
		20 µM	78.5 ± 1.0	1.5 ± 0.6	11.4 ± 0.7	8.7 ± 0.3	12.9
		50 µM	57.7 ± 2.2	1.3 ± 0.5	16.2 ± 2.5	25 ± 0.9	17.5
	4	2 µM	93.9 ± 2.1	1.0 ± 0.8	3.4 ± 1.2	1.8 ± 0.0	4.4
		10 µM	75.2 ± 2.2	1.2 ± 0.5	12.0 ± 2.0	11.7 ± 4.7	13.2
		20 µM	50.2 ± 10.8	1.0 ± 0.4	15.1 ± 3.3	33.8 ± 14.5	16.0
		50 µM	5.0 ± 6.6	0.0 ± 0.0	43.4 ± 3.7	51.5 ± 10.3	43.4
	5	200 µM	86.6 ± 7.6	1.3 ± 0.7	7.9 ± 5.3	4.2 ± 2.2	9.2
	6	200 µM	92.8 ± 2.2	1.4 ± 0.8	3.6 ± 1.5	2.1 ± 0.5	5.1
	7	200 µM	87.4 ± 6.6	1.3 ± 0.4	8.1 ± 5.5	3.2 ± 1.2	9.4
	8	200 µM	87.3 ± 8.3	1.2 ± 0.3	8.7 ± 6.9	2.7 ± 1.4	9.9
	9	200 µM	87.2 ± 7.2	1.6 ± 1.0	8.4 ± 5.5	2.9 ± 1.8	7.3
	10	200 µM	15.7 ± 6.0	5.5 ± 3.2	46.3 ± 3.2	32.4 ± 12.3	51.8

Electrophoretic dsDNA plasmid assay

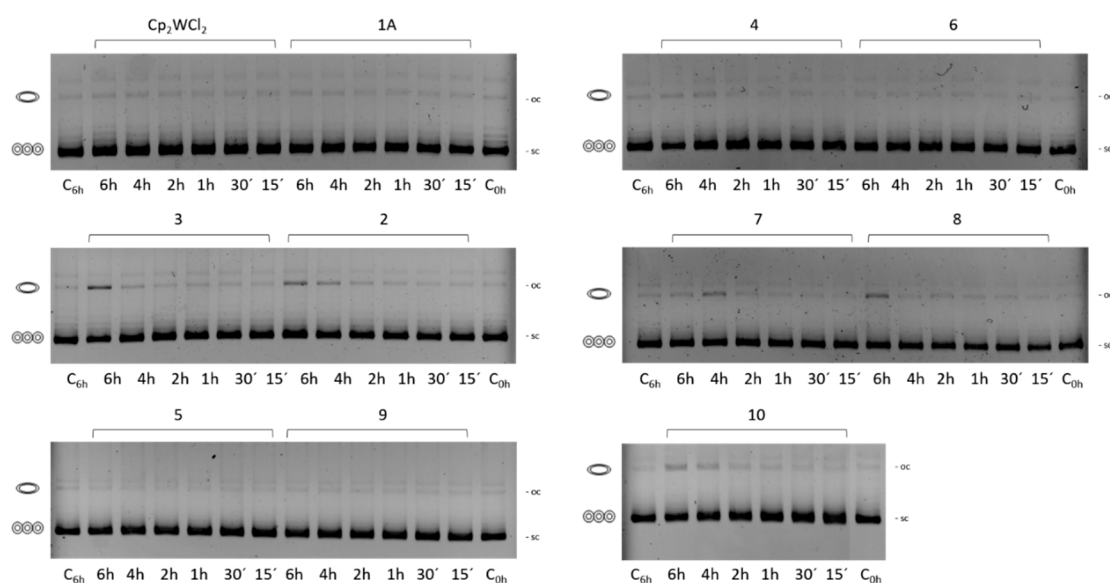


Figure S46: Electropherograms of plasmid DNA interactions with 50 µM of compounds Cp₂WCl₂ and **1A–10**. The pUC19 plasmid was incubated with the complexes for different time periods (from 15 min to 6 h) at 37 °C and the different plasmid forms were separated by agarose gel electrophoresis. ‘C_{0h}’ corresponds to an untreated, non-modified plasmid DNA control at the time point of gel loading (0 h), while ‘C_{6h}’ corresponds to an untreated control for the total duration of the experiment (6 h); oc, open circular; sc, supercoiled.

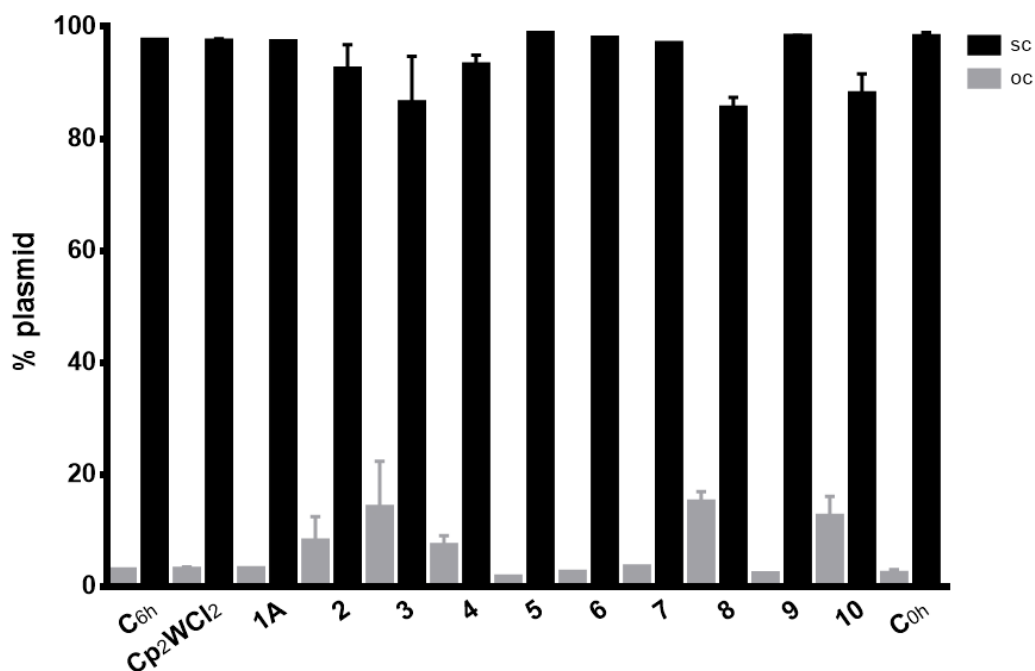


Figure S47: Bands of Figure S46 were quantitatively analyzed by the ImageJ 1.51j8 software. The black columns correspond to the open-circular (oc) form and grey columns to the supercoiled (sc) form of the pUC19 plasmid, presented as percentages of total plasmid DNA content.

References

63. Scaffidi-Domianello, Y. Y.; Legin, A.; Jakupec, M. A.; Arion, V. B.; Kukushkin, V. Y.; Galanski, M. S.; Keppler, B. K., Synthesis, characterization, and cytotoxic activity of novel potentially pH-sensitive nonclassical platinum(II) complexes featuring 1,3-dihydroxyacetone oxime ligands. *Inorg. Chem.* **2011**, 50(21), 10673-10681.

5. Concluding Discussion

Anticancer drug design and development is a complex process that is built up on recent advances in the elucidation of the biological mechanisms driving carcinogenesis, structure-activity relationship studies of a vast number of chemical leads, identification of promising compounds for clinical evaluation, as well as the conduct and analysis of clinical trials. Options for chemical synthesis are almost limitless, but they should be thoughtful, creative, and appropriate. The present work has focused on three different groups of novel metal-based (Ru, Os and W) agents, establishing their anticancer activity and elucidating aspects of their mode of action *in vitro*.

Ground-breaking studies of antineoplastic metallodrugs focused on platinum(II) agents, as a result of serendipitous observations. Cisplatin bearing two amines as non-leaving groups and two chloride ions as leaving groups with planar-square geometry and cis configuration was the first metal-based anticancer drug. Cisplatin has since been intensively studied and the general mechanism of action has been proposed, involving the activation by hydrolysis (replacement of Cl⁻ ions by water molecules) followed by binding to nuclear DNA. Cisplatin interacts with nucleophilic sites of DNA, producing a number of distorting lesions, mainly on guanine (G) by formation of monoadducts, inter- and intra-strand cross-links. Treatment with cisplatin produces a number of toxicities, while the overall therapeutic efficacy is hampered by the emergence of acquired resistance [74]. To overcome those limitations, carboplatin and oxaliplatin were subsequently designed as representatives of the second and third generation of platinum-based compounds, respectively. Carboplatin, bearing bidentate cyclobutene dicarboxylate as the leaving group, shows greater water-solubility, higher stability and slower reactivity compared to cisplatin, producing overall milder side effects [74]. Oxaliplatin with the more lipophilic 1,2-diaminocyclohexane ligand (non-leaving group) and oxalate (leaving group) partially overcomes resistance compared to cisplatin, forms fewer DNA adducts than cisplatin but is highly effective, especially at inhibiting DNA synthesis.

Other metallodrugs, e.g. of ruthenium, osmium or tungsten, were initially developed with the intention to mimic the mode of action of platinum antineoplastic agents. Although DNA was assumed as their target, it was found that the novel species mostly bind to proteins (e.g. serum proteins in blood), suggesting a significantly different pharmacology compared to platinum drugs [84, 97].

Ruthenium(III) (pro)drugs, and subsequently ruthenium(II), osmium(II) and other metallodrugs have been investigated. The two main chemical properties of Ru(III) drugs are: (1) they can be activated in the cellular environment to the more active Ru(II) species by

reduction, especially under low oxygen partial pressure (hypoxia), acidic pH and high levels of glutathione (GSH), which collectively result in greater selectivity for malignant tissues and sparing of normal cells; (2) Fe(III) and Ru(III) are chemically very similar, therefore Fe(III) can be replaced by Ru(III), exerting a “Trojan horse” mechanism [74]. The two most studied Ru(III) agents, NAMI-A and BOLD-100, interact with DNA to a lesser extent than platinum drugs and exhibit interactions with blood serum proteins such as transferrin and albumin [98]. Furthermore, BOLD-100 induces oxidative stress through the interaction with cytosolic proteins and endoplasmic reticulum (ER) stress by targeting the major cellular chaperone GRP78, ultimately inducing apoptosis through the intrinsic mitochondrial pathway [74, 84]. Additionally, it has been reported that BOLD-100 triggers immunogenic cell death (ICD) signatures in colon cancer multicellular spheroids, indicated by relocation of calreticulin on the cell membrane and secretion of high mobility group box 1 (HMGB1) and ATP [99]. Ruthenium(II) compounds on the other hand are not activated by reduction but by hydrolysis of the labile metal-halido (e.g. Ru-Cl) bond. It has been shown that the N-heterocycles or η^6 -arene moiety stabilize the lower oxidation state of ruthenium [79, 84, 100]. These compounds with half-sandwich piano-stool structures have, beside the arene, three binding sites which can be occupied by: (1) one monodentate and two halido ligands, (2) one bidentate and one halido ligand (as in Mokesch *et al.*, 2020) or (3) one tridentate ligand (as in Cseh *et al.*, 2022). One of the most studied drug candidates of the Ru-arene group is RAPTA-C, which triggers cell cycle arrest in G2/M phase, induces apoptosis *via* the mitochondrial pathway by upregulation of c-Jun NH₂-terminal kinase (JNK) and DNA damage [84, 100]. Osmium(II) compounds have several distinct features compared to ruthenium analogues such as: increased inertness resulting in slower ligand exchange kinetics, with the formed Os-aqua species being more acidic than the Ru-aqua species, and osmium as a softer Lewis acid preferably coordinating to biomolecules [84, 101]. The organometallic half-sandwich compound RM175 and its osmium analogue AFAP51 are among of the most studied couples. RM175 preferentially targets DNA at the guanine-N7 position and interacts with biological nucleophiles, e.g. L-cysteine, L-methionine and cytochrome-C, ultimately inducing apoptosis, while exhibiting anti-metastatic activity [84, 102]. AFAP51 showed more potent *in vitro* antiproliferative activity, while RM175 was more active in reducing metastasis *in vivo*, highlighting the key role of the metal centre [102]. Studies have shown the different mode of action of arene-osmium complexes depending on the chelating ligands such as (N,N-) and (N,O-) chelators, which mostly target DNA, while those with (N,S-) chelators act as enzyme inhibitors, highlighting the variety of mechanisms brought about by the different ligands [101].

In the first project presented in this thesis ruthenium(II) and osmium(II) complexes with 4'-substituted 2-phenylbenzothiazoles in piano-stool configuration were evaluated. The synthesised (C,N-) metallacycles contain a bidentate bioactive ligand, namely 2-phenylbenzothiazole, which is a heterocyclic ring system with a thiazole moiety. Benzothiazoles have been extensively studied and exert antiviral, antimicrobial, antioxidant, antidiabetic and anticancer activity. The mode of action of benzothiazole and its derivatives has been well described, including anticancer mechanisms such as tyrosine kinase inhibition (through MAPK, PI3-AKT and JNK pathways), topoisomerase I & II inhibition, induction of apoptosis through the activation of ER and/or mitochondrial signalling pathways mediated by ROS production [105]. The delineated structure-activity relationships (SAR) suggest that an aryl substitution on position 2 substantially improves the solubility and anticancer activity, and the amide substituted 2-phenylbenzothiazoles confer anticancer activity in several cell lines, highlighting the importance of phenyl ring substituents [105]. The antiproliferative activity of six ligands (2-phenylbenzothiazole and 2-(4-fluorophenyl/chlorophenyl/methylphenyl/methoxyphenyl/aminophenyl)benzothiazole) and their ruthenium and osmium complexes was evaluated in three different human cancer cell lines. The ligands exhibited moderate cytotoxicity in the micromolar range, while coupling with ruthenium and osmium resulted in increased cytotoxicity; especially osmium(II) compounds yielded IC₅₀ values in the submicromolar range. Complexes with fluorophenyl and aminophenyl moieties were selected for further biological evaluation. The two ruthenium-containing complexes showed greater apoptosis induction in SW480 cells at the same concentration compared to osmium analogues. Cellular accumulation in SW480 cells was determined by ICP-MS measurements, which showed a similar range of cellular metal content for all four organometallics. Furthermore, the aminophenyl-bearing ligand and its ruthenium(II) analogue were investigated by fluorescence microscopy, making use of inherent fluorescent properties and revealing localization in the acidic lysosome compartments. These data suggest that the anticancer activity of organoruthenium and -osmium compounds is highly dependent on the ligand incorporated. Os metallodrugs tend to be more potent or equipotent to Ru analogues upon (C,N-) coordination, as also shown by Riedl *et al.* [106, 107].

In the second project naphthoquinone-based ruthenium(II) complexes were synthesized, prompted by previous publications reporting on the anticancer activity of naphthoquinone-based organometallics [108-111]. Naphthoquinones are a prominent class of quinones, which are ubiquitous in nature, occurring in a range of organisms (animals, plants, bacteria, *etc.*) and show similarities to the clinically used anthracyclines doxorubicin and daunorubicin which are quinone derivatives. Besides their anticancer properties quinones have also been

reported to possess antibacterial, antifungal and anti-inflammatory activity. Their anticancer mode of action is multifaced and comprises the generation of ROS, the inhibition of critical enzymes such as NQO1 (NADPH: quinone oxidoreductase 1), MALT1 (mucosa-associated lymphoid tissue lymphoma translocation protein) and DNA topoisomerases, the repression of telomerase activity, the regulation of the tumour suppressor p53, apoptosis induction through ER stress and tumour-associated inflammation [112]. In this project a naphthoquinone moiety (phthiocol) together with azole yielded a tridentate (*N,O,O*–) ligand with increased stability, which coordinates to the ruthenium metal centre substituted with different arenes (*p*-cymene, benzene, toluene, indane, biphenyl, hexamethylbenzene (HMB)). The short- (24 h and 96 h) and long-term (11 days) cytotoxicity assays revealed that the most stable complexes with *p*-cymene and biphenyl arene are the most cytotoxic, followed by a complex with HMB. An unexpected finding from the *in vitro* cytotoxicity studies was that the SW480 colon carcinoma cells were more sensitive to all compounds than the otherwise broadly chemo-sensitive CH1/PA-1 ovarian teratocarcinoma cells. Cellular uptake studies revealed that the *p*-cymene and biphenyl arene complexes yielded the highest total ruthenium content in treated SW480 cells, in accordance with their higher lipophilicity. These two compounds readily induced apoptosis in exposed SW480 cells. In contrast no ROS generation and interactions with DNA were observed under the same experimental conditions. All tested compounds had a greater impact on cell cycle distribution in SW480 cells, compared to CH1/PA-1 cells. Both NQO1 enzyme activity and its inhibition was prominent in SW480 cells, while CH1/PA-1 cells were devoid of the enzyme activity. Collectively, results suggest that ruthenium organometallics exhibit a mode of action distinct from that of platinum complexes, attributed to their distinct structural features.

Metallocenes were introduced into experimental cancer therapy when titanocene dichloride (Cp_2TiCl_2) bearing structural similarities to cisplatin was developed by Köpf-Meier and Köpf. It showed promising results in phase I trials but did not progress past phase II due to low response in metastatic renal-cell carcinoma and metastatic breast cancer, which was attributed to poor solubility in aqueous milieu and hydrolytic instability. It was reported that Cp_2TiCl_2 binds to DNA, inhibits DNA synthesis as well as topoisomerase activity and induces apoptosis [94, 103]. Other metallocene dihalides with structural similarities to Cp_2TiCl_2 with the general formula Cp_2MX_2 (M: Mo, W, V and X: halides or pseudo-halides) exhibited potent activity in several human and murine cancer cell lines. Aspects of the mechanism of action of Cp_2MoCl_2 have been intensively studied and include the inhibition of cell proliferation-regulating protein kinase C (PKC), coordination of $\text{Cp}_2\text{Mo}^{2+}$ to cysteine residues of tubulin, formation of stable adducts with glutathione (GSH) and weak interaction with DNA [95]. Subsequent efforts aiming to improve the anticancer activity of metallocenes include two

structural modification strategies: (1) replacement of ancillary halides (X) by hydrophobic ligands (e.g. bidentate) to modulate stability and biological activity and/or (2) functionalization of the cyclopentadienyl ring with hydrophobic or hydrophilic groups and bioactive molecules. These modifications have resulted in improved biological activity of metallocenes, e.g. increased cytotoxicity of $\text{Cp}_2\text{Mo}(\text{malonate})$ and $[\text{Cp}_2\text{Mo}(\text{maltolato})]\text{Cl}$ complexes compared to the mother compound Cp_2MoCl_2 [95]. Meléndez and coworkers synthesized Cp_2WCl_2 derivatives bearing 3-hydroxy-4-pyronato ligands and tungstenocenes with (O,O-) bidentate ligands, which were shown to possess enhanced cytotoxic potency compared to the parent complex Cp_2WCl_2 and molybdenocene analogues [93].

In the third project tungstenocenes bearing various bioactive bidentate ligands were developed and evaluated. These compounds were additionally investigated in 3D cell culture models (multicellular HCT116, HT29 and MCF-7 spheroids). Kandoller *et al.* had previously reported on the improved stability under physiological conditions and increased cytotoxicity of molybdenocenes by incorporating bioactive chelating ligands, compared to molybdocene dichloride [113]. The same concept of replacing the halido ligands was applied in this project studying tungstenocenes with a diverse set of biologically active (O,O-), (O,S-) and (O,N-) chelates: maltol, flavonol, allomaltol, ethylmaltol and their thionated modifications, deferiprone and picolinic acid. All of these ligands bind in a bidentate manner *via* their (O,O-), (O,S-) or (O,N-) donor atoms to the central metal atoms, as previously shown in the context of other organometallics [93, 114-119]. The cytotoxicity studies performed for this project showed that complexes with flavonol and (more so) with thioflavonol chelates are the most potent in cancer cells, mirroring previous findings on molybdenocenes with (thio)flavonol ligands [113, 119]. The novel tungstenocenes were tested for their ROS generation and apoptosis induction potential and their DNA interactions. Some complexes with (O,O-) chelates (maltol, flavonol and ethylmaltol) and one with a (S,O-) chelate (thioethylmaltol) were able to increase the ROS levels in treated cells. Only a subset of the studies complexes (bearing thiomaltol, flavonol, thioflavonol, thioallomaltol or thioethylmaltol ligands) directly interacted with the DNA and were capable of producing DNA single-strand breaks, as shown in an assay employing naked plasmid DNA. Induction of apoptosis as detected by flow cytometry analysis was observed only for the most cytotoxic compounds. The two flavonol or thioflavonol bearing complexes were further tested in 3D multicellular spheroid models. Treatment with them strongly affected spheroid growth (inhibiting or completely abolishing it) and markedly changed their morphology. Immunofluorescence imaging of HCT116 spheroids stained for the KI67 proliferation marker revealed a decreased number of proliferative cells in the treated samples. Qualitative bioimaging of $^{182}\text{W}^+$, $^{23}\text{Na}^+$, $^{24}\text{Mg}^+$, $^{31}\text{P}^+$ and $^{66}\text{Zn}^+$ distribution was performed in cryosections of treated HCT116

spheroids by LA-ICP-TOF-MS. The intensity map of the metal contents in the spheroids showed differential distribution patterns between the two complexes, with the thioflavone bearing complex exhibiting greater cellular uptake. Taken together, the (S,O-) chelate bearing complexes were more active *in vitro* than their (O,O-) and (N,O-) counterparts.

Collectively, following the biological evaluation of the three classes of novel metal complexes under consideration in this thesis, a number of structure-activity relationships were established, and the most potent analogues were identified. By employing several biological assays in different cellular models including 3D spheroid models, novel aspects of the mode of action and cellular pharmacology of ruthenium, osmium and tungsten complexes were revealed, highlighting the novelty and potential of these classes of compounds for further development as anticancer agents.

6. References

1. Siegel, R.L.; Miller, K.D.; Wagle, N.S.; Jemal, A., Cancer statistics, 2023. *CA Cancer J Clin.* **2023**, *73*(1), 17–48. DOI: 10.3322/caac.21763.
2. Cancer Today - International Agency for Research on Cancer. [accessed on 2023. 04.13.]; Available from: <https://gco.iarc.fr/today/online-analysis-pie>.
3. Fane, M.; Weeraratna, A.T., How the ageing microenvironment influences tumour progression. *Nat Rev Cancer.* **2020**, *20*(2), 89–106. DOI: 10.1038/s41568-019-0222-9.
4. Rubin, J.B., The spectrum of sex differences in cancer. *Trends Cancer.* **2022**, *8*(4), 303–15. DOI: 10.1016/j.trecan.2022.01.013.
5. Loosen, S.H.; Roderburg, C.; Jordens, M.S.; Fluegen, G.; Luedde, T.; Kostev, K., Overweight and Obesity Determine the Risk for Gastrointestinal Cancer in a Sex-Dependent Manner: A Retrospective Cohort Study of 287,357 Outpatients in Germany. *Cancers (Basel).* **2022**, *14*(4). DOI: 10.3390/cancers14040931.
6. Fritz, A.; Percy, C.; Jack, A.; Shanmugaratnam, K.; Sobin, L.; Parkin, M.D.; Whelan, S., *International Classification of Diseases for Oncology*. 1st revision (ICD-O-3), 3rd ed. 2013: World Health Organization. 252 pages.
7. Hanahan, D.; Weinberg, R.A., The Hallmarks of Cancer. *Cell.* **2000**, *100*, 57–70. DOI: 10.1016/S0092-8674(00)81683-9.
8. Hanahan, D.; Weinberg, R.A., Hallmarks of cancer: the next generation. *Cell.* **2011**, *144*(5), 646–74. DOI: 10.1016/j.cell.2011.02.013.
9. Hanahan, D., Hallmarks of Cancer: New Dimensions. *Cancer Discov.* **2022**, *12*(1), 31–46. DOI: 10.1158/2159-8290.CD-21-1059.
10. Fares, J.; Fares, M.Y.; Khachfe, H.H.; Salhab, H.A.; Fares, Y., Molecular principles of metastasis: a hallmark of cancer revisited. *Signal Transduct Target Ther.* **2020**, *5*(1), 28. DOI: 10.1038/s41392-020-0134-x.
11. Liberti, M.V.; Locasale, J.W., The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem Sci.* **2016**, *41*(3), 211–218. DOI: 10.1016/j.tibs.2015.12.001.
12. Simiczyjew, A.; Dratkiewicz, E.; Mazurkiewicz, J.; Zietek, M.; Matkowski, R.; Nowak, D., The Influence of Tumor Microenvironment on Immune Escape of Melanoma. *Int J Mol Sci.* **2020**, *21*(21). DOI: 10.3390/ijms21218359.
13. Negrini, S.; Gorgoulis, V.G.; Halazonetis, T.D., Genomic instability--an evolving hallmark of cancer. *Nat Rev Mol Cell Biol.* **2010**, *11*(3), 220–8. DOI: 10.1038/nrm2858.
14. Costa, P.; Sales, S.L.A.; Pinheiro, D.P.; Pontes, L.Q.; Maranhao, S.S.; Pessoa, C.D.O.; Furtado, G.P.; Furtado, C.L.M., Epigenetic reprogramming in cancer: From diagnosis to treatment. *Front Cell Dev Biol.* **2023**, *11*, 1116805. DOI: 10.3389/fcell.2023.1116805.
15. Henke, E.; Nandigama, R.; Ergun, S., Extracellular Matrix in the Tumor Microenvironment and Its Impact on Cancer Therapy. *Front Mol Biosci.* **2019**, *6*, 160. DOI: 10.3389/fmolb.2019.00160.

16. Huang, J.; Zhang, L.; Wan, D.; Zhou, L.; Zheng, S.; Lin, S.; Qiao, Y., Extracellular matrix and its therapeutic potential for cancer treatment. *Signal Transduct Target Ther.* **2021**, *6*(1), 153. DOI: 10.1038/s41392-021-00544-0.
17. Poltavets, V.; Kochetkova, M.; Pitson, S.M.; Samuel, M.S., The Role of the Extracellular Matrix and Its Molecular and Cellular Regulators in Cancer Cell Plasticity. *Front Oncol.* **2018**, *8*, 431. DOI: 10.3389/fonc.2018.00431.
18. Karamanos, N.K.; Piperigkou, Z.; Passi, A.; Gotte, M.; Rousselle, P.; Vlodavsky, I., Extracellular matrix-based cancer targeting. *Trends Mol Med.* **2021**, *27*(10), 1000–1013. DOI: 10.1016/j.molmed.2021.07.009.
19. Cox, T.R., The matrix in cancer. *Nat Rev Cancer.* **2021**, *21*(4), 217–238. DOI: 10.1038/s41568-020-00329-7.
20. Jinka, R.; Kapoor, R.; Sistla, P.G.; Raj, T.A.; Pande, G., Alterations in Cell-Extracellular Matrix Interactions during Progression of Cancers. *Int J Cell Biol.* **2012**, *2012*, 219196. DOI: 10.1155/2012/219196.
21. Deville, S.S.; Cordes, N., The Extracellular, Cellular, and Nuclear Stiffness, a Trinity in the Cancer Resistome-A Review. *Front Oncol.* **2019**, *9*, 1376. DOI: 10.3389/fonc.2019.01376.
22. Jean, C.; Gravelle, P.; Fournie, J.J.; Laurent, G., Influence of stress on extracellular matrix and integrin biology. *Oncogene.* **2011**, *30*(24), 2697–706. DOI: 10.1038/onc.2011.27.
23. Kesh, K.; Gupta, V.K.; Durden, B.; Garrido, V.; Mateo-Victoriano, B.; Lavania, S.P.; Banerjee, S., Therapy Resistance, Cancer Stem Cells and ECM in Cancer: The Matrix Reloaded. *Cancers (Basel).* **2020**, *12*(10). DOI: 10.3390/cancers12103067.
24. Jensen, C.; Teng, Y., Is It Time to Start Transitioning From 2D to 3D Cell Culture? *Front Mol Biosci.* **2020**, *7*, 33. DOI: 10.3389/fmolb.2020.00033.
25. Habanjar, O.; Diab-Assaf, M.; Caldefie-Chezet, F.; Delort, L., 3D Cell Culture Systems: Tumor Application, Advantages, and Disadvantages. *Int J Mol Sci.* **2021**, *22*(22). DOI: 10.3390/ijms222212200.
26. Huang, B.W.; Gao, J.Q., Application of 3D cultured multicellular spheroid tumor models in tumor-targeted drug delivery system research. *J Control Release.* **2018**, *270*, 246–259. DOI: 10.1016/j.jconrel.2017.12.005.
27. Langhans, S.A., Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. *Front Pharmacol.* **2018**, *9*, 6. DOI: 10.3389/fphar.2018.00006.
28. Edmondson, R.; Broglie, J.J.; Adcock, A.F.; Yang, L.J., Three-Dimensional Cell Culture Systems and Their Applications in Drug Discovery and Cell-Based Biosensors. *Assay and Drug Development Technologies.* **2014**, *12*(4), 207–218. DOI: 10.1089/adt.2014.573.
29. Doctor, A.; Seifert, V.; Ullrich, M.; Hauser, S.; Pietzsch, J., Three-Dimensional Cell Culture Systems in Radiopharmaceutical Cancer Research. *Cancers (Basel).* **2020**, *12*(10). DOI: 10.3390/cancers12102765.
30. Zanoni, M.; Pignatta, S.; Arienti, C.; Bonafè, M.; Tesei, A., Anticancer drug discovery using multicellular tumor spheroid models. *Expert Opinion on Drug Discovery.* **2019**, *14*(3), 289–301. DOI: 10.1080/17460441.2019.1570129.

31. Cui, X.; Hartanto, Y.; Zhang, H., Advances in multicellular spheroids formation. *J R Soc Interface*. **2017**, *14*(127). DOI: 10.1098/rsif.2016.0877.
32. Jubelin, C.; Munoz-Garcia, J.; Griscom, L.; Cochonneau, D.; Ollivier, E.; Heymann, M.F.; Vallette, F.M.; Oliver, L.; Heymann, D., Three-dimensional in vitro culture models in oncology research. *Cell Biosci*. **2022**, *12*(1), 155. DOI: 10.1186/s13578-022-00887-3.
33. Azimian Zavareh, V.; Rafiee, L.; Sheikholeslam, M.; Shariati, L.; Vaseghi, G.; Savoji, H.; Haghjooy Javanmard, S., Three-Dimensional in Vitro Models: A Promising Tool To Scale-Up Breast Cancer Research. *ACS Biomater Sci Eng*. **2022**, *8*(11), 4648–4672. DOI: 10.1021/acsbiomaterials.2c00277.
34. Pinto, B.; Henriques, A.C.; Silva, P.M.A.; Bousbaa, H., Three-Dimensional Spheroids as In Vitro Preclinical Models for Cancer Research. *Pharmaceutics*. **2020**, *12*(12). DOI: 10.3390/pharmaceutics12121186.
35. Kamatar, A.; Gunay, G.; Acar, H., Natural and Synthetic Biomaterials for Engineering Multicellular Tumor Spheroids. *Polymers (Basel)*. **2020**, *12*(11). DOI: 10.3390/polym12112506.
36. Alghuwainem, A.; Alshareeda, A.T.; Alsowayan, B., Scaffold-Free 3-D Cell Sheet Technique Bridges the Gap between 2-D Cell Culture and Animal Models. *Int J Mol Sci*. **2019**, *20*(19). DOI: 10.3390/ijms20194926.
37. De, S.; Singh, N., Advancements in Three Dimensional In-Vitro Cell Culture Models. *Chem Rec*. **2022**, *22*(9), e202200058. DOI: 10.1002/tcr.202200058.
38. Lee, K.H.; Kim, T.H., Recent Advances in Multicellular Tumor Spheroid Generation for Drug Screening. *Biosensors (Basel)*. **2021**, *11*(11). DOI: 10.3390/bios11110445.
39. Hainline, K.M.; Gu, F.; Handley, J.F.; Tian, Y.F.; Wu, Y.; de Wet, L.; Vander Griend, D.J.; Collier, J.H., Self-Assembling Peptide Gels for 3D Prostate Cancer Spheroid Culture. *Macromol Biosci*. **2019**, *19*(1), e1800249. DOI: 10.1002/mabi.201800249.
40. Merry, C.L.R.; Farnie, G.; Clarke, R.B.; Grabowska, A.M.; Thompson, J.L.; Slater, C.E.; Spence, K.; Jones, S.; Meade, K.A.; Lis-Slimak, K.; Morgan, R.L.; Ashworth, J.C., Preparation of a User-Defined Peptide Gel for Controlled 3D Culture Models of Cancer and Disease. *Journal of Visualized Experiments*. **2020**(166). DOI: 10.3791/61710.
41. Passaniti, A.; Kleinman, H.K.; Martin, G.R., Matrigel: history/background, uses, and future applications. *Journal of Cell Communication and Signaling*. **2021**, *16*(4), 621–626. DOI: 10.1007/s12079-021-00643-1.
42. Igarashi, Y.; Sasada, T., Cancer Vaccines: Toward the Next Breakthrough in Cancer Immunotherapy. *J Immunol Res*. **2020**, *2020*, 5825401. DOI: 10.1155/2020/5825401.
43. Sudhakar, A., History of Cancer, Ancient and Modern Treatment Methods. *J Cancer Sci Ther*. **2009**, *1*(2), 1–4. DOI: 10.4172/1948-5956.100000e2.
44. Lane, T., A short history of robotic surgery. *Ann R Coll Surg Engl*. **2018**, *100*(6_sup), 5–7. DOI: 10.1308/rcsann.suppl.5.

45. Gianfaldoni, S.; Gianfaldoni, R.; Wollina, U.; Lotti, J.; Tchernev, G.; Lotti, T., An Overview on Radiotherapy: From Its History to Its Current Applications in Dermatology. *Open Access Maced J Med Sci.* **2017**, *5*(4), 521–525. DOI: 10.3889/oamjms.2017.122.
46. Chargari, C.; Deutsch, E.; Blanchard, P.; Gouy, S.; Martelli, H.; Guerin, F.; Dumas, I.; Bossi, A.; Morice, P.; Viswanathan, A.N.; Haie-Meder, C., Brachytherapy: An overview for clinicians. *CA Cancer J Clin.* **2019**, *69*(5), 386-401. DOI: 10.3322/caac.21578.
47. Li, X.; Lovell, J.F.; Yoon, J.; Chen, X., Clinical development and potential of photothermal and photodynamic therapies for cancer. *Nat Rev Clin Oncol.* **2020**, *17*(11), 657–674. DOI: 10.1038/s41571-020-0410-2.
48. Deng, X.; Shao, Z.; Zhao, Y., Solutions to the Drawbacks of Photothermal and Photodynamic Cancer Therapy. *Adv Sci (Weinh).* **2021**, *8*(3), 2002504. DOI: 10.1002/advs.202002504.
49. Smith, C.E.P.; Prasad, V., Targeted Cancer Therapies. *Am Fam Physician.* **2021**, *103*(3), 155–163.
50. Lee, Y.T.; Tan, Y.J.; Oon, C.E., Molecular targeted therapy: Treating cancer with specificity. *Eur J Pharmacol.* **2018**, *834*, 188–196. DOI: 10.1016/j.ejphar.2018.07.034.
51. Dougan, M.; Dranoff, G., Immune therapy for cancer. *Annu Rev Immunol.* **2009**, *27*, 83–117. DOI: 10.1146/annurev.immunol.021908.132544.
52. Johdi, N.A.; Sukor, N.F., Colorectal Cancer Immunotherapy: Options and Strategies. *Front Immunol.* **2020**, *11*, 1624. DOI: 10.3389/fimmu.2020.01624.
53. Liu, Z.; Shi, M.; Ren, Y.; Xu, H.; Weng, S.; Ning, W.; Ge, X.; Liu, L.; Guo, C.; Duo, M.; Li, L.; Li, J., et al., Recent advances and applications of CRISPR-Cas9 in cancer immunotherapy. *Mol Cancer.* **2023**, *22*(1), 35. DOI: 10.1186/s12943-023-01738-6.
54. Balon, K.; Sheriff, A.; Jackow, J.; Laczmanski, L., Targeting Cancer with CRISPR/Cas9-Based Therapy. *Int J Mol Sci.* **2022**, *23*(1). DOI: 10.3390/ijms23010573.
55. Draganescu, M.; Carmocan, C., Hormone Therapy in Breast Cancer. *Chirurgia (Bucur).* **2017**, *112*(4), 413–417. DOI: 10.21614/chirurgia.112.4.413.
56. Desai, K.; McManus, J.M.; Sharifi, N., Hormonal Therapy for Prostate Cancer. *Endocr Rev.* **2021**, *42*(3), 354–373. DOI: 10.1210/endrev/bnab002.
57. Tilsed, C.M.; Fisher, S.A.; Nowak, A.K.; Lake, R.A.; Lesterhuis, W.J., Cancer chemotherapy: insights into cellular and tumor microenvironmental mechanisms of action. *Front Oncol.* **2022**, *12*, 960317. DOI: 10.3389/fonc.2022.960317.
58. WHO Collaborating Center for Drug Statistics Methodology. [accessed on 2023. 07.14.]; Available from: https://www.whocc.no/atc_ddd_index/?code=L01&showdescription=no.
59. Lind, M.J., Principles of cytotoxic chemotherapy. *Medicine.* **2011**, *39*(12), 711–716. DOI: 10.1016/j.mpmed.2011.09.009.
60. Kondo, N.; Takahashi, A.; Ono, K.; Ohnishi, T., DNA damage induced by alkylating agents and repair pathways. *J Nucleic Acids.* **2010**, *2010*, 543531. DOI: 10.4061/2010/543531.
61. Alqahtani, F.Y.; Aleanizy, F.S.; El Tahir, E.; Alkahtani, H.M.; AlQuadeib, B.T., Paclitaxel. *Profiles Drug Subst Excip Relat Methodol.* **2019**, *44*, 205–238. DOI: 10.1016/bs.podrm.2018.11.001.

62. Kumar, A., Vincristine and Vinblastine: A Review. *Int J Med Pharmaceut Sci.* **2016**, 6(1), 23–30.
63. Pommier, Y., Topoisomerase I inhibitors: camptothecins and beyond. *Nat Rev Cancer.* **2006**, 6(10), 789–802. DOI: 10.1038/nrc1977.
64. Sritharan, S.; Sivalingam, N., A comprehensive review on time-tested anticancer drug doxorubicin. *Life Sci.* **2021**, 278, 119527. DOI: 10.1016/j.lfs.2021.119527.
65. Bhullar, K.S.; Lagaron, N.O.; McGowan, E.M.; Parmar, I.; Jha, A.; Hubbard, B.P.; Rupasinghe, H.P.V., Kinase-targeted cancer therapies: progress, challenges and future directions. *Mol Cancer.* **2018**, 17(1), 48. DOI: 10.1186/s12943-018-0804-2.
66. Rosenberg, B.; Vancamp, L.; Trosko, J.E.; Mansour, V.H., Platinum Compounds: A New Class of Potent Antitumour Agents. *Nat. Cell Biol.* **1969**, 222, 385–386.
67. Rosenberg, B., Platinum coordination complexes in cancer chemotherapy. *Naturwissenschaften.* **1973**, 60, 399–406.
68. Ciarimboli, G., Anticancer Platinum Drugs Update. *Biomolecules.* **2021**, 11(11). DOI: 10.3390/biom11111637.
69. Zhang, C.; Xu, C.; Gao, X.; Yao, Q., Platinum-based drugs for cancer therapy and anti-tumor strategies. *Theranostics.* **2022**, 12(5), 2115–2132. DOI: 10.7150/thno.69424.
70. Alassadi, S.; Pisani, M.J.; Wheate, N.J., A chemical perspective on the clinical use of platinum-based anticancer drugs. *Dalton Trans.* **2022**, 51(29), 10835–10846. DOI: 10.1039/d2dt01875f.
71. Zhou, J.; Kang, Y.; Chen, L.; Wang, H.; Liu, J.; Zeng, S.; Yu, L., The Drug-Resistance Mechanisms of Five Platinum-Based Antitumor Agents. *Front Pharmacol.* **2020**, 11, 343. DOI: 10.3389/fphar.2020.00343.
72. Johnstone, T.C.; Suntharalingam, K.; Lippard, S.J., The Next Generation of Platinum Drugs: Targeted Pt(II) Agents, Nanoparticle Delivery, and Pt(IV) Prodrugs. *Chem Rev.* **2016**, 116(5), 3436–86. DOI: 10.1021/acs.chemrev.5b00597.
73. Mahmud, K.M.; Niloy, M.S.; Shakil, M.S.; Islam, M.A., Ruthenium Complexes: An Alternative to Platinum Drugs in Colorectal Cancer Treatment. *Pharmaceutics.* **2021**, 13(8). DOI: 10.3390/pharmaceutics13081295.
74. Lucaciu, R.L.; Hangan, A.C.; Sevastre, B.; Oprean, L.S., Metallo-Drugs in Cancer Therapy: Past, Present and Future. *Molecules.* **2022**, 27(19). DOI: 10.3390/molecules27196485.
75. Antonarakis, E.S.; Emadi, A., Ruthenium-based chemotherapeutics: are they ready for prime time? *Cancer Chemother Pharmacol.* **2010**, 66(1), 1–9. DOI: 10.1007/s00280-010-1293-1.
76. Alessio, E., Thirty Years of the Drug Candidate NAMI-A and the Myths in the Field of Ruthenium Anticancer Compounds: A Personal Perspective. *European Journal of Inorganic Chemistry.* **2016**, 2017(12), 1549–1560. DOI: 10.1002/ejic.201600986.
77. Cseh, K.; Geisler, H.; Stanojkowska, K.; Westermayr, J.; Brunmayr, P.; Wenisch, D.; Gajic, N.; Hejl, M.; Schaier, M.; Koellensperger, G.; Jakupec, M.A.; Marquetand, P., et al., Arene Variation of Highly Cytotoxic Tridentate Naphthoquinone-Based Ruthenium(II) Complexes

- and In-Depth In Vitro Studies. *Pharmaceutics*. **2022**, *14*(11). DOI: 10.3390/pharmaceutics14112466.
78. Gasser, G.; Ott, I.; Metzler-Nolte, N., Organometallic anticancer compounds. *J. Med. Chem.* **2011**, *54*(1), 3–25. DOI: 10.1021/jm100020w.
 79. Lee, S.Y.; Kim, C.Y.; Nam, T.G., Ruthenium Complexes as Anticancer Agents: A Brief History and Perspectives. *Drug Des Devel Ther.* **2020**, *14*, 5375–5392. DOI: 10.2147/DDDT.S275007.
 80. Swaminathan, S.; Karvembu, R., Dichloro Ru(II)-p-cymene-1,3,5-triaza-7-phosphaadamantane (RAPTA-C): A Case Study. *ACS Pharmacol Transl Sci.* **2023**, *6*(7), 982–996. DOI: 10.1021/acsptsci.3c00085.
 81. Sonkar, C.; Sarkar, S.; Mukhopadhyay, S., Ruthenium(ii)-arene complexes as anti-metastatic agents, and related techniques. *RSC Med Chem.* **2022**, *13*(1), 22–38. DOI: 10.1039/d1md00220a.
 82. Meszaros, J.P.; Kandoller, W.; Spengler, G.; Prado-Roller, A.; Keppler, B.K.; Enyedy, E.A., Half-Sandwich Rhodium Complexes with Releasable N-Donor Monodentate Ligands: Solution Chemical Properties and the Possibility for Acidosis Activation. *Pharmaceutics*. **2023**, *15*(2). DOI: 10.3390/pharmaceutics15020356.
 83. Zhang, P.; Huang, H., Future potential of osmium complexes as anticancer drug candidates, photosensitizers and organelle-targeted probes. *Dalton Trans.* **2018**, *47*(42), 14841–14854. DOI: 10.1039/c8dt03432j.
 84. Meier-Menches, S.M.; Gerner, C.; Berger, W.; Hartinger, C.G.; Keppler, B.K., Structure-activity relationships for ruthenium and osmium anticancer agents - towards clinical development. *Chem Soc Rev.* **2018**, *47*(3), 909–928. DOI: 10.1039/c7cs00332c.
 85. Hildebrandt, J.; Hafner, N.; Kritsch, D.; Gorls, H.; Durst, M.; Runnebaum, I.B.; Weigand, W., Highly Cytotoxic Osmium(II) Compounds and Their Ruthenium(II) Analogues Targeting Ovarian Carcinoma Cell Lines and Evading Cisplatin Resistance Mechanisms. *Int J Mol Sci.* **2022**, *23*(9). DOI: 10.3390/ijms23094976.
 86. Köpf, H.; Köpf-Maier, P., Titanocene Dichloride – The First Metallocene with Cancerostatic Activity. *Angew. Chem. Int. Ed. Engl.* **1979**, *18*(6), 477–478. DOI: 10.1002/anie.197904771.
 87. Gomez-Ruiz, S.; Maksimovic-Ivanic, D.; Mijatovic, S.; Kaluderovic, G.N., On the discovery, biological effects, and use of Cisplatin and metallocenes in anticancer chemotherapy. *Bioinorg. Chem. Appl.* **2012**, *2012*, 140284. DOI: 10.1155/2012/140284.
 88. Köpf-Maier, P., Complexes of Metals Other than Platinum as Antitumour Agents. *Eur. J. Clin. Pharmacol.* **1994**, *47*(1), 1–16. DOI: 10.1007/BF00193472.
 89. Köpf-Maier, P.; Köpf, H., Vanadocen-dichlorid - ein weiteres Antitumor-Agens aus der Metallocenreihe / Vanadocene Dichloride - Another Antitumor Agent from the Metallocene Series. *Zeitschrift für Naturforschung B.* **1979**, *34*(6), 805–807. DOI: 10.1515/znb-1979-0609.
 90. Köpf-Maier, P.; Leitner, M.; Voigtländer, R.; Köpf, H., Molybdocene Dichloride as an Antitumor Agent. *Z Naturforsch C.* **1979**, *34*(12), 1174–1176.

91. Köpf-Maier, P.; Leitner, M.; Köpf, H., Tumor Inhibition by Metallocenes: Antitumor Activity of Niobocene and Tungstocene Dichlorides. *J. Inorg. Nucl. Chem.* **1980**, *42*(12), 1789–1791. DOI: 10.1016/0022-1902(80)80166-7.
92. Kuo, L.Y.; Kanatzidis, M.G.; Sabat, M.; Tipton, A.L.; Marks, T.J., Metallocene Antitumor Agents. Solution and Solid-State Molybdenocene Coordination Chemistry of DNA Constituents. *J. Am. Chem. Soc.* **1991**, *113*(24), 9027–9045. DOI: 10.1021/ja00024a002.
93. Dominguez-Garcia, M.; Ortega-Zuniga, C.; Meléndez, E., New tungstenocenes containing 3-hydroxy-4-pyrone ligands: antiproliferative activity on HT-29 and MCF-7 cell lines and binding to human serum albumin studied by fluorescence spectroscopy and molecular modeling methods. *J. Biol. Inorg. Chem.* **2013**, *18*(2), 195–209. DOI: 10.1007/s00775-012-0964-2.
94. Meléndez, E., Metallocenes as Target Specific Drugs for Cancer Treatment. *Inorg. Chim Acta.* **2012**, *393*, 36–52. DOI: 10.1016/j.ica.2012.06.007.
95. Meléndez, E., Bioorganometallic Chemistry of Molybdenocene Dichloride and Its Derivatives. *J. Organomet. Chem.* **2012**, *706–707*, 4–12. DOI: 10.1016/j.jorganchem.2012.02.006.
96. Gianferrara, T.; Bratsos, I.; Alessio, E., A categorization of metal anticancer compounds based on their mode of action. *Dalton Trans.* **2009**(37), 7588-98. DOI: 10.1039/b905798f.
97. Jakupiec, M.A.; Galanski, M.S.; Arion, V.B.; Hartinger, C.G.; Keppler, B.K., Antitumour metal compounds: more than theme and variations. *Dalton Trans.* **2008**(2), 183–94. DOI: 10.1039/b712656p.
98. Alessio, E.; Messori, L., NAMI-A and KP1019/1339, Two Iconic Ruthenium Anticancer Drug Candidates Face-to-Face: A Case Story in Medicinal Inorganic Chemistry. *Molecules.* **2019**, *24*(10). DOI: 10.3390/molecules24101995.
99. Wernitznig, D.; Kiakos, K.; Del Favero, G.; Harrer, N.; Machat, H.; Osswald, A.; Jakupiec, M.A.; Wernitznig, A.; Sommergruber, W.; Keppler, B.K., First-in-class ruthenium anticancer drug (KP1339/IT-139) induces an immunogenic cell death signature in colorectal spheroids in vitro. *Metallomics.* **2019**, *11*(6), 1044–1048. DOI: 10.1039/c9mt00051h.
100. Anthony, E.J.; Bolitho, E.M.; Bridgewater, H.E.; Carter, O.W.L.; Donnelly, J.M.; Imberti, C.; Lant, E.C.; Lermite, F.; Needham, R.J.; Palau, M.; Sadler, P.J.; Shi, H., et al., Metallodrugs are unique: opportunities and challenges of discovery and development. *Chem Sci.* **2020**, *11*(48), 12888–12917. DOI: 10.1039/d0sc04082g.
101. Hanif, M.; Babak, M.V.; Hartinger, C.G., Development of anticancer agents: wizardry with osmium. *Drug Discov Today.* **2014**, *19*(10), 1640–8. DOI: 10.1016/j.drudis.2014.06.016.
102. Bergamo, A.; Masi, A.; Peacock, A.F.; Habtemariam, A.; Sadler, P.J.; Sava, G., In vivo tumour and metastasis reduction and in vitro effects on invasion assays of the ruthenium RM175 and osmium AFAP51 organometallics in the mammary cancer model. *J Inorg Biochem.* **2010**, *104*(1), 79–86. DOI: 10.1016/j.jinorgbio.2009.10.005.
103. Ott, I.; Gust, R., Non platinum metal complexes as anti-cancer drugs. *Arch Pharm (Weinheim).* **2007**, *340*(3), 117–26. DOI: 10.1002/ardp.200600151.

104. Štarha, P.; Trávníček, Z., Non-platinum complexes containing releasable biologically active ligands. *Coordination Chemistry Reviews*. **2019**, *395*, 130–145. DOI: 10.1016/j.ccr.2019.06.001.
105. Pathak, N.; Rath, E.; Kumar, N.; Kini, S.G.; Rao, C.M., A Review on Anticancer Potentials of Benzothiazole Derivatives. *Mini Rev Med Chem*. **2020**, *20*(1), 12–23. DOI: 10.2174/1389557519666190617153213.
106. Riedl, C.A.; Hejl, M.; Klose, M.H.M.; Roller, A.; Jakupec, M.A.; Kandioller, W.; Keppler, B.K., N- and S-donor leaving groups in triazole-based ruthena(ii)cycles: potent anticancer activity, selective activation, and mode of action studies. *Dalton Trans*. **2018**, *47*(13), 4625–4638. DOI: 10.1039/c8dt00449h.
107. Riedl, C.A.; Flocke, L.S.; Hejl, M.; Roller, A.; Klose, M.H.; Jakupec, M.A.; Kandioller, W.; Keppler, B.K., Introducing the 4-Phenyl-1,2,3-Triazole Moiety as a Versatile Scaffold for the Development of Cytotoxic Ruthenium(II) and Osmium(II) Arene Cyclometalates. *Inorg Chem*. **2017**, *56*(1), 528–541. DOI: 10.1021/acs.inorgchem.6b02430.
108. Kubanik, M.; Kandioller, W.; Kim, K.; Anderson, R.F.; Klapproth, E.; Jakupec, M.A.; Roller, A.; Sohnel, T.; Keppler, B.K.; Hartinger, C.G., Towards targeting anticancer drugs: ruthenium(ii)-arene complexes with biologically active naphthoquinone-derived ligand systems. *Dalton Trans*. **2016**, *45*(33), 13091–103. DOI: 10.1039/c6dt01110a.
109. Mészáros, J.P.; Geisler, H.; Poljarević, J.M.; Roller, A.; Legina, M.S.; Hejl, M.; Jakupec, M.A.; Keppler, B.K.; Kandioller, W.; Enyedy, É.A., Naphthoquinones of natural origin: Aqueous chemistry and coordination to half-sandwich organometallic cations. *Journal of Organometallic Chemistry*. **2020**, *907*. DOI: 10.1016/j.jorganchem.2019.121070.
110. Geisler, H.; Wernitznig, D.; Hejl, M.; Gajic, N.; Jakupec, M.A.; Kandioller, W.; Keppler, B.K., Novel phthiocol-based organometallics with tridentate coordination motif and their unexpected cytotoxic behaviour. *Dalton Trans*. **2020**, *49*(5), 1393–1397. DOI: 10.1039/c9dt04462k.
111. Geisler, H.; Westermayr, J.; Cseh, K.; Wenisch, D.; Fuchs, V.; Harringer, S.; Plutzar, S.; Gajic, N.; Hejl, M.; Jakupec, M.A.; Marquetand, P.; Kandioller, W., Tridentate 3-Substituted Naphthoquinone Ruthenium Arene Complexes: Synthesis, Characterization, Aqueous Behavior, and Theoretical and Biological Studies. *Inorg Chem*. **2021**, *60*(13), 9805–9819. DOI: 10.1021/acs.inorgchem.1c01083.
112. Pereyra, C.E.; Dantas, R.F.; Ferreira, S.B.; Gomes, L.P.; Silva-Jr, F.P., The diverse mechanisms and anticancer potential of naphthoquinones. *Cancer Cell Int*. **2019**, *19*, 207. DOI: 10.1186/s12935-019-0925-8.
113. Kandioller, W.; Reikersdorfer, M.; Theiner, S.; Roller, A.; Hejl, M.; Jakupec, M.A.; Malarek, M.S.; Keppler, B.K., The Impact of Leaving Group Variation on the Anticancer Activity of Molybdenocenes. *Organometallics*. **2018**, *37*(21), 3909–3916. DOI: 10.1021/acs.organomet.8b00582.
114. Domotor, O.; Aicher, S.; Schmidlehner, M.; Novak, M.S.; Roller, A.; Jakupec, M.A.; Kandioller, W.; Hartinger, C.G.; Keppler, B.K.; Enyedy, E.A., Antitumor pentamethylcyclopentadienyl

- rhodium complexes of maltol and allomaltol: synthesis, solution speciation and bioactivity. *J Inorg Biochem.* **2014**, *134*, 57–65. DOI: 10.1016/j.jinorgbio.2014.01.020.
115. Hackl, C.M.; Legina, M.S.; Pichler, V.; Schmidlehner, M.; Roller, A.; Domotor, O.; Enyedy, E.A.; Jakupec, M.A.; Kandioller, W.; Keppler, B.K., Thiomaltol-Based Organometallic Complexes with 1-Methylimidazole as Leaving Group: Synthesis, Stability, and Biological Behavior. *Chemistry.* **2016**, *22*(48), 17269–17281. DOI: 10.1002/chem.201603206.
 116. Legina, M.S.; Nogueira, J.J.; Kandioller, W.; Jakupec, M.A.; Gonzalez, L.; Keppler, B.K., Biological evaluation of novel thiomaltol-based organometallic complexes as topoisomerase IIalpha inhibitors. *J Biol Inorg Chem.* **2020**, *25*(3), 451–465. DOI: 10.1007/s00775-020-01775-2.
 117. Kandioller, W.; Kurzwernhart, A.; Hanif, M.; Meier, S.M.; Henke, H.; Keppler, B.K.; Hartinger, C.G., Pyrone derivatives and metals: From natural products to metal-based drugs. *J. Organomet. Chem.* **2011**, *696*(5), 999–1010. DOI: 10.1016/j.jorganchem.2010.11.010.
 118. Berasaluce, I.; Cseh, K.; Roller, A.; Hejl, M.; Heffeter, P.; Berger, W.; Jakupec, M.A.; Kandioller, W.; Malarek, M.S.; Keppler, B.K., The First Anticancer Tris(pyrazolyl)borate Molybdenum(IV) Complexes: Tested in Vitro and in Vivo-A Comparison of O,O-, S,O-, and N,N-Chelate Effects. *Chem. Eur. J.* **2020**, *26*(10), 2211–2221. DOI: 10.1002/chem.201903605.
 119. Fuchs, V.; Cseh, K.; Hejl, M.; Vician, P.; Neuditschko, B.; Meier-Menches, S.M.; Janker, L.; Bileck, A.; Gajic, N.; Kronberger, J.; Schaier, M.; Neumayer, S., et al., Highly Cytotoxic Molybdenocenes with Strong Metabolic Effects Inhibit Tumour Growth in Mice. *Chem. Eur. J.* **2022**, *29*(4), e202202648. DOI: 10.1002/chem.202202648.

7. List of Scientific Contributions

7.1. List of all peer-reviewed publications

1. Göschl, S.; Schreiber-Brynzak, E.; Pichler, V.; Cseh, K.; Heffeter, P.; Jungwirth, U.; Jakupec, M. A.; Berger, W.; Keppler, B. K. Comparative studies of oxaliplatin-based platinum(IV) complexes in different *in vitro* and *in vivo* tumor models. *Metallomics*, **2017**, 9(3), 309–322, DOI: 10.1039/c6mt00226a
2. Sommerfeld, N. S.; Gülzow, J.; Roller, A.; Cseh, K.; Jakupec, M. A.; Grohmann, A.; Galanski, M. S.; Keppler, B. K. Antiproliferative Copper(II) and Platinum(II) Complexes with Bidentate N,N-Donor Ligands. *European Journal of Inorganic Chemistry*, **2017**, 24, 3115–3124. DOI: 10.1002/ejic.201700416
3. Meier, S. M.; Kreutz, D.; Winter, L.; Klose, M. H. M.; Cseh, K.; Weiss, T.; Bileck, A.; Alte, B.; Mader, J. C.; Jana, S.; Chatterjee, A.; Bhattacharyya, A.; Hejl, M.; Jakupec, M. A.; Heffeter, P.; Berger, W.; Hartinger, C. G.; Keppler, B. K.; Wiche, G.; Gerner, C. An Organoruthenium Anticancer Agent Shows Unexpected Target Selectivity For Plectin. *Angewandte Chemie International Edition*, **2017**, 56, 8267, DOI: 10.1002/anie.201702242
4. Sommerfeld, N. S.; Strohhofer, D.; Cseh, K.; Theiner, S.; Jakupec, M. A.; Koellensperger, G.; Galanski, M. S.; Keppler, B. K. Platinum(IV) Complexes Featuring Axial Michael Acceptor Ligands – Synthesis, Characterization, and Cytotoxicity. *European Journal of Inorganic Chemistry*, **2017**, 34, 4049–4054. DOI: 10.1002/ejic.201700753
5. Höfer, D.; Cseh, K.; Hejl, M.; Roller, A.; Jakupec, M.A.; Galanski, M.; Keppler, B. K. Synthesis, characterization, cytotoxic activity, and ¹⁹F NMR spectroscopic investigations of (OC-6-33)-diacetato(ethane-1,2-diamine)bis(3,3,3-trifluoropropanoato)platinum(IV) and its platinum(II) counterpart. *Inorganica Chimica Acta*, **2019**, 490, 190–199, DOI: 10.1016/j.ica.2019.02.017

6. Berasaluce, I.; Cseh, K.; Roller, A.; Hejl, M.; Heffeter, P.; Berger, W.; Jakupec, M. A.; Kandioller, W.; Malarek, M. S.; Keppler, B. K. The First Anticancer Tris(pyrazolyl)borate Molybdenum(IV) Complexes: Tested in Vitro and in Vivo—A Comparison of O,O -, S,O -, and N ,N- Chelate Effects. *Chemistry A European Journal*, **2019**, 26(10), 2211–2221, DOI: 10.1002/chem.201903605

7. Ma, T.; Yang, P.; Dammann, I.; Lin, Z.; Mougharbel, A. S.; Li, M.-X.; Adăscăliței, F.; Mitea, R.; Silvestru, C.; Thorstenson, C.; Ullrich, M. S.; Cseh, K.; Jakupec, M. A.; Keppler, B. K.; Donalisio, M.; Cavalli, R.; Lembo, D.; Kortz. Tetra-(*p*-tolyl)antimony(III)-Containing Heteropolytungstates, $[(p\text{-tolyl})\text{Sb}^{\text{III}}]_4(\text{A-}\alpha\text{-XW}_9\text{O}_{34})_2]^{n-}$ (X = P, As, or Ge): Synthesis, Structure, and Study of Antibacterial and Antitumor Activity. *Inorganic Chemistry*, **2020**, 59(5), 2978–2987, DOI: 10.1021/acs.inorgchem.9b03322

8. Mokesch, S.; Cseh, K.; Geisler, H.; Hejl, M.; Klose, M. H. M.; Roller, A.; Meier-Menches, S. M.; Jakupec, M. A.; Kandioller, W.; Keppler, B. K. Investigations on the Anticancer Potential of Benzothiazole-Based Metallacycles. *Frontiers in Chemistry*, **2020**, 8, DOI:10.3389/fchem.2020.00209

9. Wernitznig, D.; Meier-Menches, S. M.; Cseh, K.; Theiner, S.; Wenisch, D.; Schweikert, A.; Jakupec, M. A.; Koellensperger, G.; Wernitznig, A.; Sommergruber, W.; Keppler, B. K. Plecstatin-1 induces an immunogenic cell death signature in colorectal tumour spheroids. *Metallomics*, **2020**, 12(12), 2121–2133, DOI: 10.1039/d0mt00227e

10. Geisler, H.; Westermayr, J.; Cseh, K.; Wenisch, D.; Fuchs, V.; Harringer, S.; Plutzar, S.; Gajic, N.; Hejl, M.; Jakupec, M. A.; Marquetand, P.; Kandioller, W. Tridentate 3-Substituted Naphthoquinone Ruthenium Arene Complexes: Synthesis, Characterization, Aqueous Behavior, and Theoretical and Biological Studies. *Inorganic Chemistry*, **2021**, 60(13) 9805–9819, DOI: 10.1021/acs.inorgchem.1c01083

11. Liu, X.; Wenisch, D.; Barth, M.-C.; Cseh, K.; Kowol, C. R.; Jakupec, M. A.; Gibson, D.; Keppler, B. K.; Weigand, W. Novel oxaliplatin (IV) complexes conjugated with ligands bearing pendant 1, 2-dithiolane/1, 2-diselenolane/cyclopentyl motifs. *Dalton Transactions*, **2022**, 51(44), 16824–16835, DOI: 10.1039/D2DT02217F

12. Liu, X.; Barth, M.-C.; Cseh, K.; Kowol, C. R.; Jakupec, M. A.; Keppler, B. K.; Gibson, D.; Weigand, W. Oxoplatin-Based Pt(IV) Lipoate Complexes and Their Biological Activity. *Chemistry & Biodiversity*, **2022**, 19(10), e202200695, DOI: 10.1002/cbdv.202200695

13. Cseh, K.; Geisler, H.; Stanojkovska, K.; Westermayr, J.; Brunmayr, P.; Wenisch, D.; Gajic, N.; Hejl, M.; Schaier, M.; Koellensperger, G.; Jakupec, M. A.; Marquetand, P.; Kandioller, W. Arene Variation of Highly Cytotoxic Tridentate Naphthoquinone-Based Ruthenium(II) Complexes and In-Depth In Vitro Studies *Pharmaceutics*, **2022**, 14(11), 2466, DOI: 10.3390/pharmaceutics14112466

14. Fuchs, V.; Cseh, K.; Hejl, M.; Vician, P.; Neuditschko, B.; Meier-Menches, S. M.; Janker, L.; Bileck, A.; Gajic, N.; Kronberger, J.; Schaier, M.; Neumayer, S.; Köllensperger, G.; Gerner, C.; Berger, W.; Jakupec, M. A.; Malarek, M. S.; Keppler, B. K. Highly Cytotoxic Molybdenocenes with Strong Metabolic Effects Inhibit Tumour Growth in Mice. *Chemistry–A European Journal*, **2023**, 29(4), e202202648, DOI: 10.1002/chem.202202648

15. Lerchbammer-Kreith, Y.; Sommerfeld, N. S.; Cseh, K.; Jiang, X. W.; Odunze, U.; Schätzlein, A. G.; Uchegbu, I. F.; Galanski, M. S.; Jakupec, M. A.; Keppler, B. K. Platinum(IV) loaded degraded glycol chitosan as efficient platinum(IV) drug delivery platform. *Pharmaceutics*, **2023**, 15(4), 1050, DOI: 10.3390/pharmaceutics15041050

16. Cseh, K.; Berasaluce, I.; Fuchs, V.; Banc, A.; Schweikert, A.; Prado-Roller, A.; Hejl, M.; Wernitznig, D.; Koellensperger, G.; Jakupec, M. A.; Kandioller, W.; Malarek, M. S.; Keppler, B. K. Anticancer tungstenocenes with a diverse set of (O,O–) (O,S–) and (O,N–) chelates – a detailed biological study using an improved evaluation *via* 3D spheroid models. *Pharmaceutics*, **2023**, 15(7), 1875, DOI:10.3390/pharmaceutics15071875

17. Maier, T.; Wutschitz, J.; Gajic, N.; Prado-Roller, A.; Hejl, M.; Cseh, K.; Jakupec, M. A.; Galanski, M. S.; Keppler, B. K. Not the usual suspect – an unexpected organometallic product during the synthesis of a cytotoxic platinum(II) complex. Revised manuscript is submitted to Dalton Transactions on 15. 07. 2023.

18. Pósa, V.; Federa, A.; Cseh, K.; Wenisch, D.; Spengler, G.; May, N. V.; Prado-Roller, A.; Jakupec, M. A.; Keppler, B. K.; Kowol, C. R.; Enyedy, É. A. A comparative study on complexation of the anticancer iron chelator VLX600 with essential metal ions. Manuscript in preparation.

19. Brynzak-Schreiber, E.; Schögl, E.; Bapp, C.; Cseh, K.; Kopatz, V.; Jakupec, M. A.; Weber, A.; Lange, T.; Toca-Herrera, J. L.; del Favero, G.; Wadsak, W.; Kenner, L.; Pichler, V. Microplastics in Motion: Their Role in Cell Migration and Distribution During Cancer Cell Division, Manuscript in preparation.

7.2. List of poster contributions

1. Wernitznig, D.; Cseh, K.; Kiakos, K.; Harrer, N.; Machat, H.; Jakupec, M. A.; Sommergruber, B. K. KP1339 induces apoptosis and autophagy in colon cancer cells in vitro: A 3-Dimensional perspective. *Frontiers in Medicinal Chemistry*, Jena, Germany, **2018**
2. Brynzak-Schreiber, E.; Schögl, E.; Cseh, K.; Kopatz, V.; Weber, A.; Toca-Herrera, J. H.; Jakupec, M. A.; Kenner, L.; Pichler, V. Accumulation and effects of micro- and nanoplastics in colon carcinoma cells. *First Vienna Summit on Microplastics and Health 2022*, Vienna, Austria, **2022**
3. Brynzak-Schreiber, E.; Schögl, E.; Cseh, K.; Kopatz, V.; Legin, A.; Jakupec, M. A.; Weber, A.; Toca-Herrera, J. H.; del Favero, G.; Kiss, E.; Wadsak, W.; Kenner, L.; Pichler, V. Optical imaging of intra- and extracellular micro- and nanoplastic during cell division. *18th European Molecular Imaging Meeting | EMIM 2023*, Salzburg, Austria, **2023**