



universität
wien

DISSERTATION

Tumor-inhibierende Ru^{II} (aren) Komplexe mit
Flavonoid-Liganden – Auf dem Weg zu "multi-target"
Chemotherapeutika

Mag. Andrea Kurzwernhart

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer. nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt:

A 791 419

Dissertationsgebiet lt. Studienblatt:

Dr.-Studium der Naturwissenschaften (Dissertationsgebiet: Chemie)

Betreuerin / Betreuer:

O. Univ.-Prof. Dr. Dr. Bernhard Keppler



universität
wien

Ph.D. Thesis

Tumor-inhibiting Ru^{II}(arene) Complexes with Flavonoid
Ligands – On the Way to Multi-targeted Anticancer Agents

Mag. Andrea Kurzwernhart

Submitted in part fulfillment of the requirements for the degree
Doctor of Sciences (Dr. rer. nat.)

Supervisor

O. Univ.-Prof. Dr. Dr. Bernhard K. Keppler

Vienna, February 2012

Für meine Familie

„Was immer Du tun kannst oder träumst es zu können, fang damit an.“

(Johann Wolfgang von Goethe)

Acknowledgements

I would like to thank

O. Prof. Dr. Dr. Keppler for the possibility to work in his group on this very interesting and challenging topic.

My supervisors A/Prof. Christian G. Hartinger and Dr. Wolfgang Kandioller for their help, support and creative discussions (especially about T-shirt design) during my whole thesis.

Our NMR team for measuring countless NMR spectra; Alexander Roller and Prof. Arion for X-ray data collection and structure refinement; Dr. Michael Jakupec, Dr. Caroline Bartel, Dr. Gerhard Mühlgassner and MSc. Maria Novak for performing the MTT assays; and the team of Prof. Doris Marko, especially Dipl.-LMChem. Simone Bächler for the decatenation assays.

Elfriede Limberger for patiently managing all kinds of administrative issues and problems.

All colleagues, especially Claudia, Jelena, Masha, Michi, Leo, Luki, Paul, Sam and Wolfgang for the nice conversations and fruitful discussions, and of course all current and former members of Lab6@AT for the great time and pleasant working atmosphere.

My friends Eva, Raji, Helena, Martina & Martin, René and Sabine for very nice times and non-chemical distraction whenever it was needed.

My cute little rabbits Floh, Franzl, Gretchen and Sini for simply being there and looking sweet when I'm coming home.

Last, but not least I deeply thank my whole family for their support and care throughout my whole life and my partner Christoph for his love and patience.

This Ph.D. Thesis is based on the following publications, which are presented in the original format:

“Pyrone Derivatives and Metals: From Natural Products to Metal-based Drugs”

Wolfgang Kandioller, Andrea Kurzwernhart, Muhammad Hanif, Samuel M. Meier, Helena Henke, Bernhard K. Keppler, Christian G. Hartinger, *Journal of Organometallic Chemistry* **2011**, 696, 999-1010.

“Targeting the DNA-topoisomerase Complex in a Double-strike Approach with a Topoisomerase Inhibiting Moiety and Covalent DNA Binder”

Andrea Kurzwernhart, Wolfgang Kandioller, Caroline Bartel, Simone Bächler, Robert Trondl, Gerhard Mühlgassner, Michael A. Jakupiec, Vladimir B. Arion, Doris Marko, Bernhard K. Keppler, Christian G. Hartinger, *Chemical Communications* **2012**, 48, 4839–4841.

“Structure–Activity Relationships of Targeted Ru^{II}(η^6 -*p*-Cymene) Anticancer Complexes with Flavonol-Derived Ligands”

Andrea Kurzwernhart, Wolfgang Kandioller, Simone Bächler, Caroline Bartel, Sanela Martić, Magdalena Buczkowska, Gerhard Mühlgassner, Michael A. Jakupiec, Heinz-Bernhard Kraatz, Patrick J. Bednarski, Vladimir B. Arion, Doris Marko, Bernhard K. Keppler, and Christian G. Hartinger, *Journal of Medicinal Chemistry* **2012**, 55, 10512–10522.

“3-Hydroxyflavones vs. 3-Hydroxyquinolinones: Structure–Activity Relationships and Stability Studies on Ru^{II}(arene) Anticancer Complexes with Biologically Active Ligands”

Andrea Kurzwernhart, Wolfgang Kandioller, Éva A. Enyedy, Maria Novak, Michael A. Jakupiec, Bernhard K. Keppler and Christian G. Hartinger, *Dalton Transactions* **2013**, DOI: 10.1039/C2DT32206D.

Abstract

Cancer is worldwide a major cause of death and many patients are still not treatable or do not respond to standard chemotherapeutics such as cisplatin, often because of intrinsic or acquired resistance. Furthermore severe side effects limit the applicability of many anti-cancer drugs in clinical use. To overcome these drawbacks, there are intensive research efforts focused on the development of novel chemotherapeutics and new approaches, such as multi-targeted therapy. In the class of metal-based drugs, Ruthenium compounds are considered promising drug candidates and two Ru^{III} complexes, NAMI-A and KP1019, are already in clinical trials. Within the last 20 years, the class of Ru^{II}(arene) complexes gained more and more interest as kinetics, reactivity and pharmacological properties can be fine-tuned by variation of the ligand system. Two important examples for this substance class are ethylenediamine-derived Ru^{II}(arene) complexes and the so-called RAPTA-type compounds which are both at an advanced preclinical development stage.

One approach to design multi-targeted anticancer drugs is to link bioactive ligands to metal moieties. Flavonoids are natural components of plants and exhibit a wide range of biological properties such as antioxidant, anti-inflammatory, and also anticancer activity.

Within this Ph.D. thesis, a series of novel Ru^{II}(arene) complexes, bearing biologically active flavonoid ligands, were synthesized and characterized by standard analytical methods, including X-ray diffraction analysis. The stability and behavior in aqueous solution was studied and *pK_a* values for both complexes and free ligands were determined. The *in vitro* anti-cancer activity of the compounds was examined in the human cancer cell lines CH1 (ovarian carcinoma), SW480 (colon carcinoma) and A549 (non-small cell lung carcinoma) by means of the colorimetric MTT assay and in human urinary bladder (5637), human large cell lung (LCLC-103H) and human pancreatic carcinoma (DAN-G) with the crystal violet assay; and structure–activity relationships were derived. In order to find possible biological targets, the interactions with small biomolecules (5'-GMP and various amino acids) and the inhibitory activity on CDK2 and topoisomerase II α were investigated, and flow cytometry analyses of the cell cycle were conducted.

Zusammenfassung

Krebs ist eine der häufigsten Todesursachen weltweit und ist im Falle vieler Patienten nicht heilbar, zumeist aufgrund von erworbenen oder intrinsischen Resistenzen gegen die verwendeten Standardchemotherapeutika wie zum Beispiel Cisplatin. Außerdem limitieren oft starke Nebenwirkungen die Anwendbarkeit dieser Medikamente. Deshalb wird intensiv an der Entwicklung neuartiger Chemotherapeutika und an neuen Ansätzen wie der zielgerichteten („targeted“) Therapie geforscht. Im Feld der metall-basierten Chemotherapeutika gelten Rutheniumverbindungen als vielversprechende Kandidaten und zwei Ru^{III} Komplexe, NAMI-A und KP1019, befinden sich bereits in klinischen Studien. In den letzten 20 Jahren, gewann die Klasse der Ru^{II}(Aren) Komplexe mehr und mehr an Interesse, da deren Kinetik, Reaktivität und pharmakologische Eigenschaften leicht durch Variation des Ligandensystems beeinflusst werden können. Ein Beispiel sind Ethylendiamin- und die sogenannten RAPTA-Komplexe, welche sich derzeit in frühen präklinischen Studien befinden.

„Multi-targeted“ Verbindungen können unter anderem durch Verknüpfung von Metallzentren mit biologisch aktiven Liganden hergestellt werden. Flavonoide sind natürliche bioaktive Pflanzeninhaltsstoffe, die antioxidative, entzündungshemmende, antimikrobielle, und auch krebshemmende Wirkung aufweisen.

Im Rahmen dieser Doktorarbeit wurde eine Reihe neuer Ru^{II}(Aren) Komplexe mit Flavonoidliganden hergestellt und mittels analytischer Standardmethoden, sowie Kristallstrukturanalyse charakterisiert. Die Stabilität und das Verhalten in wässriger Lösung wurde untersucht und pK_s Werte für sowohl die Komplexe als auch die freien Liganden bestimmt. Die *in vitro* Aktivität wurde in den humanen Krebszelllinien CH1 (Eierstockkarzinom), SW480 (Dickdarmkarzinom) und A549 (nicht-kleinzelliges Bronchialkarzinom) mittels MTT Assay und im humanen Blasenkarzinom (5637), großzelligen Bronchialkarzinom (LCLC-103H) and Pankreaskarzinom (DAN-G) mittels Crystal violet Assay bestimmt und daraus Struktur/Aktivitäts-Beziehungen abgeleitet. Zur Bestimmung möglicher biologischer Ziele wurde die Wechselwirkung mit kleinen Biomolekülen (5'-GMP und einigen Aminosäuren) und die Hemmung von CDK2 und der Topoisomerase II α untersucht, sowie eine Durchflusszytometrie durchgeführt.

Table of Contents

Abbreviations	15
1. Introduction.....	17
1.1. Cancer	17
1.1.1. Some Facts	17
1.1.2. The Causes of Cancer	18
1.1.3. Pathophysiology of Cancer.....	19
1.2. Approaches for Cancer Therapy	22
1.2.1. Chemotherapy	23
1.3. Metal-based Drugs	24
1.3.1. Platinum(II) Anticancer Drugs.....	24
1.4. (Multi-)Targeted Chemotherapeutics	27
1.5. Ruthenium-based Chemotherapeutics.....	31
1.5.1. Ruthenium(III) Compounds in Clinical Trials.....	31
1.5.2. Ruthenium(II)–Arene Complexes.....	33
1.6. Flavonoids.....	38
1.6.1. Medicinal Properties of Flavonoids	38
1.6.2. Metal-Flavonoid Complexes	40
2. Research justification.....	45
References.....	46
3. Published Results.....	53
3.1. Pyrone Derivatives and Metals: From Natural Products to Metal-based Drugs	53
3.2. Targeting the DNA-topoisomerase Complex in a Double-strike Approach with a Topoisomerase Inhibiting Moiety and Covalent DNA Binder	67
3.3. Structure–Activity Relationships of Targeted Ru ^{II} (η^6 - <i>p</i> -Cymene) Anticancer Complexes with Flavonol-Derived Ligands	81
3.4. 3-Hydroxyflavones vs. 3-Hydroxyquinolinones: Structure–Activity Relationships and Stability Studies on Ru ^{II} (arene) Anticancer Complexes with Biologically Active Ligands	103
4. Conclusions and Outlook.....	125
Curriculum vitae	129

Abbreviations

1D/2D NMR	one- or two-dimensional NMR spectroscopy
acac	acetylacetonate
5'-AMP	adenosine 5'-monophosphate
bcl-2	B-cell lymphoma 2
°C	degree Celsius
CDK	cyclin-dependent kinase
d	doublet (NMR)
D ₂ O	deuterated water
CDCl ₃	deuterated chloroform
δ	chemical shift (NMR)
(d ₆ -) dmsO	(deuterated) dimethyl sulfoxide
DNA	2'-deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
<i>e.g.</i>	<i>exempli gratia</i> (for example)
EGF(R)	epidermal growth factor (receptor)
en	ethylenediamine
eq	equivalent
<i>et al.</i>	<i>et alii</i> (and others)
<i>etc.</i>	<i>et cetera</i> (and other things)
FDA	US Food and Drug Administration
Gly	glycine
5'-GMP	guanosine 5'-monophosphate
h	hours
HIV	human immunodeficiency virus
Hz	Hertz
IC ₅₀	drug concentration that causes 50% inhibition (of cell growth)
J	coupling constant (NMR)
Cys	L-cysteine
His	L-histidine
Met	L-methionine

M	molar
m	multiplet (NMR)
mAb	monoclonal antibody
μ M	micromolar
nM	nanomolar
NMR	nuclear magnetic resonance
pH	pondus Hydrogenii (power of hydrogen)
pK _a	log K _a (acid dissociation constant)
ppm	parts per million
PTA	1,3,5-triaza-7-phosphoadamantane
RNA	ribonucleic acid
ROS	reactive oxygen species
s	singlet (NMR)
TKI	tyrosine kinase inhibitor
UV	ultraviolet
VIS	visible
VEGF(R)	vascular endothelial growth factor (receptor)
WHO	World Health Organization

1. Introduction

1.1. Cancer

1.1.1. Some Facts

Cancer is one of the major causes of death worldwide, besides infectious diseases, malnutrition, cardiac diseases and war. In 2008, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred worldwide, thereof 3.4 million incidences and 1.8 million deaths in Europe.^[1] Taken Austria as an example, cardiovascular diseases led to 42% of all deaths, followed by cancer as the second most prevalent cause of death (26%), similar to Europe (Figure 1).^[2]

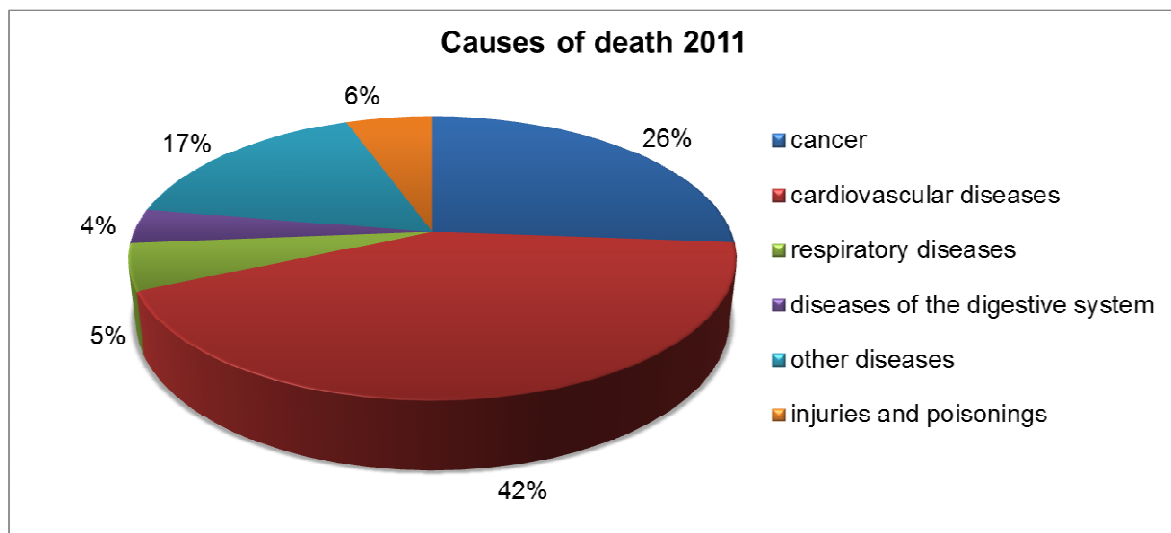


Figure 1. Causes of death in Austria in 2011.^[2]

Cancer is not a modern disease and the global cancer burden is continuously rising, mainly due to the permanent growth of the world's population and the proceeding ageing, especially in more developed countries.^[1] Another reason is the increasing adoption of cancer-associated behaviors particularly smoking, physical inactivity, and unhealthy diets.^[3] Thus it is estimated that the global cancer incidences and related deaths will at least double by 2030.^[1]

In Europe, the most common cancer diagnosed in men is lung cancer followed by prostate cancer and colorectal cancer, which lead also to the most frequent cancer-related deaths in men. In women the most common incident form of cancer is breast cancer which is also the most frequent cancer cause of death in women, followed by colorectal and lung cancer (Figure 2). Especially in the case of breast cancer in women but also of prostate cancer in men, it is obvious that the number of incidences differs significantly from the number of deaths, which can be attributed to early diagnosis, successful therapy or improved prevention.^[1]

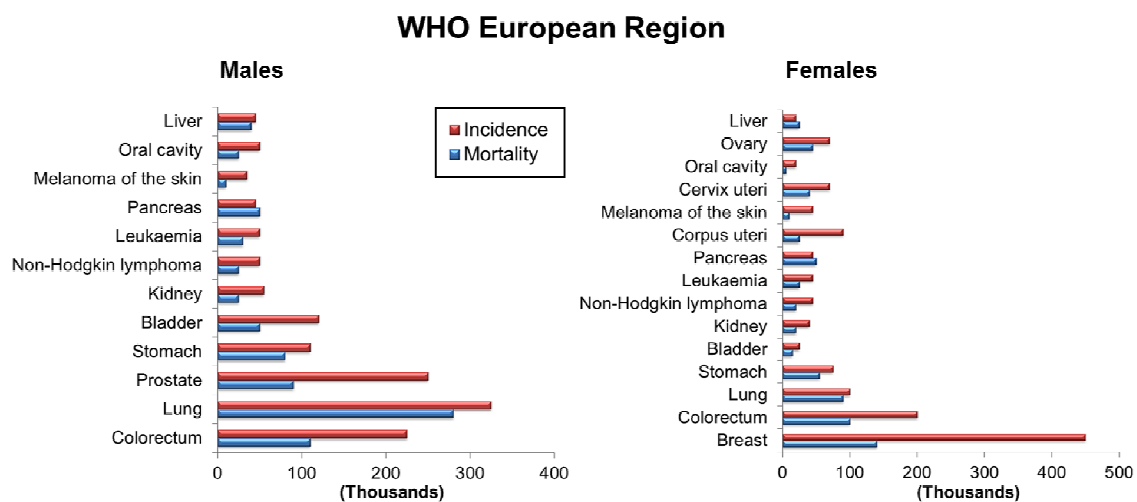


Figure 2. Cancer Incidence and Mortality recorded by the World Health Organization in the European Region.^[1]

1.1.2. The Causes of Cancer

For the development of cancer, multiple genetic changes are necessary (Figure 3), which lead to the loss of control over a number of processes. Thus cancer increases with age because more time for the accumulation of initiating mutations has elapsed and the effect of molecular repair mechanism and the immune system is reduced.^[4]

Beside point-mutations which may be produced in any cell division event due to faulty DNA replication or repair, changes in the genetic material of an organism can be caused by several external factors, so-called mutagens.

Mutagens are for example:

- Carcinogenic chemicals: react with and modify the DNA,^[5] *e.g.* arsenic, chromates and asbestos (used in industry), aflatoxins (produced from mold fungus) and polycyclic aromatic hydrocarbons (PAHs, are found in high levels in grilled meat and tobacco smoke).^[4]
- Physical carcinogens: ionizing radiation (causes chromosome breakage and rearrangements), and UV light (main reason for the occurrence of melanomas).
- Viruses: insert their RNA or DNA into the host genome and thereby disrupt the genetic function, *e.g.* papilloma virus or HIV.^[5]

Furthermore, naturally occurring tumor promoters (co-carcinogens) are involved, which do not cause cancer on their own, but stimulate further cell division at a later state of carcinogenesis (Figure 3), *e.g.* phorbol esters (from plant oil), several components in tobacco smoke and hormones.^[4]

1.1.3. Pathophysiology of Cancer

Cancer cells are characterized by two inheritable properties, which convert them into a malignant, often mortal disease:

- 1) Uncontrolled multiplication of cells: normal cells can only divide a limited number of times, afterwards they stop dividing except for replacement of dead cells. Tumor cells in turn have no limitation on the number of cell divisions and can replicate without mitotic signals from other cells or fail to self-destruct after DNA damage. This aberrant behavior leads to a, possibly excessive, growth of cells and the formation of a solid (benign) tumor (Figure 3), which can mostly be removed surgically.
- 2) Invasion of other tissues and metastasis: a tumor becomes cancerous (malignant), if the cells begin to invade adjacent tissues, break off and migrate in the blood or lymph systems and form secondary tumors (metastasis, Figure 3).^[6]

Cancer types can be divided into

- carcinomas: arise from epithelial cells
- sarcomas: transformed cells of mesenchymal origin (bone, cartilage, fat, muscle, vascular, or hematopoietic tissues)
- leukemia: cancer of the blood or bone marrow.^[5,6]

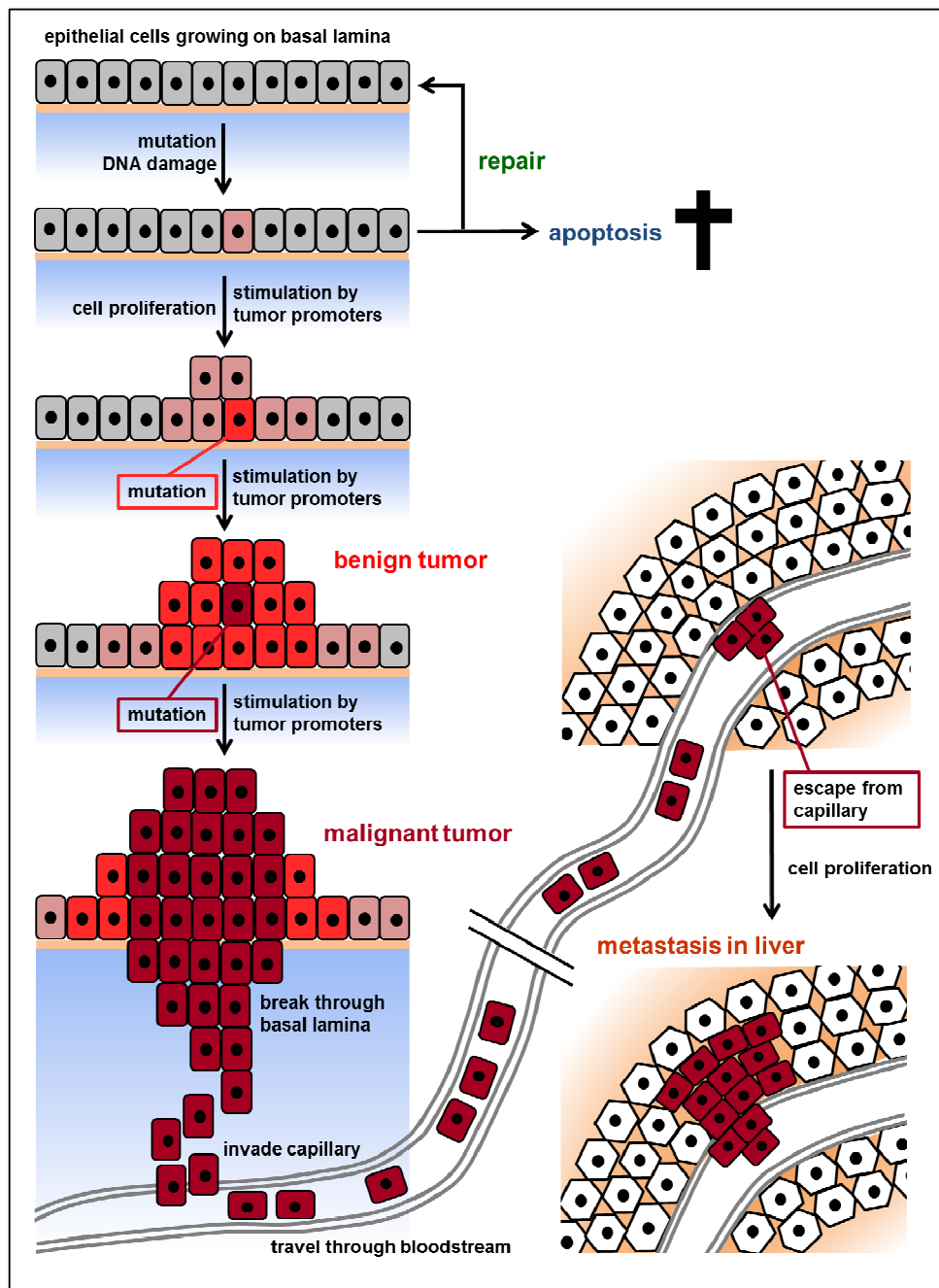


Figure 3. Multi-step process of cancer development (adapted from ref. [6]).

Cancer cells acquire a number of features, which provide them with a competitive advantage over normal cells:

- 1) Cell growth, viability and division is independent from the signals of other cells
- 2) Evade apoptosis and immune destruction
- 3) Unlimited proliferation
- 4) Invasive behavior due to the lack of cell adhesion molecules (cadherins)
- 5) Ability to survive, proliferate in extrinsic tissues and form metastases
- 6) Sustained angiogenesis for oxygen and nutrition supply
- 7) Limit energy metabolism largely to glycolysis [6,7,8]

Underlying the acquisition of these properties are genetic instabilities that lead to an increased mutation rate in certain genes and inflammation, which additionally promotes these features.[8]

Critical mutations usually affect proto-oncogenes which become overactive and as a result overexpress certain proteins or tumor suppressor genes whose functions are destroyed:

- A mutation in a proto-oncogene leads to a so-called oncogene, which consequently gives abnormal control signals and thus initiates uncontrolled cell division or causes inappropriate cell survival. An example is the ras protein, which is involved into the activation of growth factors. A mutation in the ras protein gene leads to a hyperactive protein production and thus to an abnormal cell cycle control.
- Tumor-suppressor genes normally protect cells against uncontrolled cell division. Mutations in these genes cause the removal of their protective effect and the cells are more likely to progress to the cancerous state. A large percentage of all cancers are attributed to a defective p53 gene. In normal cells p53 gets activated as a result of DNA damage and starts to produce the p53 protein which causes interruption of the cell cycle until the damage is repaired or, when DNA is irreparable, initialize the apoptotic pathway leading to controlled cell death. In the absence of a functional p53 gene, there is no cell cycle arrest and no apoptosis signal, so that cells with abnormal DNA can further replicate.[5,6]

1.2. Approaches for Cancer Therapy

Due to the multitude of different cancer types, there are a number of treatment options, also depending upon the location and stage of the cancer and also the patient's general health state. Among the most common methods currently in use are:

1. Surgery: is the primary method for cancer treatment. Tumor size can be reduced prior to other treatments or even completely removed.
2. Radiation therapy: application of ionizing radiation either from an external source or internal sources (brachytherapy) in order to destroy or shrink the tumor. The radiation causes irreversible DNA damage which leads to apoptosis of the affected cells. This method has severe side effects as there is no discrimination between malignant and normal cells leading to damage of epithelial surfaces, swelling of soft tissues and infertility.
3. Chemotherapy: treatment with cytotoxic antineoplastic agents which basically interfere with cell replication of malignant cells and force them into apoptosis. Due to lack of specificity for malignant cells, also healthy cells are affected leading to severe side effects such as nephro- and hepatotoxicity (damage of kidneys and liver, respectively), immune- and myelosuppression or hair loss.
4. Immunotherapy: relatively new field of cancer treatment in which the patient's immune system is stimulated by the injection of killed cancer cells, tumor antigens or interferons and cytokines.
5. Hormonal therapy: exploits the fact that some tumor types are sensitive to certain hormones (*e.g.* estrogen-dependent breast cancer) which can be blocked by the application of drugs that inhibit the production or activity of these hormones.

The ultimate goal of cancer therapy is the complete removal of the tumor and potential existing metastasis. To maximize the success of tumor therapy, it is common to combine two

or more treatment methods or to use a combination of certain chemotherapeutic agents that complement each other (combination therapy).^[9]

1.2.1. Chemotherapy

There are several classes of chemotherapeutics, which can be distinguished by their structures and mechanisms of action:

- Alkylating agents: impair cell function by alkylation of DNA, RNA and proteins and therefore inhibit replication. Examples are triazines, nitrogen mustards, alkyl sulfonates and nitrosoureas.
- Metal-based drugs: Depending on their structure there are numerous different mechanisms of action. An important example are the platinum(II) agents cisplatin, carboplatin and oxaliplatin which induce structural changes of DNA by covalent binding of the metal center to nucleic acids leading to cell death.
- Antimetabolites: compete with or substitute biomolecules involved in DNA and RNA synthesis and thus alter the critical pathways of nucleotide synthesis. Examples are folic acid-, purine- and pyrimidine analogues (*e.g.* 5-fluorouracil).
- Topoisomerase inhibitors: Topoisomerases are essential enzymes that maintain the topology and integrity of DNA. Inhibition of topoisomerase interferes with all central DNA processing steps such as replication, transcription, translation and recombination (see Chapter 1.6.1.), *e.g.* etoposide, topotecan.

Other approaches are antitumor antibiotics, hormone inhibitors, mitosis inhibitors, monoclonal antibodies, signal transduction and enzyme inhibitors. Among the latest methods are nanoparticles,^[10] special targeted therapies (see Chapter 1.4.) and gene therapy (therapy to block the expression of oncogenes or even replace missing or defective tumor-suppressor genes).^[11]

1.3. Metal-based Drugs

Metals play important roles in living systems as they perform a variety of tasks, *e.g.* iron in the transport of oxygen in hemoglobin, zinc as a structural component in zinc finger proteins or as natural component of insulin, and iron, nickel, manganese, copper, magnesium *etc.* as catalytic active sites in metalloenzymes. Although the use of metals for medical purposes has been reported already 5000 years ago,^[12] the pharmaceutical market has traditionally been dominated by organic substances. But within the last 40-50 years, bioinorganic chemistry is becoming a more and more important new area of medicinal chemistry. It offers access to novel drugs with completely new mechanisms of action and the possible treatment of diseases which have been incurable.^[13] In the first decade of the 20th century, Paul Ehrlich, who is now seen as the founder of chemotherapy, and Sahachiro Hata developed the arsenic-containing compound Salvarsan for the successful treatment of syphilis.^[12] However, the interest in the discipline medicinal inorganic chemistry was finally stimulated by the success of cisplatin, which is now one of the most frequently used anti-cancer drugs.^[13]

1.3.1. Platinum(II) Anticancer Drugs

The antitumor activity of *cis*-diamminedichloridoplatinum(II) (cisplatin, Figure 4) was accidentally discovered by Barnett Rosenberg in the late 1960s and finally approved by the FDA in 1978.^[14] It is remarkably effective in a broad range of tumor types including testicular, ovarian, lung, cervical, endometrial, bladder and esophageal cancer. Cisplatin is administered intravenously in a sodium chloride containing solution and activated intracellularly by aquation of the chloride leaving groups. It is suggested that the compound enters the cell through passive diffusion^[15] and possibly *via* active uptake by a copper transport protein,^[16] where it causes DNA damage by coordination of the metal to the *N7* atoms of purine nucleobases, finally leading to apoptosis.^[15]

Driven by the impact of cisplatin on cancer therapy, great efforts have been made on the development of numerous new derivatives with improved pharmacological properties, but only carboplatin has received worldwide approval so far.

Cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II) (carboplatin, Figure 4) shows much less side effects than cisplatin, but a comparable activity profile and is used as standard therapy in ovarian cancer and is also often applied in non-small and small-cell lung cancer.

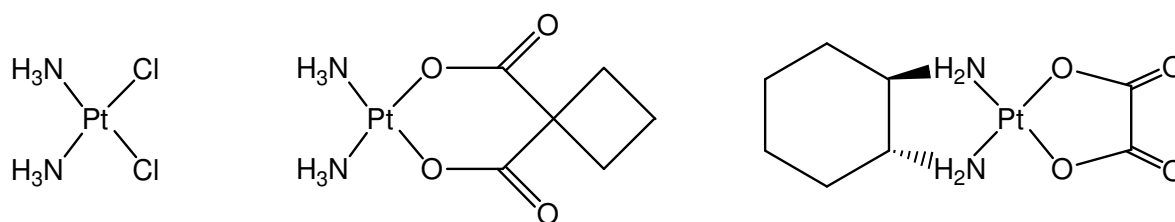


Figure 4. The platinum(II) compounds cisplatin, carboplatin and oxaliplatin (from left to right).

The third Pt^{II} drug, which is in worldwide clinical use,^[17] is [(1*R*,2*R*)-diaminocyclohexane]]oxalatoplatinum(II) (oxaliplatin, Figure 4). Oxaliplatin is active in advanced colorectal cancer, in which cis- and carboplatin are inactive.^[15]

Despite the high antitumor activity of platinum(II) compounds, the number of severe side effects, such as nephro- and neurotoxicity, ototoxicity, nausea and vomiting,^[15] has to be kept in mind, as well as intrinsic and acquired resistances which also hamper the applicability of this substance class.^[18]

To overcome these drawbacks the development of novel chemotherapeutics with a varying activity profile and different modes of action, higher selectivity towards tumor cells and therefore lower toxicity is essential for the improvement of cancer chemotherapy. Consequently, more and more attention is focused on the investigation of non-platinum complexes and organometallics.

The inorganic compounds Trisenox® (As₂O₃)^[19] and Gallium(III) nitrate^[20,21] are already approved in cancer therapy for the treatment of acute promyelocytic leukemia and cancer-associated hypercalcemia, respectively.^[22]

Butotitane (cis-diethoxybis(1-phenylbutane-1,3-dionato-κ²O¹,O²)titanium(IV), Figure 5) was among the first non-platinum metal-based anticancer agents studied in clinical trials, but was abandoned after phase I due to formulation issues, whereas titanocene dichloride,

which was the first organometallic transition metal compound in clinical investigations, was abandoned in phase II trials as no advantages over other treatment regimens were observed.^[22,23]

Two germanium compounds, namely germanium-132 (carboxyethylgermanium sesquioxide) and spirogermanium (*N*-(3-dimethylaminopropyl)-2-aza-8,8-diethyl-8-germaspiro-4,5-decane dihydrochloride, Figure 5) reached phase II clinical trials but were abandoned. The reasons for their failure were not clearly stated.^[22]

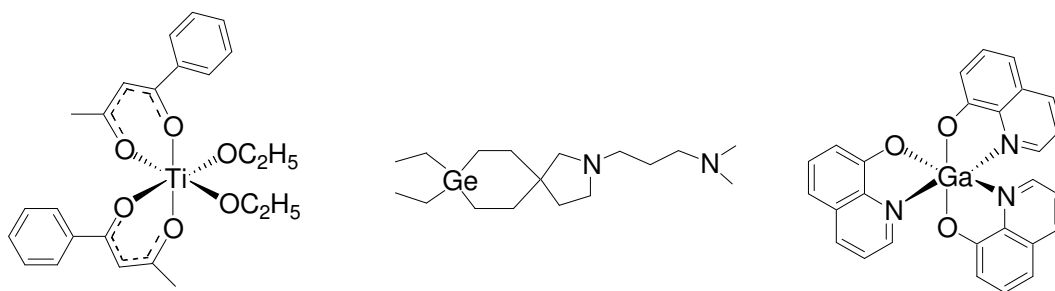


Figure 5. Structures of budotitan (left), spirogermanium (center) and KP46 (right).

Intensive research is currently ongoing in the field of ruthenium and gallium compounds as the Ru^{III} complexes KP1019 and NAMI-A (see Chapter 1.5.1.) and the Ga^{III} compounds tris(8-quinolinolato)gallium(III) (KP46, Figure 5) and Ga maltolate ^[24] are investigated in clinical trials.^[21] Other non-platinum agents currently in clinical investigations are 4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsonous acid (GSAO), motexafin gadolinium (MGd) and tetrathiomolybdate (TM).^[25]

1.4. (Multi-)Targeted Chemotherapeutics

The development of metal-based anticancer drugs has been mainly governed by the aim to prepare compounds similar to cisplatin and its next-generation analogues. Thus, the synthesis of novel metal-based drugs was focused on the improvement of DNA binding properties in order to interfere with the cancer cells' replication and mitotic processes. Nevertheless, many cancer types are still not treatable due to intrinsic or acquired resistances and the huge variability that exists among different tumor types, often because of additional mutations during tumor development. Furthermore the efficacy of established anticancer drugs is mostly affected by dose-dependent toxicity and side effects. This is why there is a need for new individualized drugs, for which the antitumor effect is achieved by selective target interaction of a special determinant of a tumor cell rather than with unselective targets, such as DNA, which is shared by all kinds of cells.

Today's knowledge on cancer genomics and tumor cell biology suggests the triggering of targets that are selectively expressed or overexpressed by cancer cells and do not occur in healthy tissues as promising strategies in drug development, termed targeted therapy. These targets could be proteins, special membrane receptors or components of crucial biochemical signaling pathways that are controlling *e.g.* cell proliferation, growth, angiogenesis or apoptosis. The aim of this approach is the development of highly effective treatments with far higher specificity and potentially reduced toxicity and side effects.^[11,26,27]

There are already a series of organic targeted therapeutics used in routine cancer treatment. The first clinically available targeted agent was imatinib mesylate, which inhibits BCR-ABL (a specific chromosomal abnormality associated with chronic myelogenous leukemia), the platelet-derived growth factor receptor (PDGFR) and c-KIT (a known proto-oncogene) tyrosine kinases. Nowadays most of the clinically available targeted organic drugs target one of two key growth factors crucial for tumor growth and development, namely the epidermal growth factor (EGF) or the vascular endothelial growth factor (VEGF). Cell growth, proliferation, and differentiation or vasculogenesis and angiogenesis, respectively, are stimulated by binding of the respective growth factor to its receptor, namely the EGF receptor (EGFR) or the VEGF receptor (VEGFR). Gefitinib and erlotinib (Figure 6) are both tyrosine kinase inhibitors (TKIs) which both inhibit the epidermal

growth factor receptor (EGFR) and are approved for the treatment of advanced or metastatic non-small cell lung cancer after failure of the prior chemotherapy regimen.

Another approach in targeted therapy is the application of monoclonal antibodies (mAb) that attach to tumor cells and mark them for attack by the immune system or bind directly to a specific growth factor (*e.g.* VEGF) and thereby inhibit receptor binding and activation, *e.g.* bevacizumab.^[11,28] Presently there are 33 FDA-approved mAbs and TKIs for metastatic cancer and one for the adjuvant treatment of solid tumors.^[29] Notably, many of the newer organic targeted agents inhibit more than one receptor tyrosine kinase, such as vandetanib which inhibits EGFR and VEGFR tyrosine kinase activity and therefore has the ability to block both tumor proliferation and angiogenesis.^[28]

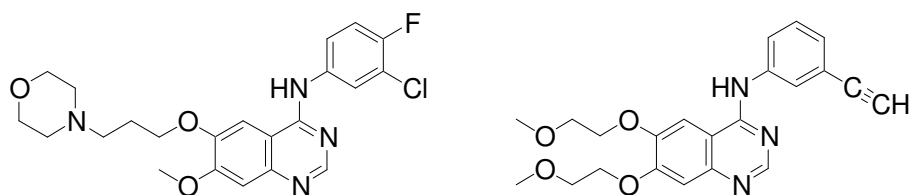


Figure 6. Structures of gefitinib (left) and erlotinib (right).

However, many agents that are directed against individual molecular targets were found to be less active often due to acquired resistances developed by the biological system during treatment or because some of the targets such as signal transduction pathways exist in normal tissue as well. Consequently most monotherapies cannot fully cure such a complex disease as cancer or are only effective in a few types of cancer. This limitation can be overcome by attacking the disease on multiple targets.^[26,30] Therefore, drug combinations are used as standard therapy schemes for the treatment of cancer (*e.g.* FOLFOX = combination of folinic acid, fluorouracil and oxaliplatin for the treatment of colorectal cancer), but which often do not overcome the side effects of the individual drugs.^[15]

A few years ago, the generation of multi-target drugs was emerging in which multiple pharmacophores combined in single molecules impact simultaneously multiple molecular targets. This can be accomplished by the usage of a mixture of separate active compounds or of a single compound which is able to fulfill multiple actions.

There are three therapeutic types of multi-target drugs:

- ❖ Components of the molecule impact separate targets and create a combination effect. The targets can be in the same or separate biological pathways within one cell, or even in separate tissues.
- ❖ One component alters the metabolism of the second pharmaceutically active component or blocks an efflux pump or another resistance mechanism.
- ❖ The components bind on separate sites on the same target and create a combination effect which increases the pharmacological action.^[30]

The concept of multi-target drugs offers several advantages over the “classic” chemotherapeutics, as for example altered pharmacological properties, metabolism and resistance development, tunable antitumor properties, the possibility of an “intramolecular” combination therapy and also the design of drugs with selective targeted properties.^[30]

One approach to prepare multifunctional drugs is the combination of the effect of a transition metal, *e.g.* binding to the DNA or redox activity and thereby induced ROS formation, with the efficacy of biologically active organic molecules.^[31,32] The addition of a ferrocenyl moiety to selected bioactive polyaromatic phenols, amines or amides has already been shown to potentiate their antiproliferative activity against breast and prostate cancer cells.^[33] An important example is ferrocifen, in which Gérard Jaouen combined the tamoxifen structure, which is an antagonist of the estrogen receptor and widely used for the treatment of hormone-dependent breast cancer, with the redox properties of ferrocene (Figure 7). This approach led to an increase in the antiproliferative activity in hormone-dependent breast cancer cells, but interestingly also to activity in hormone-independent breast cancer cells.^[31]

The complexation of $\text{Co}_2(\text{CO})_6$, which itself exhibits interesting bioactivity, with a derivative of the anti-inflammatory drug aspirin (o-acetylsalicylic acid, AAS) led to a compound (Co-AAS, Figure 7) with promising antiproliferative properties. Co-AAS was shown to inhibit cyclooxygenase-activity (an enzyme which is involved in inflammation processes), caspase-3 activity (which plays a role in apoptosis), and showed antiangiogenic effects in developing zebrafish embryos.^[34]

Another example are the Ru^{II}(arene) complexes of Meggers *et al.* resembling the staurosporine structure, a naturally occurring kinase inhibitor (Figure 7). This design strategy led to stable inhibitors for different protein kinases with activity against melanoma cell lines.^[35]

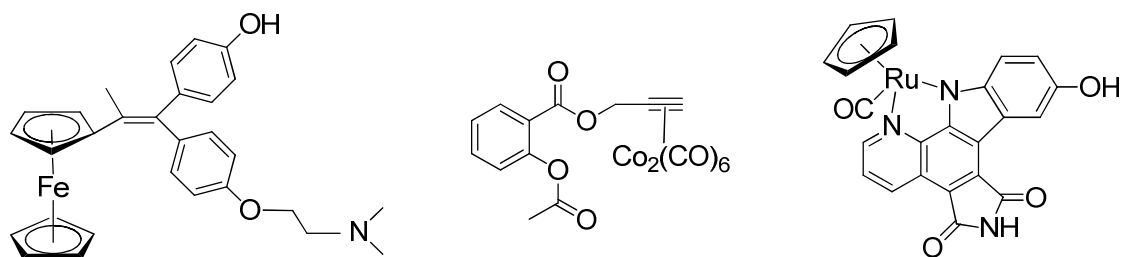


Figure 7. Ferrocifen (left), Co-AAS (center) and a staurosporine-derived Ru^{II}(arene) complex (right).

1.5. Ruthenium-based Chemotherapeutics

The well-developed synthetic chemistry of ruthenium provides the possibility for many approaches to new metallopharmaceuticals.^[36] There are several properties which make it well suited for medicinal application:

- ✓ additional coordination sites: the octahedral geometry of Ru complexes offers more coordination sites than the square-planar Pt^{II} complexes.^[20]
- ✓ the range of accessible oxidation states: Ru^{II}, Ru^{III} and Ru^{IV} are accessible under physiological conditions.
- ✓ the rate of ligand exchange: Ru^{II} and Ru^{III} complexes have similar ligand exchange kinetics as Pt^{II} compounds, which appears to be an important determinant of biological activity.
- ✓ the ability to mimic iron: Ru is able to bind to many biomolecules including transferrin and albumin instead of the naturally occurring Fe which leads to a lower toxicity and a different mode of action.
- ✓ higher selectivity: Cancer cells are very rapidly dividing, thus have a greater need for Fe. It is supposed that the Ru drug will preferentially be enriched in cancer cells and less of it will reach healthy cells.^[37]

1.5.1. Ruthenium(III) Compounds in Clinical Trials

Two Ru^{III} complexes, namely NAMI-A and KP1019 (Figure 8), are already under development in clinical trials with promising results. Imidazolium *trans*-[tetrachlorido(imidazole)(dimethylsulfoxide)ruthenate(III)] (NAMI-A) is selectively effective against lung metastases of solid metastasizing tumors with an activity significantly greater than that of cisplatin while having far less severe side-effects.

The mechanism of action of NAMI-A seems to be a combination of multiple interactions outside and inside of cancer cells, finally leading to a cell cycle stop at the G₂/M pre-mitotic phase and the initiation of apoptosis. Furthermore the drug exerts antiangiogenic activity and regulates the actin-dependent adhesion and cytoskeleton remodeling with inhibition of invasion and metastasis.^[38]

Indazolium *trans*-[tetrachloridobis(1H-indazole)ruthenate(III)] (KP1019) showed promising activity against several types of tumors in preclinical studies including colorectal tumors.^[38,39] A phase I clinical trial has already been completed with five of six evaluable patients suffering from different types of solid tumors showing stable disease and only mild treatment-related toxicities.^[40]

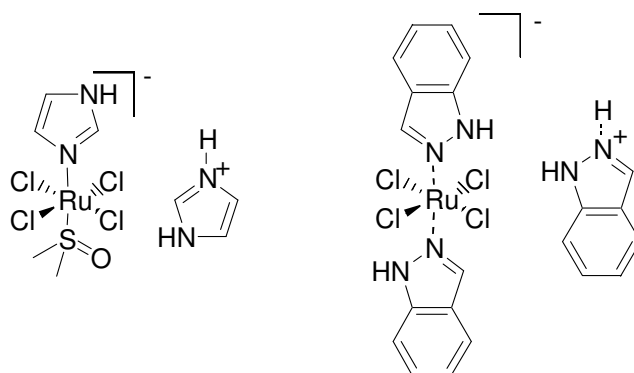


Figure 8. The ruthenium(III) complexes NAMI-A (left) and KP1019 (right).

KP1019 is administered intravenously,^[38] but the exact mechanism of action is not yet fully understood. It is known that the drug strongly binds to serum proteins such as albumin and transferrin which is supposed to be important for the specific accumulation in the tumor. Inside the tumor cells, the Ru^{III} species is released and reduced to Ru^{II} ^[39,40] according to the “Activation by Reduction” hypothesis. It is assumed that Ru^{III} complexes serve as pro-drugs and are activated by reduction to their active Ru^{II} species owing to the hypoxic and slightly acid conditions in solid tumors.^[36] The specific accumulation in tumor cells and the “Activation by Reduction” would also explain the low toxicity of this drug.

KP1019-induced cell death is thought to be caused by activation of apoptosis *via* the mitochondrial pathway by depolarization of the mitochondrial membranes, down-modulation of endogenous bcl-2 and activation of caspase-3,^[39] although the participation of DNA interactions cannot be excluded.^[38]

Because of the poor aqueous solubility of KP1019, the dose escalation studies in the clinical trial setup could not be continued and neither the maximum tolerated dose nor the optimal dose were reached. Thus, its 30-times better soluble sodium salt, KP1339, is used in further clinical trials with similarly promising results.^[40]

1.5.2. Ruthenium(II)–Arene Complexes

In 1980 the first Ru^{II} complex was studied for cytotoxic properties. It was a Ru analogue of cisplatin, *cis*-Ru(NH₃)₄Cl₂, but although anticancer active, it turned out to be not soluble enough for pharmaceutical use. In the following years, a large number of Ru^{II} compounds were tested for anticancer properties, including *cis*- and *trans*-[Ru(DMSO)₄X₂] (X = Br, Cl)^[41] and complexes bearing larger organic ligands, in particular polypyridyl complexes such as *cis*-Ru(*N,N*-bipy)₂Cl₂ and *mer*-Ru(*N,N,N*-terpy)Cl₃ (bipy = 2,2'-bipyridine, terpy = 2,2':6'2'' - terpyridine).^[42]

In 1985, Gérard Jaouen, who has been working with organometallic carbonyl complexes, introduced the term “bioorganometallic chemistry” ^[43] and thereby also stimulated the search for organometallic drugs.

Within the last 20 years, the class of organometallic Ru^{II}(η^6 -arene) complexes has gained more and more interest and was intensively investigated.^[44] These half-sandwich, so-called “piano-stool” complexes (Figure 9) offer multiple possibilities for drug design by modification of the arene ligand and functionalization of the three remaining coordination sites (X, Y and Z) by introduction of either three monodentate, one monodentate and a bidentate or even one tridentate chelating ligand. Depending on the coordinating ligands, neutral or charged complexes could be obtained. These features provide an ability to control the reactivity of these compounds and fine-tune pharmacological properties such as cell-uptake, possible interactions with biomolecules and finally the mechanism of action.^[31]

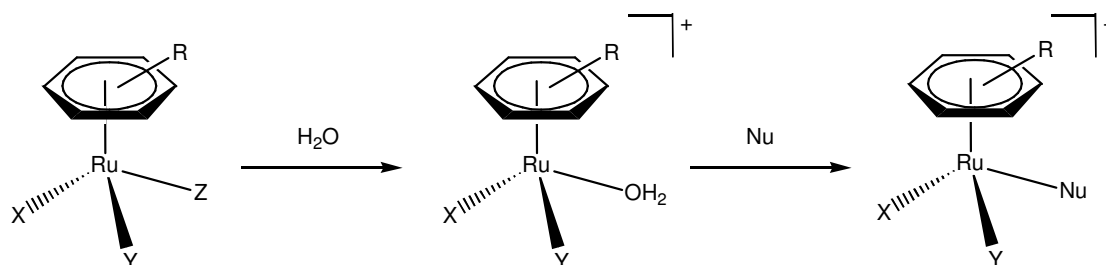


Figure 9. General structure of half-sandwich Ru^{II}(arene) complexes (R = H, functional group, alkyl, aryl; X, Y, Z = ligands), formation of aqua species and reaction with nucleophiles (Nu).

The arene ligand stabilizes Ru^{II} in its oxidation state and also provides a lipophilic face, which might promote interactions with biomolecules and the passive transport *via* diffusion of the Ru^{II} compound through the cell membranes.^[45] The coordination of one halide, most frequently chloride, to the ruthenium center acts as a so-called leaving group, which is replaced by an aqua ligand in aqueous solution to give [Ru(η^6 -arene)(X)(Y)(OH₂)]ⁿ⁺. This aqua complex is supposed to be the reactive species, ready to react with biological nucleophiles such as DNA, amino acids or other biomolecules (Figure 9).^[46] The hydrolysis reaction can often be suppressed by addition of sodium chloride. Since the extracellular chloride concentration is higher than the intracellular chloride levels, it is supposed that the compounds are activated by aquation as soon as they enter the cells, equivalent to cisplatin.^[45] Most examples of this substance class are able to bind nuclear DNA with a high affinity for the N7 of guanine, forming monofunctional adducts,^[46] but this is not always regarded as their major mechanism of action. Instead, in many cases the coordinated ligand system determines the mechanism of action, as for example by interaction with certain enzymes.^[32] This will be discussed later on in this chapter.

The field of anticancer Ru^{II}(arene) complexes was pioneered by the work of Peter Sadler *et al.* on complexes containing ethylenediamine (en) ligands and Paul Dyson *et al.* with the so-called RAPTA-type compounds (Figure 10) which are both at an advanced preclinical development stage.^[44] RAPTA complexes bear a PTA (1,3,5-triaza-7-phosphoadamantane) moiety which causes these compounds to be selectively activated in the hypoxic milieu of solid tumors. RAPTA-T (Figure 10), one of the lead compounds, is only weak cytotoxic, but very selective for tumor cells and able to lower the cells' ability to migrate and metastasize *in vitro*. The compound has also shown some activity *in vivo* in a solid metastasizing tumor model.^[47]

RAPTA-C, the *p*-cymene analogue of RAPTA-T, induces DNA damage, but the compound family is also able to inhibit cathepsin B and thioredoxin reductase, two enzymes that are believed to be targets in cancer therapy.^[48] RAPTA-C also induces apoptosis and slows down cell division.^[49]

Several approaches have been tried to improve the cytotoxicity of this compound class. If PTA is replaced by 3,5,6-bicyclophosphite- α -D-glucofuranoside ligands, RAPTA analogues with enhanced cytotoxicity are obtained due to the increased lipophilicity.^[50]

Coupling the arene ligand to human serum albumin (HSA) increases the cellular accumulation and thus improves the selectivity *in vitro*.^[51] Tethering ethacrynic acid to the arene resulted in a multi-targeted compound with glutathione-S-transferase inhibitory activity, which is accompanied by a cleavage of the enzyme inhibiting fragment from the metal moiety which can target a second biomolecule as for example DNA. As glutathione S-transferase catalyzes the binding of xenobiotics to glutathione in order to remove them from the metabolism, this concept provides a therapy for multi-drug resistant tumors.^[52,53]

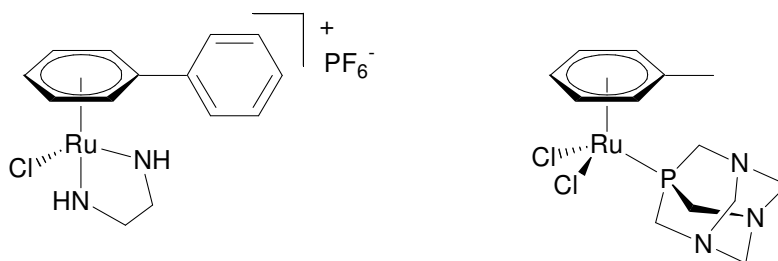


Figure 10. Structures of the Sadler type compound *RM175* (left) and *RAPTA-T* by Dyson *et al.* (right).

The cationic $\text{Ru}^{\text{II}}(\text{arene})$ complexes containing ethylenediamine chelating ligands show very high cytotoxicities *in vitro*, especially as hexafluorophosphate salts (IC_{50} in the low μM range). Substitution of the chloride leaving group with iodide had only little effect on the *in vitro* anticancer activity. As opposed to this, variation of the arene ligand from benzene to *p*-cymene to biphenyl (*RM175*, Figure 10) to dihydroanthracene to tetrahydroanthracene led to a large increase in their growth inhibitory activity depending on increasing size and hydrophobicity.^[54,55] The $\text{Ru}^{\text{II}}(\text{arene})$ ethylenediamine moiety binds preferentially to the *N7* of guanine residues in double-helical DNA, supported by a hydrogen bond formed between *NH* of en and *O6* of guanine,^[55] while in the case of biphenyl, dihydroanthracene and tetrahydroanthracene ligands also non-covalent hydrophobic interactions seem to be involved, which may include arene intercalation and minor groove binding.^[56] Subsequently *RM175* showed to induce apoptosis by modulation of the p53-p21-bax pathway.^[57] *RM175* also exhibits high activity *in vivo* in an A2780 (human ovarian carcinoma) xenograft^[54] and against MCa mammary carcinoma including reduction of metastasis.^[58]

Beside the possible influence of the arene ligand on anticancer activity, the type of chelating ligand plays an important role and has a strong influence on the chemical, physical and

biological properties of Ru^{II}(arene) compounds. Substitution of ethylenediamine as neutral *N,N*-chelating ligand by *O,O*-chelates such as acetylacetonate (acac; Figure 11) leads to significant changes in the selectivity for DNA bases, the electronic properties of the ruthenium center and the behavior of the leaving group. [Ru(η^6 -arene)(acac)Cl] complexes exhibit higher affinity for adenine than for guanine and show an increased rate and extent of hydrolysis.^[59]

Ru^{II}(arene) complexes with the *O,O*-chelating ligand maltol (Figure 11) exhibit moderate anticancer activity and again preferentially bind to the *N7* of guanine bases. In contrast to the ethylenediamine complexes, variation of the arene ligand and the halido leaving group has no influence on cytotoxic activity. Interestingly, changing the *O,O*-chelating maltol backbone to the *S,O*-thiomaltol motif led to compounds with improved activities and stabilities with higher sensitivity to the colorectal carcinoma cell line SW480 instead of the generally more sensitive ovarian cancer cell line CH1.^[60]

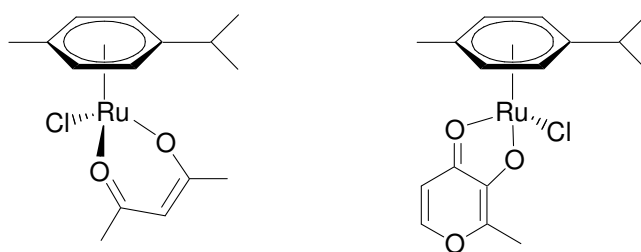


Figure 11. Ru^{II}(arene) compounds with the *O,O*-chelating ligands acac (right) and maltol (left).

As shown for platinum anticancer agents,^[61] it has also been expected for ruthenium complexes that multinuclearity could possibly improve the activity of anticancer drug candidates. The first reported dinuclear Ru^{II}(arene) complex, which showed higher activity than the corresponding monomers, contained a pyridone-derived linker (Figure 12) and showed interesting cytotoxic activity *in vitro* dependent on the spacer length and therefore on lipophilicity. The complexes are able to bind to transferrin *in vitro* and may act as interhelical and DNA-protein cross-linking agents.^[62,63]

Dinuclear Ru^{II}(arene) complexes with 2,3-bis(2-pyridyl)pyrazine as chelating ligands show potential for photodynamic therapy. The indane derivative (Figure 12) dissociates upon UV or visible light irradiation, which is visualized by fluorescence suitable for fluorescence imaging, and subsequently forms strong diruthenium DNA adducts leading to cell death.^[64]

Tetranuclear $\text{Ru}^{\text{II}}(\text{arene})$ complexes containing a porphyrin scaffold combine the photodynamic action of porphyrin with the cytotoxic activity of ruthenium. The linking of ruthenium to the heterocyclic system increases the solubility of the ligand and also the selectivity for cancer cells.^[65] More recently also higher nuclear $\text{Ru}^{\text{II}}(\text{arene})$ complexes and ruthenium cages have been investigated, in which the latter may work as a kind of “Trojan Horse” to deliver a hydrophobic metal-containing host molecule to the cancer cell while also the cage itself is cytotoxic.^[44]

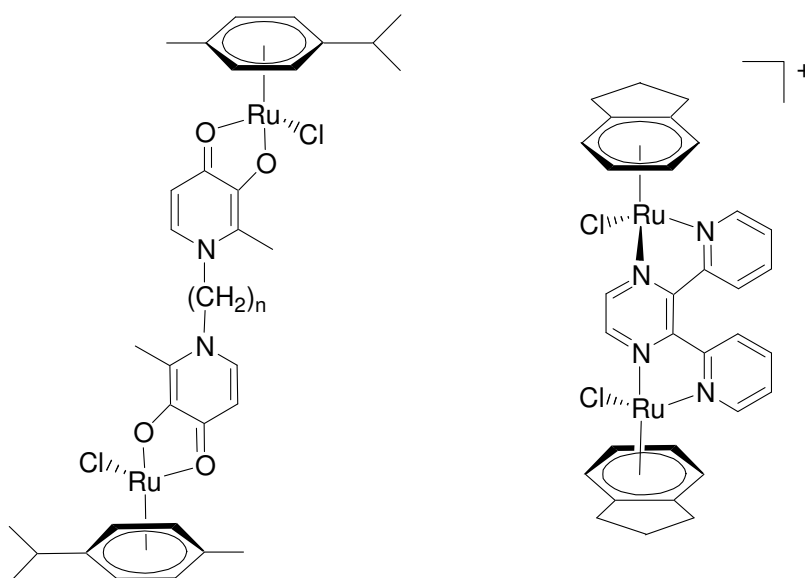


Figure 12. Dinuclear $\text{Ru}^{\text{II}}(\text{arene})$ complexes with a pyridone-derived linker (left) and 2,3-bis(2-pyridyl)pyrazine as a bischelating ligand (right).

1.6. Flavonoids

Flavonoids are polyphenolic secondary plant metabolites found in fruits, vegetables, leaves and flowers. They are responsible for providing colors attractive to plant pollinators, protection from microorganisms and UV radiation and are also involved in photosensitisation, energy transfer, plant growth, control of respiration and photosynthesis, morphogenesis and sex determination.^[66,67] The compound class can be divided into flavones, flavonols, flavanones, flavanonols, flavanols, isoflavones and anthocyanidins, dependent on their general structure (Figure 13). The basic structural feature of flavonoids is the 2-phenylbenzo[α]pyrane moiety consisting of two benzene rings (A and B) which are linked by a heterocyclic pyrane ring (C). While flavones and flavonols have a double bond between the C2 and C3 position, flavanones and flavanonols are characterized by a saturated three carbon chain in ring C. The latter possess an keto group at the R₄ position, in contrast to the flavanols which contain a hydroxyl group at position R₃ or R₄, while the other position remains saturated. In plants, flavonoids are mostly conjugated to sugars, primarily glucose, rhamnose and rutinose.^[68]

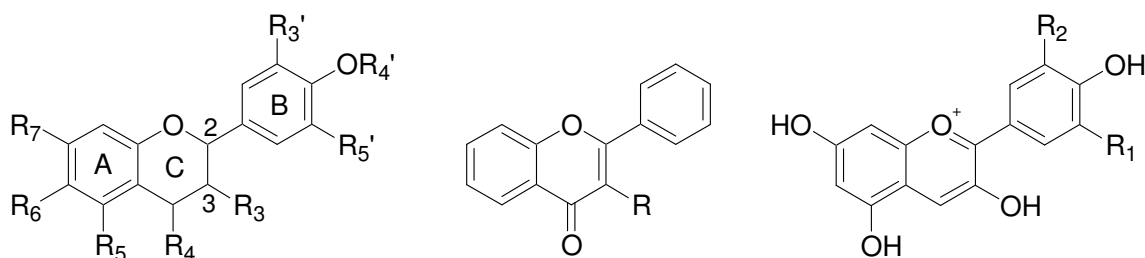


Figure 13. Basic flavonoid structure (left), general structures of flavones (R=H) and flavonols (R=OH) (center) and of anthocyanidins (right).

1.6.1. Medicinal Properties of Flavonoids

As a common constituent of the daily human diet, these natural products are long-since known for their beneficial effects on health such as antioxidant and antiradical activity, anti-inflammatory, antimicrobial and also anticarcinogenic properties.

Their antioxidant activity protects the human body from reactive oxygen species (ROS), which can be produced during the normal oxygen metabolism or are induced by an exoge-

nous damage. ROS and free radical-mediated diseases include atherosclerosis, neuronal degeneration, cancer and rheumatoid arthritis.^[69] The flavonoid structure acts as direct ROS or radical scavenger or is oxidized itself, resulting in a more stable and less-reactive radical. The inhibition of xanthine oxidase, which is a source of oxygen free radicals, seems also to play a role in the antioxidant mechanisms of flavonoids.^[66] Furthermore the compounds seem to interact with lipid and protein compounds of the cell membranes and thus protect the cells from oxidative damages by lipid peroxidation.^[69]

The antiviral activity of flavonoids, as for example against the human immunodeficiency virus (HIV), comes mainly from the inhibition of several enzymes and the inhibition of HIV entry into the cells. Their antibacterial activity was shown to be due to various different mechanisms of action as for example the inhibition of nucleic acid synthesis possibly by intercalation or hydrogen bond formation to nucleobases or by inhibition of DNA gyrase as shown in the case of *Escherichia coli*.^[67]

The inhibition of cyclooxygenase and lipoxygenase is thought to play an important role in the anti-inflammatory activity of flavonoids. These enzymes are inflammatory mediators involved in the release of arachidonic acid which is a starting point for a general inflammatory response.^[66]

The anticarcinogenic activity of flavonoid compounds has already been shown in numerous *in vitro* and *in vivo* studies. One example is the flavonol quercetin, which exerted a dose-dependent inhibitory effect *in vitro* on the proliferation of human colon cancer cell lines and also in a breast cancer cell line in low μM to nM concentrations. The compound showed promising anticancer activity against skin, breast, lung and prostate cancer *in vivo*,^[68] however, the mechanism of action is not fully understood yet. The scavenging of ROS, which can damage DNA and thus may initiate cancer, is one possible reason for their protective effect.^[66] On the other hand, some flavonoids have also shown to inhibit cell proliferation and angiogenesis, which is often found unregulated in cancer.^[70] A possible mechanism of action might be the inhibition of several enzymes such as cyclin-dependent kinases,^[71] tyrosine kinases^[72] and protein kinase C.^[73]

Another target of flavonoids are topoisomerase I^[74] and II,^[75] leading to the inhibition of DNA proliferation. DNA topoisomerases are enzymes that solve topological problems caused in nearly every process involving DNA such as transcription, replication and DNA

repair. DNA topology is changed by the introduction of transient breaks into the phosphodiester backbone of DNA, allowing the DNA to be untangled or unwound. After the release of torsion stress, the DNA strands are reconnected by the enzyme. Topoisomerases can be classified as type I or type II depending on if they induce single- or double-strand breaks, respectively. During this process, the enzymes are covalently bound to DNA *via* an active tyrosine residue. This intermediate is called “cleavable complex”. Depending on their mechanism of action, there are two different types of topoisomerase inhibitors:

- Catalytic inhibitors: bind to the enzyme prior to the formation of the cleavable complex and inhibit its formation, thus DNA remains tangled, *e.g.* genistein.
- Topoisomerase poisons: bind to the cleavable complex after it has been formed, therefore prohibit the release or the reconnection of the DNA strand and consequently lead to a strand break, *e.g.* etoposide.

DNA topoisomerases are often overexpressed in many types of cancer, thus constitute important targets for cancer therapy, and inhibitors such as doxorubicin, mitoxantrone and etoposide are already routinely used in the clinic.^[74,76]

1.6.2. Metal-Flavonoid Complexes

Flavonoids are able to form stable complexes with several *p*-, *d*-, and *f*- electron metals *via* the 3-hydroxy or 5-hydroxy groups and 4-carbonyl group on the C-ring or *via* a 3',4'-dihydroxy system located on the B-ring. The chelation of metal ions can be employed for their analysis in different kinds of samples using various techniques. For the determination of trace metal ion contents in biological samples, often pretreatment steps, such as preconcentration or selective separation of the analyte, are necessary. For this purpose, solid-phase extraction systems using chelating resins that contain covalently bound flavonoid molecules as functional groups, have already been successfully applied, as for example in the separation and preconcentration of Mn^{II}, Co^{II}, Ni^{II}, Cu^{II} and Zn^{II} with silica glass functionalized with quercetin. Quercetin-modified carbon paste electrodes are investigated for the simultaneous voltammetric determination of Co, Pb and Zn.

Flavonoids are UV- and fluorescence active and exhibit two major absorption bands in the UV/VIS region at 320-385 nm corresponding to the B ring and at 240-280 nm which is as-

sociated to the A ring. The absorption bands of the corresponding metal complexes are shifted to the long-wavelength region due to the extension of the conjugated system by the complexation. This property can be applied for the analytical determination of metals by UV-VIS spectrophotometry and spectrofluorimetry. The flavonoids morin and quercetin are already used as chromogenic agents for the determination of Al^{III} , Cr^{III} , Fe^{III} , Ge^{IV} , Zr^{IV} , Hf^{IV} , W^{VI} and Mo^{VI} .^[77] The complex formation of aluminium(III) chloride with 3-hydroxyflavone can be used as quantitative sensor for fluoride and acetate ions as the exchange of the chlorido ligands by these anions turns off the fluorescence signal of the complex.^[78]

In the human body, the chelation of metal ions by flavonoids prevents the metal-catalyzed generation of free radicals and their subsequent reactions and thus protects biologically active molecules from oxidative stress. So it has been shown that 3- and 5-hydroxychromones prevent DNA damage by hydroxyl radicals out of the iron-mediated Fenton reaction due to competitive binding to Fe^{II} and oxidation to Fe^{III} *in vitro*.^[79] Experimental data even show that the complexation of flavonoids to metal ions increases their biological activities. The complexes of Fe^{II} , Fe^{III} , Cu^{II} and Zn^{II} with rutin and dihydroquercetin turned out to be more effective radical scavengers than the uncomplexed flavonoids due to the introduction of additional superoxide dismutating centers.^[80] In the same way, the Cu^{II} complex of the flavanone glycoside naringin (Figure 14) showed higher antioxidant, anti-inflammatory and antitumor activity than free naringin without a reduction in cell viability.^[81]

Thus metal-flavonoid complexes are investigated for the treatment of a range of diseases such as some bacterial and viral infections, but also diabetes mellitus and cancer. Complexes of morin with La^{III} , Gd^{III} and Lu^{III} have shown to inhibit bacterial strains like *E. coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, which are known to cause bacterial infections like intestinal and bladder infections, pulmonary inflammation and skin infections, respectively.^[82]

The flavonoid kaempferol exhibits anti-diabetes and insulin-mimetic activity due to its anti-hyperglycemic effect. These properties have been shown to be increased by complexation with vanadium(IV) oxide.^[83]

Huntington's disease, a neurodegenerative disorder connected with lesions in the striatum of the brain, is characterized by increased Cu^{II} levels which lead to an aggregation of the protein huntingtin. The flavonoid epigallocatechin-gallate, which can be extracted from green tea, is a copper chelator and used to modulate early disorders in huntingtin folding. β -Thalassemia is a hereditary disease because of abnormal hemoglobin synthesis leading to hemolysis, anemia and severe iron overload. Excess of body iron ions causes increased production of ROS which can cause damage to major organs and especially the cardiovascular system. A standard cure is the application of iron chelators such as deferoxamine and deferiprone, which cause however various side effects. On the search for new effective drugs, the flavonoid baicalin has also been shown to bind non-heme iron and remove it from the organism.^[82]

The cisplatin derived complex of two synthetic 3-aminoflavone ligands (Figure 14) causes cell necrosis and apoptosis and has a toxic effect on leukemia cells in mice, while being much less cytotoxic to normal cells. Its proposed main mechanism is the induction of DNA breakage, but it has also been shown to decrease the expression of p53 and BAX proteins.^[84]

The before mentioned Cu^{II} -naringin complex (Figure 14) leads to cell death in murine fibroblast, murine melanoma and human chronic myeloid leukemia cell lines at 50 μM and 100 μM concentrations within 24 h.^[81]

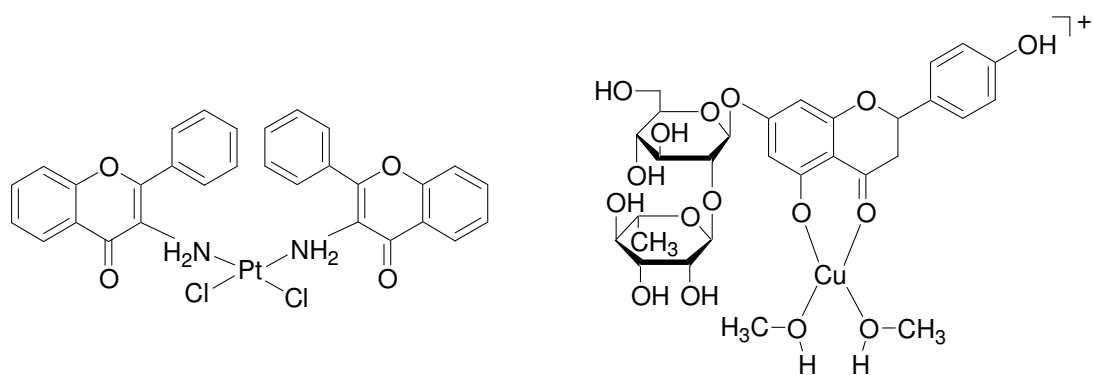


Figure 14. 3-Aminoflavone derived *cis* - Pt(II) complex (left) and Cu(II) complex of naringin (right).

Transition metal (Zn^{II} , Mn^{II} , Cu^{II} , Co^{II} , Ni^{II}) complexes of isoflavone (Figure 15) possess higher growth inhibitory effects than the free ligand, with the Mn^{II} and Ni^{II} derivatives showing the highest antitumor activity and selectivity and were shown to be even more

effective than cisplatin against selected cell lines. Furthermore, DNA flow cytometric analysis demonstrated that the complexes cause a significant G2/M phase arrest, which then progressed to early apoptosis.^[85]

Linking ferrocene to different flavonoid structures such as chalcones, aurones, flavones, flavonols (Figure 15) and flavanones led to compounds with clearly increased cytotoxic activity on murine melanoma cells. Furthermore, the ferrocenyl flavones showed anti-vascular activity on endothelial cells at submicromolar concentrations.^[86]

Ru^{II} complexes with flavanone ligands can be prepared *in situ* by reaction of ruthenium(III) chloride with 3-aminoflavone and showed high cytotoxicity towards the cisplatin-resistant cell lines EJcisR (human bladder carcinoma) and L1210R (mouse leukemia cells) and are as active as cisplatin in the respective sensitive cell lines, while being much less toxic on human lymphocytes.^[87] Recently, also $\text{Ru}^{\text{II}}(\text{dmsO})(\text{chlorido})$ complexes of 3-hydroxychalcones and -flavones (Figure 15) have been published and were found to exhibit cytotoxic activity in Dalton Lymphoma cell lines.^[88]

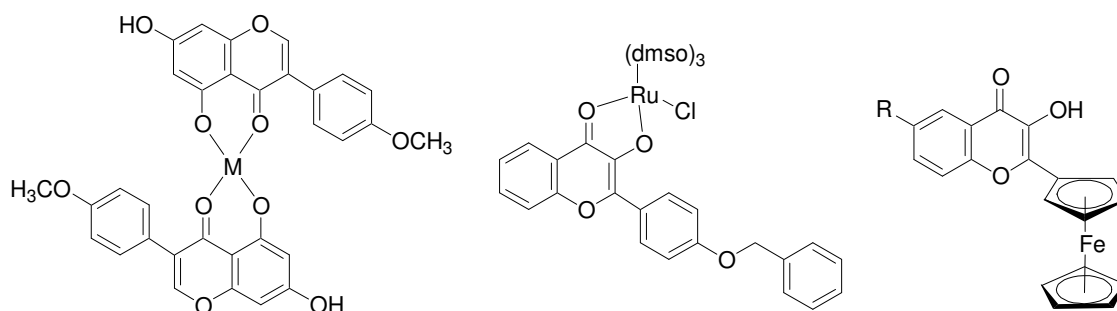


Figure 15. Structures of transition metal complexes ($\text{M} = \text{Zn}^{\text{II}}, \text{Mn}^{\text{II}}, \text{Cu}^{\text{II}}, \text{Co}^{\text{II}}, \text{Ni}^{\text{II}}$) with isoflavone (left), a 3-hydroxyflavone derived $\text{Ru}^{\text{II}}(\text{dmsO})(\text{Cl})$ complex (center) and a ferrocenyl-substituted flavonol ($\text{R} = \text{H}, \text{Br}, \text{OMe}$) (right).

2. Research justification

Within this Ph.D. thesis, a series of novel Ru^{II}(arene) complexes, bearing flavonoids as chelating ligand system, has been prepared. Ruthenium compounds are considered promising candidates for anticancer drug development with two representatives being already in clinical trials with very promising results. The application of hydroxypyrrone complexes as potential anticancer agents has already been extensively studied in our lab.^[60,89,90] These ligands share the same *O,O*-coordination motif with flavonols. The hydroxypyrrone complexes, as with Ru^{II}(arene) complexes in general, have been shown to bind covalently to DNA, mostly via the *N7* atom of guanine bases.^[60] Flavonoids as natural components of plants are known for their beneficial effects on health due to their antioxidant, anti-inflammatory, antiviral and anticarcinogenic properties. Also a few examples of metal-flavonoid complexes are reported to exhibit biological properties. However, these studies often did not aim to correlate their activity to the inhibition of specific biological targets.

By linking flavonoid ligands to Ru^{II}(arene) moieties, we aimed for compounds which can act on multiple targets by combination of the DNA-binding properties of the metal center with the versatile biological activity of the natural substances. This strategy, namely linking metal fragments to biologically active molecules, has already resulted in interesting compounds with novel modes of action and promising activity profiles.^[33,34,51] Furthermore ligand systems derived from natural compounds such as flavonoids often offer additional advantageous properties such as an advantageous toxicity profile.

As flavonoids act mainly by the inhibition of enzymes, the interaction of the complexes with potential target molecules such as CDK2 and human topoisomerase II α were investigated, and flow cytometry analyses of the cell cycle were conducted. Due to the intrinsic fluorescence of the ligand system, it was also possible to perform preliminary fluorescence studies on the compounds' distribution and enrichment in the cells by fluorescence confocal laser scanning microscopy.

The concept of multi-targeted anticancer drugs, in which components of a molecule impact multiple separate targets, could be an approach for single-molecular combination therapy and a promising strategy to overcome drug resistance and to overcome drawbacks of current chemotherapeutics in general.

References

- ¹ WHO, International Agency for Research on Cancer, World Cancer Report 2008.
- ² Statistik Austria, Causes of death 2011.
- ³ A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, David F., Global Cancer Statistics. *CA Cancer J Clin* **2011**, *61*, 69–90.
- ⁴ F. Lang, Pathophysiologie und Pathobiochemie – Eine Einführung, 4. Auflage, **1990**, Ferdinand Enke Verlag Stuttgart.
- ⁵ W. H. Elliot, D. C. Elliot, Biochemistry and Molecular Biology, 3rd Edition, **2005**, Oxford University Press.
- ⁶ B. Alberts et al., Molecular Biology of the Cell, 4th Edition, **2002**, New York, Garland.
- ⁷ D. Hanahan, R. A. Weinberg. The Hallmarks of Cancer. *Cell* **2000**, *100*, 57–70.
- ⁸ D. Hanahan, R. A. Weinberg. The Hallmarks of Cancer: The Next Generation. *Cell* **2004**, *144*, 646–674.
- ⁹ C. M. Haskell, Cancer Treatment, 5th Edition, **2001**, Saunders, Philadelphia.
- ¹⁰ C. H. Takimoto, E. Calvo, Cancer Management: A Multidisciplinary Approach, 11th Edition, **2008**, CMPMedica.
- ¹¹ E. Segota, R. M. Bukowski, The Promise of Targeted Therapy: Cancer Drugs become more specific. *Clev Clin J Med* **2004**, *71*, 551–560.
- ¹² C. Orvig, M. J. Abrams, Medicinal Inorganic Chemistry: Introduction. *Chem Rev* **1999**, *99*, 2201–2203.
- ¹³ Z. Guo, P. J. Sadler, Metals in Medicine. *Angew Chem Int Ed* **1999**, *38*, 1512–1531.
- ¹⁴ L. Kelland, The Resurgence of Platinum-based Cancer Chemotherapy. *Nat Rev Cancer* **2007**, *7*, 573–584.
- ¹⁵ M. A. Jakupec, M. Galanski, B. K. Keppler, Tumour-inhibiting Platinum Complexes–State of the Art and Future Perspectives. *Rev Physiolog Biochem Pharmacol* **2003**, *146*, 1–53.
- ¹⁶ X. Du, X. Wang, H. Lia, Hongzhe Sun, Comparison between Copper and Cisplatin Transport mediated by Human Copper Transporter 1 (hCTR1). *Metallomics* **2012**, *4*, 679–685.
- ¹⁷ N. J. Wheate, S. Walker, G. E. Craig, R. Oun, The Status of Platinum Anticancer Drugs in the Clinic and in Clinical Trials. *Dalton Trans* **2010**, *39*, 8113–8127.
- ¹⁸ P. Heffeter, U. Jungwirth, M. Jakupec, C. Hartinger, M. Galanski, L. Elbling, M. Micksche, B. Keppler, W. Berger, Resistance against Novel Anticancer Metal Compounds: Differences and Similarities. *Drug Resistance Updates* **2008**, *11*, 1–16.
- ¹⁹ K. H. Antman, Introduction: The History of Arsenic Trioxide in Cancer Therapy. *The Oncologist* **2001**, *6*, 1–2.
- ²⁰ M. J. Clarke, F. Zhu, D. R. Frasca, Non-Platinum Chemotherapeutic Metallopharmaceuticals. *Chem Rev* **1999**, *99*, 2511–2533.
- ²¹ M. Galanski, V. B. Arion, M. A. Jakupec, B. K. Keppler, Recent Developments in the Field of Tumor-Inhibiting Metal Complexes. *Curr Pharmaceut Design* **2003**, *9*, 2078–2089.
- ²² I. Bratsos, T. Gianferrara, E. Alessio, C. G. Hartinger, M. A. Jakupec, B. K. Keppler, Ruthenium and Other Non-Platinum Anticancer Compounds, in Bioinorganic Medicinal Chemistry (ed E. Alessio), **2011**, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany. DOI: 10.1002/9783527633104.ch5

- ²³ P. Köpf-Maier, Complexes of Metals other than Platinum as Antitumour Agents. *Eur J Clin Pharmacol* **1994**, *47*, 1–16.
- ²⁴ L. R. Bernstein, T. Tanner, C. Godfrey, B. Noll, Chemistry and Pharmacokinetics of Gallium Maltolate, a Compound with high oral Gallium Bioavailability. *Metal-based Drugs* **2000**, *7*, 33–47.
- ²⁵ U. Jungwirth, C. R. Kowol, B. K. Keppler, C. G. Hartinger, W. Berger, P. Heffeter, Anticancer Activity of Metal Complexes: Involvement of Redox Processes. *Antioxid Redox Sign* **2011**, *15*, 1085–1127.
- ²⁶ B. Dong, Y.-M. Zhu, Molecular-targeted Therapy for Cancer. *Chin J Cancer* **2010**, *29*, 340–345.
- ²⁷ G. Sava, G. Jaouen, E. A. Hillard, A. Bergamo, Targeted Therapy vs. DNA-adduct Formation-guided Design: Thoughts about the Future of Metal-based Anticancer Drugs. *Dalton Trans* **2012**, *41*, 8226–8234.
- ²⁸ R. S. Herbst, Role of Novel Targeted Therapies in the Clinic. *Brit J Cancer* **2005**, *92*, 21–27.
- ²⁹ D. E. Zogas, C. S. Katsios, M. Tzaphlidou, D. H. Roukos, Targeted Therapy: Overcoming Drug Resistance with Clinical Cancer Genome. *Expert Rev Anticancer Ther* **2012**, *12*, 861–864.
- ³⁰ G. R. Zimmermann, J. Lehar, C. T. Keith, Multi-Target Therapeutics: When the Whole is greater than the Sum of the Parts. *Drug Discovery Today* **2007**, *12*, 34–42.
- ³¹ E. Meggers, Exploring Biologically Relevant Space with Metal Complexes. *Curr Opin Chem Biol* **2007**, *11*, 287–292.
- ³² G. S. Smith, B. Therrien, Targeted and multifunctional arene ruthenium chemotherapeutics. *Dalton Trans* **2011**, *40*, 10793–10800.
- ³³ D. Hamels, P. M. Dansette, E. A. Hillard, S. Top, A. Vessieres, P. Herson, G. Jaouen, D. Mansuy, Ferrocenyl Quinone Methides as Strong Antiproliferative Agents: Formation by Metabolic and Chemical Oxidation of Ferrocenyl Phenols. *Angew Chem Int Ed* **2009**, *48*, 9124–9126.
- ³⁴ I. Ott, B. Kircher, C. P. Bagowski, D. H. W. Vlecken, E. B. Ott, J. Will, K. Bensdorf, W. S. Sheldrick, R. Gust, Modulation of the Biological Properties of Aspirin by Formation of a Bioorganometallic Derivative. *Angew Chem Int Ed* **2009**, *48*, 1160–1163.
- ³⁵ E. Meggers, G. E. Atilla-Gokcumen, K. Gründler, C. Frias, A. Prokop, Inert Ruthenium Half-Sandwich Complexes with Anticancer Activity. *Dalton Trans* **2009**, *48*, 10882–10888.
- ³⁶ M. J. Clarke, Ruthenium Metallopharmaceuticals. *Coord Chem Rev* **2003**, *236*, 209–233.
- ³⁷ C. S. Allardyce, P. J. Dyson, Ruthenium in Medicine: Current Clinical Uses and Future Prospects. *Platinum Metals Rev* **2001**, *45*, 62–69.
- ³⁸ A. Bergamo, C. Gaidon, J.H.M. Schellens, J.H. Beijnen, G. Sava, Approaching Tumour Therapy beyond Platinum Drugs - Status of the Art and Perspectives of Ruthenium Drug Candidates. *J Inorg Biochem* **2012**, *106*, 90–99.
- ³⁹ F. Lentz, A. Drescher, A. Lindauer, M. Henke, R. A. Hilger, C. G. Hartinger, M. E. Scheulen, C. Dittrich, B. K. Keppler, U. Jaehde, Pharmacokinetics of a Novel Anticancer Ruthenium Complex (KP1019, FFC14A) in a Phase I Dose-Escalation Study. *Anti-Cancer Drugs* **2009**, *20*, 97–103.
- ⁴⁰ P. Heffeter, K. Böck, B. Atil, M. Al, R. Hoda, W. Körner, C. Bartel, U. Jungwirth, B. K. Keppler, M. Micksche, W. Berger, G. Koellensperger, Intracellular Protein Binding Patterns of the Anticancer Ruthenium Drugs KP1019 and KP1339. *J Biol Inorg Chem* **2010**, *15*, 737–748.

- ⁴¹ E. Alessio, G. Mestroni, G. Nardin, W. M. Attia, M. Calligaris, G. Sava, S. Zorzet, Cis- and Trans-Dihalotetrakis(dimethylsulfoxide)ruthenium(II) complexes (RuX₂(DMSO)₄; X = Cl, Br): Synthesis, Structure, and Antitumor Activity. *Inorg Chem* **1988**, *27*, 4099–4106.
- ⁴² O. Novakova, J. Kasparkova, O. Vrana, P. M. Vanvliet, J. Reedijk, V. Brabec, Correlation between Cytotoxicity and DNA Binding of Polypyridyl Ruthenium Complexes. *Biochemistry* **1995**, *34*, 12369–12378.
- ⁴³ G. Jaouen, A. Vessieres, Transition Metal Carbonyl Oestrogen Receptor Assay. *Pure Appl Chem* **1985**, *57*, 1865–1874.
- ⁴⁴ G. Süss-Fink, Arene ruthenium complexes as anticancer agents. *Dalton Trans* **2010**, *39*, 1673–1688.
- ⁴⁵ R.E. Morris, R.E. Aird, P. del Socorro Murdoch, H. Chen, J. Cummings, N.D. Hughes, S. Parsons, A. Parkin, G. Boyd, D.I. Jodrell, P.J. Sadler, Inhibition of Cancer Cell Growth by Ruthenium Arene Complexes. *J Med Chem* **2001**, *44*, 3616–3621.
- ⁴⁶ A. F. A. Peacock, P. J. Sadler, Medicinal Organometallic chemistry: Designing Metal Arene Complexes as Anticancer Agents. *Chem Asian J* **2008**, *3*, 1890–1899.
- ⁴⁷ A. Bergamo, A. Masi, P. J. Dyson, G. Sava, Modulation of the Metastatic Progression of Breast Cancer with an Organometallic Ruthenium Compound. *Int J Oncol* **2008**, *33*, 1281–1289.
- ⁴⁸ A. Casini, C. Gabbiani, F. Sorrentino, M. P. Rigobello, A. Bindoli, T. J. Geldbach, A. Marrone, N. Re, C. G. Hartinger, P. J. Dyson, L. Messori, Emerging Protein Targets for Anticancer Metallodrugs: Inhibition of Thioredoxin Reductase and Cathepsin B by Antitumor Ruthenium(II)-Arene Compounds. *J Med Chem* **2008**, *51*, 6773–6781.
- ⁴⁹ S. Chatterjee, S. Kundu, A. Bhattacharyya, C. G. Hartinger, P. J. Dyson, The Ruthenium(II)-arene Compound RAPTA-C induces Apoptosis in EAC Cells through Mitochondrial and p53–JNK Pathways. *J Biol Inorg Chem* **2008**, *13*, 1149–1155.
- ⁵⁰ I. Berger, M. Hanif, A. A. Nazarov, C. G. Hartinger, R. O. John, M. L. Kuznetsov, M. Groessl, F. Schmitt, O. Zava, F. Biba, V. B. Arion, M. Galanski, M. A. Jakupiec, L. Juillerat-Jeanneret, P. J. Dyson, B. K. Keppler, In Vitro Anticancer Activity and Biologically Relevant Metabolization of Organometallic Ruthenium Complexes with Carbohydrate-Based Ligands. *Chem Eur J* **2008**, *14*, 9046–9057.
- ⁵¹ W. H. Ang, E. Daldini, L. Juillerat-Jeanneret, P. J. Dyson, Strategy To Tether Organometallic Ruthenium-Arene Anticancer Compounds to Recombinant Human Serum Albumin. *Inorg Chem* **2007**, *46*, 9048–9050.
- ⁵² W. H. Ang, L. J. Parker, A. De Luca, L. Juillerat-Jeanneret, C. J. Morton, M. Lo Bello, M. W. Parker, P. J. Dyson, Rational Design of an Organometallic Glutathione Transferase Inhibitor. *Angew Chem Int Ed* **2009**, *48*, 3854–3857.
- ⁵³ S. Chatterjee, I. Biondi, P. J. Dyson, A. Bhattacharyya, A Bifunctional Organometallic Ruthenium Drug with Multiple Modes of Inducing Apoptosis. *J Biol Inorg Chem* **2011**, *16*, 715–724.
- ⁵⁴ R. E. Aird, J. Cummings, A. A. Ritchie, M. Muir, R. E. Morris, H. Chen, P. J. Sadler, D. I. Jodrell, In vitro and in vivo Activity and Cross Resistance Profiles of Novel Ruthenium (II) Organometallic Arene Complexes in Human Ovarian Cancer. *Br J Cancer* **2002**, *86*, 1652–1657.

- ⁵⁵ H. Chen, J.A. Parkinson, R.E. Morris, P.J. Sadler, Highly Selective Binding of Organometallic Ruthenium Ethylenediamine Complexes to Nucleic Acids: Novel Recognition Mechanisms. *J Am Chem Soc*, **2003** 125, 173–186.
- ⁵⁶ O. Novakova, H. Chen, O. Vrana, A. Rodger, P.J. Sadler, V. Brabec, DNA Interactions of Monofunctional Organometallic Ruthenium(II) Antitumor Complexes in Cell-free Media. *Biochemistry* **2003**, 42, 11544–11554.
- ⁵⁷ R. L. Hayward, Q. C. Schornagel, R. Tente, J. S. Macpherson, R. E. Aird, S. Guichard, A. Habtemariam, P. Sadler, D. I. Jodrell, Investigation of the Role of Bax, p21/Waf1 and p53 as Determinants of Cellular Responses in HCT116 Colorectal Cancer Cells exposed to the Novel Cytotoxic Ruthenium(II) Organometallic Agent, RM175, *Cancer Chemother Pharmacol* **2005**, 55, 577–583.
- ⁵⁸ A. Bergamo, A. Masi, A.F.A. Peacock, A. Habtemariam, P.J. Sadler, G. Sava, 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, 79–86.
- ⁵⁹ R. Fernandez, M. Melchart, A. Habtemariam, S. Parsons, P. J. Sadler, Use of Chelating Ligands to Tune the Reactive Site of Half-Sandwich Ruthenium(II)-Arene Anticancer Complexes. *Chem Eur J* **2004**, 10, 5173–5179.
- ⁶⁰ W. Kandioller, A. Kurzwernhart, M. Hanif, S. M. Meier, H. Henke, B. K. Keppler, C. G. Hartinger, Pyrone Derivatives and Metals: From Natural Products to Metal-based Drugs. *J Org Chem* **2011**, 696, 999–1010.
- ⁶¹ C. Manzotti, G. Pratesi, E. Menta, R. D. Domenico, E. Cavaletti, H. H. Fiebig, L. R. Kelland, N. Farrell, D. Polizzi, R. Supino, G. Pezzoni, F. Zunino, BBR 3464: A Novel Triplatinum Complex, Exhibiting a Preclinical Profile of Antitumor Efficacy Different from Cisplatin. *Clin Cancer Res* **2000**, 6, 2626–2634.
- ⁶² M. G. Mendoza-Ferri, C. G. Hartinger, M. A. Mendoza, M. Groessl, A. E. Egger, R. E. Eichinger, J. B. Mangrum, N. P. Farrell, M. Maruszak, P. J. Bednarski, F. Klein, M. A. Jakupiec, A.A. Nazarov, K. Severin, B. K. Keppler, Transferring the Concept of Multinuclearity to Ruthenium Complexes for Improvement of Anticancer Activity *J Med Chem* **2009**, 52, 916–925.
- ⁶³ O. Novakova, A. A. Nazarov, C. G. Hartinger, B. K. Keppler, V. Brabec, DNA Interactions of Dinuclear Ru^{II} Arene Antitumor Complexes in Cell-free Media. *Biochem Pharmacol* **2007**, 77, 364–374.
- ⁶⁴ S. W. Magennis, A. Habtemariam, O. Novakova, J. B. Henry, S. Meier, S. Parsons, I. D. H. Oswald, V. Brabec and P. J. Sadler, Dual Triggering of DNA Binding and Fluorescence via Photoactivation of a Dinuclear Ruthenium(II) Arene Complex. *Inorg Chem* **2007**, 46, 5059–5068.
- ⁶⁵ F. Schmitt, P. Govindaswamy, G. Süß-Fink, W. H. Ang, P. J. Dyson, L. Juillerat-Jeanneret, B. Therrien, Ruthenium Porphyrin Compounds for Photodynamic Therapy of Cancer. *J Med Chem* **2008**, 51, 1811–1816.
- ⁶⁶ R. J. Nijveldt, E. van Nood, D. E. C. van Hoorn, P. G. Boelens, K. van Norren, P. A. M. van Leeuwen, Flavonoids: A Review of Probable Mechanisms of Action and Potential Applications. *Am J Clin Nutr* **2001**, 74, 418–425.
- ⁶⁷ T. P. T. Cushnie, A. J. Lamb, Antimicrobial Activity of Flavonoids. *Int J Antimicrob Agents* **2005**, 26, 343–356.
- ⁶⁸ S. N. Nichenametla, T. G. Taruscio, D. L. Barney, J. H. Exon, A Review of the Effects and Mechanisms of Polyphenolics and Cancer, *Crit Rev Food Sci Nutr* **2006**, 46, 161–183.
- ⁶⁹ S. Chaudhuri, A. Banerjee, K. Basu, B. Sengupta, P. K. Sengupta. *Int J Biol Macromol* **2007**, 41, 42–48.

- ⁷⁰ T. Fotsis, M. S. Pepper, E. Aktas, S. Breit, S. Rasku, H. Adlercreutz, K. Wähälä, R. Montesano, L. Schweigerer, Flavonoids, Dietary-derived Inhibitors of Cell Proliferation and in Vitro Angiogenesis. *Cancer Res* **1997**, *57*, 2916–2921.
- ⁷¹ J. E. Karp, B. D. Smith, L. S. Resar, J. M. Greer, A. Blackford, M. Zhao, D. Moton-Nelson, K. Alino, M. J. Levis, S. D. Gore, B. Joseph, H. Carraway, M. A. McDevitt, L. Bagain, K. Mackey, J. Briel, L. A. Doyle, J. J. Wright, M. A. Rudek, Phase 1 and Pharmacokinetic Study of Bolus-Infusion Flavopiridol followed by Cytosine Arabinoside and Mitoxantrone for Acute Leukemias. *Blood* **2011**, *117*, 3302–3310.
- ⁷² N. Teller, W. Thiele, U. Boettler, J. Sleeman, D. Marko, Delphinidin Inhibits a Broad Spectrum of Receptor Tyrosine Kinases of the ErbB and VEGFR Family. *Mol Nutr Food Res* **2009**, *53*, 1075–1083.
- ⁷³ M. Kern, G. Pahlke, K. K. Balavenkatraman, F. D. Boehmer, D. Marko, Apple Polyphenols Affect Protein Kinase C Activity and the Onset of Apoptosis in Human Colon Carcinoma Cells. *J Agric Food Chem* **2007**, *55*, 4999–5006.
- ⁷⁴ M. Habermeyer, J. Fritz, H. U. Barthelmes, M. O. Christensen, M. K. Larsen, F. Boege, D. Marko, Anthocyanidins Modulate the Activity of Human DNA Topoisomerases I and II and Affect Cellular DNA Integrity. *Chem Res Toxicol* **2005**, *18*, 1395–1404.
- ⁷⁵ O. J. Bandele, N. Osheroff, Bioflavonoids as Poisons of Human Topoisomerase II α and II β . *Biochem* **2007**, *46*, 6097–6108.
- ⁷⁶ J. A. Holden, DNA Topoisomerases as Anticancer Drug Targets: From the Laboratory to the Clinic. *Curr Med Chem Anti Canc Agents* **2001**, *1*, 1–25.
- ⁷⁷ K. Pyrzynska, A. Pekal, Flavonoids as Analytical Reagents. *Crit Rev Anal Chem* **2011**, *41*, 335–345.
- ⁷⁸ S. Sathish, G. Narayan, N. Rao, C. Janardhana, A Self-Organized Ensemble of Fluorescent 3-Hydroxyflavone-Al(III) Complex as Sensor for Fluoride and Acetate Ions. *J Fluoresc* **2007**, *17*, 1–5.
- ⁷⁹ A. M. Verdan, H. C. Wang, C. R. Garcia, W. P. Henry, J. L. Brumaghim, Iron Binding of 3-Hydroxychromone, 5-Hydroxychromone, and Sulfonated Morin: Implications for the Antioxidant Activity of Flavonols with Competing Metal Binding Sites. *J Inorg Biochem* **2011**, *105*, 1314–1322.
- ⁸⁰ V. A. Kostyuk, A. I. Potapovich, E. N. Vladyskovskaya, L. G. Korkina, I. B. A. Afanas'ev, Influence of Metal Ions on Flavonoid Protection against Asbestos-Induced Cell Injury. *Arch Biochem Biophys* **2001**, *385*, 129–137.
- ⁸¹ R. M. S. Pereira, N. E. D. Andrades, N. Paulino, A. C. H. F. Sawaya, M. N. Eberlin, M. C. Marcucci, G. M. Favero, E. M. Novak, S. P. Bydlowski, Synthesis and Characterization of a Metal Complex Containing Naringin and Cu, and its Antioxidant, Antimicrobial, Antiinflammatory and Tumor Cell Cytotoxicity. *Molecules* **2007**, *12*, 1352–1366.
- ⁸² M. Grazul, E. Budzisz, Biological Activity of Metal Ions Complexes of Chromones, Coumarins and Flavones. *Coord Chem Rev* **2009**, *253*, 2588–2598.
- ⁸³ L. H. Cazarolli, L. Zanatta, A. P. Jorge, E. de Sousa, H. Horst, V. M. Woehl, M. G. Pizzolatti, B. Szpoganicz, F. R. M. Barreto Silva, Follow-up Studies on Glycosylated Flavonoids and their Complexes with Vanadium: Their Anti-hyperglycemic Potential Role in Diabetes. *Chemico-Biol Interact* **2006**, *163*, 177–191.
- ⁸⁴ Beata Kosmider, Izabela Zawlik, Pawel P. Liberski, Regina Osiecka, Elzbieta Zyner, Justyn Ochocki, Jacek Bartkowiak, Evaluation of P53 and BAX Gene Expression and Induction of Apoptosis and Necrosis by the cis-

Pt(II) complex of 3-Aminoflavone in Comparison with *cis*-Diamminedichloroplatinum(II) (*cis*-DDP) in Human Lymphocytes. *Mutat Res* **2006**, *604*, 28–35.

⁸⁵ X. Chen, L. Tang, Y. Sun, P. Qiu, G. Liang, Syntheses, Characterization and Antitumor Activities of Transition Metal Complexes with Isoflavone. *J Inorg Biochem* **2010**, *104*, 379–384.

⁸⁶ J. Monserrat, K. N. Tiwari, L. Quentin, P. Pigeon, G. Jaouen, A. Vessièrès, G. G. Chabot, E. A. Hillard, Ferrocenyl Flavonoid-induced Morphological Modifications of Endothelial Cells and Cytotoxicity against B16 Murine Melanoma Cells. *J Organomet Chem* **2013**, DOI: 10.1016/j.jorganchem.2012.12.031.

⁸⁷ J. Ochocki, M. Kasprzak, L. Checinska, A. Erxleben, E. Zyner, L. Szmigiero, A. Garza-Ortiz, J. Reedijk, Synthesis, Single-Crystal and Solution Structure Analysis and *in vitro* Cytotoxic Activity of two Novel Complexes of Ruthenium(II) with *in situ* formed Flavanone-based Ligands. *Dalton Trans* **2010**, *39*, 9711–9718.

⁸⁸ R. Prajapati, S. Kumar Dubey, R. Gaur, R. Kumar Koiri, B. Kumar Maurya, S. Kumar Trigun, L. Mishra, Structural Characterization and Cytotoxicity Studies of Ruthenium(II)–DmsO–Chloro Complexes of Chalcone and Flavone Derivatives. *Polyhedron* **2010**, *29*, 1055–1061.

⁸⁹ W. Kandioller, C. G. Hartinger, A. A. Nazarov, J. Kasser, R. John, R.; M. A. Jakupec, V. B. Arion, P. J. Dyson, B. K. Keppler, Tuning the Anticancer Activity of Maltol-derived Ruthenium Complexes by Derivatization of the 3-Hydroxy-4-pyrone Moiety. *J Organomet Chem* **2009**, *694*, 922–929.

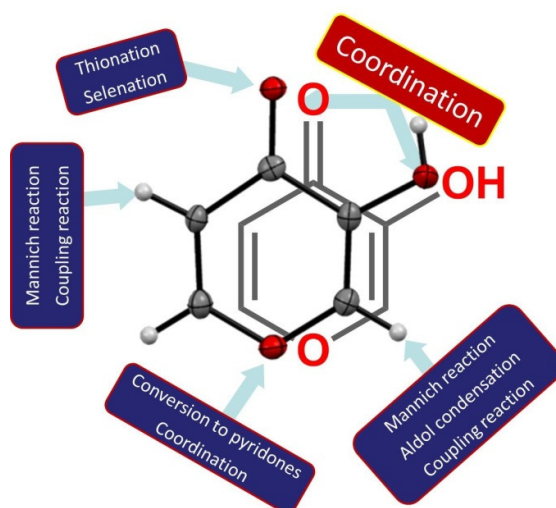
⁹⁰ W. Kandioller, C. G. Hartinger, A. A. Nazarov, C. Bartel, M. Skocic, M. A. Jakupec, V. B. Arion, B. K. Keppler, Maltol-Derived Ruthenium–Cymene Complexes with Tumor Inhibiting Properties: The Impact of Ligand–Metal Bond Stability on Anticancer Activity In Vitro. *Chem Eur J* **2009**, *15*, 12283–12291.

3. Published Results

3.1. Pyrone Derivatives and Metals: From Natural Products to Metal-based Drugs

Journal of Organometallic Chemistry **2011**, 696, 999–1010.

Graphical abstract



Recent literature on the use of (thio)pyr(id)ones in bioinorganic chemistry is summarized, highlighting in particular their ion metal chelating properties. Selected examples and different approaches using (thio)pyr(id)ones are presented and the influence of structural modifications on the chemical, physical and biological properties are discussed.



Review

Pyrone derivatives and metals: From natural products to metal-based drugs

Wolfgang Kandioller^{a,*}, Andrea Kurzwehnhart^a, Muhammad Hanif^a, Samuel M. Meier^{a,b},
Helena Henke^{a,b}, Bernhard K. Keppler^{a,b}, Christian G. Hartinger^{a,b,*}

^a University of Vienna, Institute of Inorganic Chemistry, Waehringer Str. 42, A-1090 Vienna, Austria

^b University of Vienna, Research Platform "Translational Cancer Therapy Research", Waehringer Str. 42, A-1090 Vienna, Austria

ARTICLE INFO

Article history:

Received 20 September 2010

Received in revised form

5 November 2010

Accepted 9 November 2010

Keywords:

Bioinorganic chemistry

Bioorganometallic chemistry

Medicinal chemistry

Metal complexes

(Thio)pyr(id)one ligands

ABSTRACT

Pyrone scaffolds are often present in natural products and many derivatives therefore exhibit favorable biocompatibility and toxicity profiles. Hydroxypyrones are obtained from natural sources or can be synthesized by different well established approaches and may easily be converted into the analogous thiopyrones and hydroxypyridones. These features make them well suited to drug development and other biological applications. Herein, we summarize recent literature on the use of (thio)pyr(id)ones in bioinorganic chemistry with a focus on their metal ion chelating properties. Selected examples and different approaches using (thio)pyr(id)ones are presented and the influence of structural modifications on their chemical, physical and biological properties are discussed.

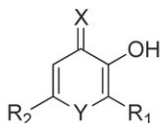
© 2010 Elsevier B.V. All rights reserved.

Contents

1. (Thio)pyr(id)ones – occurrence, synthetic approaches and biological activity	1000
1.1. Hydroxypyrones	1000
1.2. Hydroxypyridones	1001
1.3. Thio- and selenopyr(id)ones	1001
2. Coordination chemistry of pyr(id)ones and selected applications in medicinal inorganic chemistry	1001
2.1. Pyr(id)ones in the treatment of iron deficiency and overload	1002
2.2. Pyrone-based insulin mimetics	1002
2.3. Pyr(id)ones as chelators for Al ^{III} in Alzheimer's disease	1003
2.4. Pyrone complexes as diagnostic tools	1003
2.5. The anticancer drug candidate tris(maltolato)gallium(III)	1003
3. Pyr(id)one-based ruthenium and osmium complexes and their applications in bioinorganic chemistry	1004
3.1. Ru(III) coordination compounds	1004
3.2. Organometallic M ^{II} (arene) (M=Ru, Os) compounds	1004
3.2.1. Organometallic Ru(arene) complexes bearing pyrone-derived ligands and their potential as anticancer drugs	1004
3.2.2. From pyridone-based supramolecular biosensors to polynuclear anticancer agents	1006
4. Conclusions	1008
Acknowledgments	1008
References	1008

Abbreviations: cym, η^6 -*p*-cymene; DNA, 2'-deoxyribonucleic acid; en, 1,2-diaminoethane; 5'-GMP, guanosine 5'-monophosphate; mal, maltolato; pta, 1,3,5-triaza-7-phosphatricyclo[3.3.1]decane; ROS, reactive oxygen species.

* Corresponding authors. University of Vienna, Institute of Inorganic Chemistry, Waehringer Str. 42, A-1090 Vienna, Austria. Tel.: +43 1 4277 52609; fax: +43 1 4277 52680.
E-mail addresses: wolfgang.kandioller@univie.ac.at (W. Kandioller), christian.hartinger@univie.ac.at (C.G. Hartinger).



maltol: X = O, Y = O, R₁ = CH₃, R₂ = H
 ethylmaltol: X = O, Y = O, R₁ = C₂H₅, R₂ = H
 allomaltol: X = O, Y = O, R₁ = H, R₂ = CH₃
 pyromeconic acid: X = O, Y = O, R₁ = H, R₂ = H
 kojic acid: X = O, Y = O, R₁ = H, R₂ = CH₂OH
 thiomaltol: X = S, Y = O, R₁ = CH₃, R₂ = H
 thioethylmaltol: X = S, Y = O, R₁ = C₂H₅, R₂ = H
 thioallomaltol: X = S, Y = O, R₁ = H, R₂ = CH₃
 thiopyromeconic acid: X = S, Y = O, R₁ = H, R₂ = H
 deferiprone (*N*-methyl-2-methyl-3-hydroxy-4(1*H*)-pyridone): X = O, Y = NCH₃, R₁ = CH₃, R₂ = H

Fig. 1. Structures of common 3-hydroxy-4(1*H*)-(thio)pyr(id)ones.

1. (Thio)pyr(id)ones – occurrence, synthetic approaches and biological activity

1.1. Hydroxypyrones

Hydroxypyrones are natural products found in plants, but many of them can be synthesized and some are commercially available. Some important derivatives are listed in Fig. 1, including 3-hydroxy-2-methyl-4(1*H*)-pyrone (maltol). Maltol is one of the best studied compounds of this class and is known for its low toxicity and therefore favorable biocompatibility [1]. Maltol and its close analogue ethylmaltol are produced on a large scale and are used as food additives (E636 and E637, respectively) to impart the malty taste and aroma in bread, beer, cakes, etc. [2].

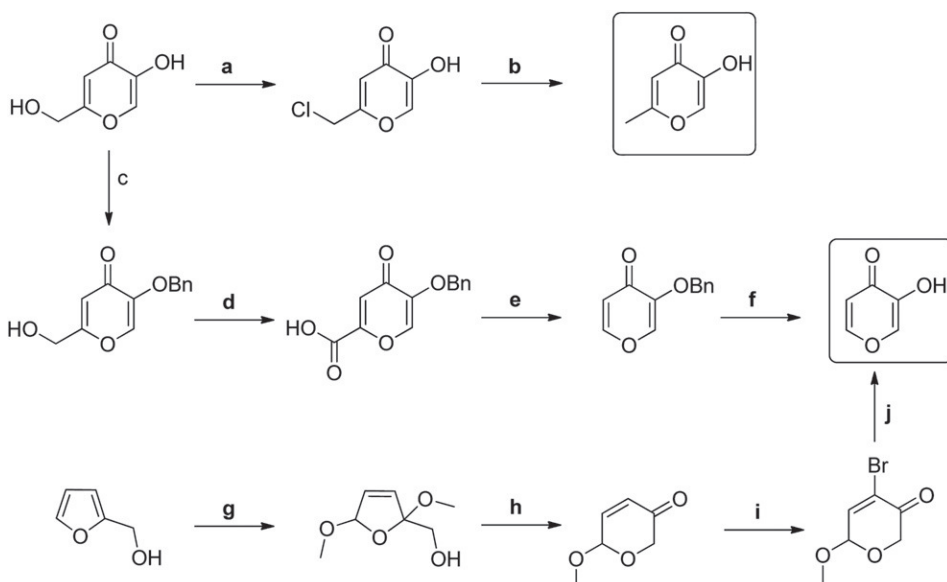
Maltol can be isolated from roasted malt, larch bark, and pine needles, by destructive distillation of carbohydrates and from various other sources, but there are also many ways of synthesizing pyrone derivatives. For example, maltol can be prepared from pyromeconic acid in a Mannich reaction by reaction with formaldehyde and piperidine and subsequent reduction using palladium on activated charcoal [3]. Another method was described by Torii

and co-workers, in which dimethoxylated furfuryl alcohol derivatives undergo ring expansion, epoxidation and finally an acid-catalyzed rearrangement [4]. The same approach is also applicable for the synthesis of pyromeconic acid and ethylmaltol. The latter can also be obtained synthetically by hydroxyethylation of comenic acid, reduction with stannous chloride in hydrochloric acid and subsequent decarboxylation [5].

Kojic acid (5-hydroxy-2-hydroxymethyl-4(1*H*)-pyrone) is a fungal metabolite, occurring during the fermentation of carbohydrates under aerobic conditions by *Aspergillus oryzae*. “Koji” is the Japanese word for steamed rice and kojic acid was first detected therein by Saito in 1907 [6]. Yabuta has investigated the influence of the substrate and of the fungi strain on the yield of the fermentation process [6,7]. Kojic acid is used as an additive in cosmetics to lighten skin color and is known to inhibit the copper-containing enzyme tyrosinase that causes melanization in humans [8,9].

The synthesis of allomaltol, 5-hydroxy-2-methyl-4(1*H*)-pyrone, was first described by Yabuta [10], a synthetic procedure that is still in use today due to its straightforward manner and its simplicity. In this procedure, kojic acid is reacted with thionylchloride, yielding chlorokojic acid and subsequent reduction with zinc in hydrochloric acid affords the desired product (Scheme 1). Although other synthetic pathways are described in literature, e.g., by Beelik and Purves [11], who obtained allomaltol *via* the reduction of dibenzyl kojic acid with zinc in glacial acetic acid, Yabuta’s method is still the most popular [12].

The preparation of pyromeconic acid, 3-hydroxy-4(1*H*)-pyrone, was first described by Ost in 1879, who obtained the compound by decarboxylation of meconic acid, 3-hydroxy-4(1*H*)-pyrone-2,6-dicarboxylic acid. Nowadays, there are two different synthetic approaches to access pyromeconic acid (Scheme 1). The first method was described by Tate and Miller [13] (improved by van der Helm and co-workers [14]), by oxidizing benzyl kojic acid with Jones reagent to yield benzyl comenic acid. Decarboxylation and deprotection afford pyromeconic acid. A second approach was published by Hider and co-workers [15], who reacted furfuryl alcohol with bromine in methanol to obtain an isomeric mixture of *cis/trans*-2,5-dihydro-2,5-dimethoxy-furanylmethanol. Treatment of the mixture with formic acid leads to ring expansion and formation of 6-methoxy-2*H*-pyran-3(6*H*)-one. Bromination in the



Scheme 1. Reaction pathways for the synthesis of allomaltol and pyromeconic acid. a) SOCl₂, b) Zn/HCl, c) BnBr, d) Jones reagent, e) *N*-methyl pyrrolidine/reflux, f) 4 M HCl, g) Br₂/MeOH, h) formic acid, i) Br₂/triethylamine and j) trifluoroacetic acid.

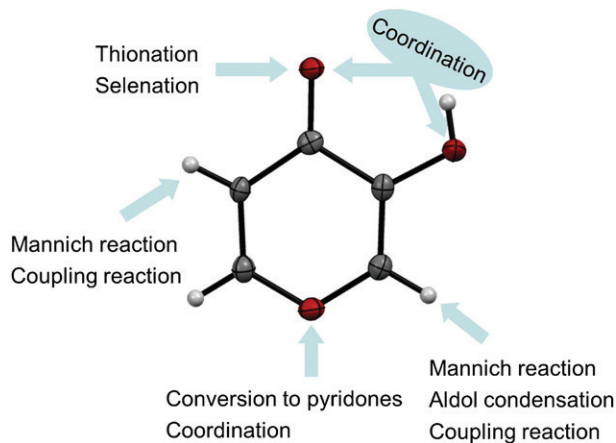


Fig. 2. Possible sites for derivatization of pyrones.

presence of trimethylamine yields the 4-bromo derivative, which is converted into pyromeconic acid in the presence of trifluoroacetic acid (Scheme 1).

3-Hydroxy-4(1H)-pyrones and their analogues are widely used as building blocks for biologically active compounds and many derivatives have been synthesized for numerous purposes and applications. The 6-membered oxygen-containing heterocycle offers possibilities of modification on different positions (Fig. 2).

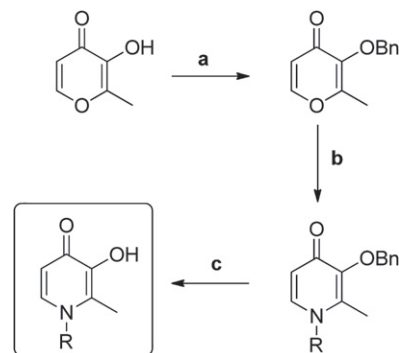
The most reactive position of 4-pyrones is position 2 (in the case of allomaltol position 6), which can undergo aldol-type condensations [15] and Mannich [16] reactions, due to the strong +M effect of the adjacent hydroxy group under alkaline conditions. The reaction with diacylperoxides or diaroylperoxides leads to insertion of alkyl or aromatic substituents [17]. Another approach to insert aromatic groups is the Suzuki reaction, where arylboronic acids are reacted in the presence of $\text{Pd}(\text{Ph})_4$ with 2-bromo-3-hydroxy-4(1H)-pyrones [18], which in turn can be prepared by bromination with bromine [19] or *N*-bromosuccinimide [20]. If position 2 of the pyrone moiety is occupied by an aliphatic residue, as in the case of maltol and ethylmaltol, then the next reactive place is position 5, which can be functionalized by a Mannich reaction [21]. Oxidation of the methyl substituent of maltol to a carboxylic acid offers another possibility to insert a new functional group into the molecule [22].

Kojic acid has the same backbone as the other pyrones, but features with its hydroxymethyl residue an additional site, which can be converted into different functional groups. For example, the aliphatic alcohol can be oxidized with Jones reagent to the carboxylic acid [23] or can be converted into chlorokojic acid by reaction with thionylchloride. Chlorokojic acid can be reacted with different nucleophiles, such as azides [24], amines [25,26], thiolates [27], phenolates, etc. [28,29]. Additionally, modifications on position 6 can be performed in the same way as described above by using aldol and Mannich reactions under the condition that positions 2 and 5 are already substituted [25,30,31].

Such modifications offer the opportunity to generate a broad library of pyrone-based substances with tunable biological properties. For example, Mannich products of kojic acid were found to exhibit anticonvulsant activity in maximal electroshock- and subcutaneous metrazol-induced seizure tests in male mice at non-toxic doses [32].

1.2. Hydroxypyridones

Hydroxypyrones can be used for the synthesis of the analogous hydroxypyridones, by reacting them with ammonia or primary



Scheme 2. The reaction pathway from maltol to hydroxypyridones. a) BnBr , b) $\text{H}_2\text{N}-\text{R}$, c) H_2 , Pd/C .

amines (including amino acids). The conversion of maltol with some aromatic amines can be done by heating both components in a sealed tube; however, the reaction is very slow and often results in low yields [33,34].

A more generally applicable approach was described by Harris et al. [35]. The protection of the hydroxyl group of hydroxypyrones via benzylation was shown to facilitate the conversion into the pyridone, with the pH value of the reaction (~ 12) being essential to obtain the desired product in high yield (Scheme 2). Cleavage of the benzyl group via hydrogenation affords the desired 4-pyridones [25,36]. These reactions can also be exploited for the synthesis of polypyridone species [37–40]. The most widely known pyridone is deferiprone (1,2-dimethyl-3-hydroxy-4(1H)-pyridone; Fig. 1), which is used for the treatment of iron overload (see Section 2.1) [41].

Both 3-hydroxy-4-pyridones and 3-hydroxy-2-pyridones can be easily *N*-functionalized, which provides the option for tuning physicochemical and biological properties, such as bioavailability after oral administration, lipophilicity, chelating ability, and metal-clearing efficiency from body tissues or organs. This feature resulted in the development of a variety of hydroxypyridone derivatives, namely bidentate [42–44], tetradentate [45,46], hexadentate [47] and octadentate [48] chelators and tethering bioactive or biological molecules including carbohydrates, amino acids, etc. [42].

1.3. Thio- and selenopyr(id)ones

Thiopyr(id)ones are a relatively new class of *S,O*-metal chelating agents, which are characterized by a higher affinity towards softer metal ions and enhanced stability of the formed complexes. The conversion of 3-hydroxy-4-pyr(id)ones to the corresponding thionated compounds may be achieved using different strategies and the most common are (i) the addition of 1 eq Lawesson's reagent [49] and (ii) the reaction with an excess of P_4S_{10} [50] under various conditions [51]. The reaction of two equivalents of Lawesson's reagent with 4-pyrones was reported to lead to the formation of dithiopyrones [52].

Recently, the synthesis of the selenium analogue of thiomaltol, although in low yield, was described by Tejchman and co-workers [53], by reacting maltol with P_4Se_{10} , generated *in situ* by the reaction of red phosphorus and grey selenium. Until now, no biological data for selenomaltol or derivatives are available and the coordination chemistry remains to be investigated.

2. Coordination chemistry of pyr(id)ones and selected applications in medicinal inorganic chemistry

3-Hydroxy-*n*-pyr(id)ones ($n=2, 4$) are widely used as *O,O*-chelating ligands due to their high affinity towards metal ions and

the stability of the formed complexes. The metal ion is embedded in a 5-membered ring structure, known for its thermodynamic stability at physiological pH values [54]. The ability to act as a bidentate ligand can be explained by the zwitterionic pseudo-aromatic character of the heterocyclic system (Fig. 3).

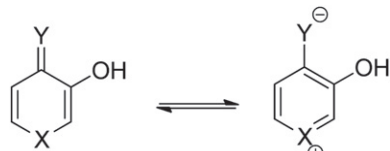


Fig. 3. Mesomeric forms of 3-hydroxy-4-(thio)pyr(id)ones, where X = N–R, O and Y = O, S.

Pyr(id)one-derived metal complexes have been described in literature over the last decades [55]. In general, pyridones form more stable complexes than the analogous pyrones (Table 1), and therefore they are used to remove metal ions, e.g., to restore iron overload disorders (see Section 2.1). On the other hand pyrone-based complexes can act as a metal ion source, due to their weaker bidentate binding, and have potential for the delivery and release of metals, fine-tunable by the substitution pattern of the pyrone backbone. The order of stability for complexes of different hydroxy-pyridonates is the following: 3-hydroxy-4-pyridonates > 3-hydroxy-2-pyridonates > 1-hydroxy-2-pyridonates [36,56,57].

New areas of applications were reported for (thio)pyr(id)ones over the last years, most of which are related to their metal ion coordinating properties. For example, kojic acid-based fluorescent probes specific for Fe^{II} were reported by Hider and co-workers [63]. The same group also studied the antimalarial activity of pyridones at non-toxic levels in mammalian cells [25]. Furthermore, macrocyclic chelators, obtained by linking several pyridone moieties, were found capable of acting as hosts for medically relevant metal ions (Ga^{III} , Fe^{III} , In^{III} , Cu^{II}) [64]. Coordination compounds of thiopyr(id)ones with Ga^{III} and In^{III} have potential in SPECT or PET imaging [50]. Zn^{II} and $\text{V}^{\text{IV}}\text{O}_2^{2+}$ derivatives showed encouraging *in vitro* results as anti-diabetic agents [65], and $\text{Mo}^{\text{VI}}\text{O}_2^{2+}$ complexes inhibit xanthine oxidase [49]. Cohen and co-workers found that (thio)pyr(id)ones have potential as inhibitors of matrix metalloproteins due to their ability to chelate zinc ions and they are considered alternatives to hydroxamic acids and derivatives [66–68]. Recently, maltol was reported to increase the solubility of platinum-based anticancer drugs upon coordination to the cisplatin $\text{Pt}(\text{NH}_3)_2$ fragment (Fig. 4) which did not alter the DNA damaging properties of the platinum complex compared to cisplatin. Similar sequence specificity and intensity of damage were found for the diammine(mal)platinum(II) (mal = maltolato) complex, which makes it potentially useful in cancer treatment [69]. Accordingly, (thio)pyr(id)ones and their complexes can be applied for a wide variety of purposes, some of which are discussed more in detail in the next sections.

Table 1
Stability constants (log β) of metal complexes based on maltol and deferiprone.

Metal ion	Log β	
	Maltol	Deferiprone
Fe^{III}	15.18 [58]	35.92 [59]
Al^{III}	12.66 [58]	32.62 [59]
$\text{V}^{\text{IV}}\text{O}_2^{2+}$	16.31 [60]	22.25 [61]
Ga^{III}	–	35.76 [59]
$\text{Ru}^{\text{II}}(\text{cym})$	9.05 [62]	11.86 [62]

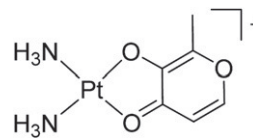


Fig. 4. Structure of a diammine(maltolato)platinum(II) complex with anticancer activity.

2.1. Pyr(id)ones in the treatment of iron deficiency and overload

Iron is an essential trace element, however, both overload and deficiency cause problems to living organisms and therefore iron levels are carefully controlled in cells (“iron homeostasis”) [70]. 3-Hydroxy-4-pyridones exhibit strong affinity towards iron [71] and can therefore be used as regulating agents for iron overload disorders. Fine control on hydrophilic/lipophilic balance can be achieved by simply introducing appropriate substituents on the endocyclic nitrogen atom of the pyridone ring without significantly changing its chelating properties [72]. Deferiprone (1,2-dimethyl-3-hydroxy-4 (1H)-pyridone; Fig. 1) was synthesized by Kontoghiorghe et al. in the early 1980ies [73] and is clinically applied in Europe since 1999 [41]. The bidentate ligand forms very selectively 3:1 chelate complexes with iron and it was found to be relatively non-toxic even at high doses (200 mg/kg) [74]. Deferiprone was the first drug for the treatment of iron overload to be administered orally. One major disadvantage of deferiprone is the fast inactivation by phase II metabolism (glucuronidation) [75]. Around 85% of the administered dose was detected in the urine converted into its O-glucuronide. These insights into the deferiprone metabolism led to the synthesis of novel pyridones, which do not undergo this type of inactivation [15].

On the other hand, many people suffer from iron deficiency and require iron supplements. The major problem for the administration of iron is that Fe^{III} (the form bound to transferrin) is potentially toxic by generating reactive oxygen species (ROS) at 5-fold higher concentrations than normal in human tissue [76]. Furthermore, Fe^{III} undergoes hydrolysis and precipitation under physiological conditions (pH ~ 7), whereas administration of Fe^{II} requires high doses to obtain relevant therapeutic effects, due to the fast oxidation [77]. Iron (III) complexes based on maltol (Fig. 5) and ethylmaltol have a series of advantages compared to administration as iron salts. These comprise higher intermediate stability, which inhibits immediate hydrolysis and the precipitation of iron; the complexes are neutral which allows passive diffusion through lipophilic membranes; and the pyrone ligands themselves are not toxic [78]. It has been shown that the *in vivo* bioavailability of pyrone-based iron(III) compounds is significantly higher compared to FeSO_4 , which allows administration of lower doses to maintain the same iron level [79].

2.2. Pyrone-based insulin mimetics

Vanadium compounds are known to have insulin enhancing properties, which can be used to treat type-2 diabetes as shown in

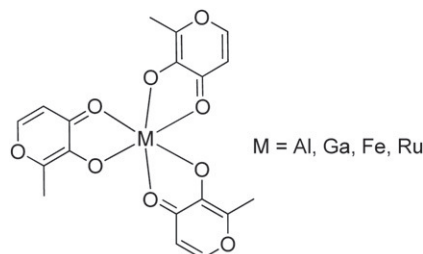


Fig. 5. General formula for Al^{III} , Ga^{III} , Fe^{III} and Ru^{III} maltolato complexes.

vitro [80] and *in vivo* [81], and [bis(maltolato)oxovanadium(IV)] (BMOV; Fig. 6) and its ethylmaltol analogue (BEOV) have been developed to clinical phase II trials [82]. Structural studies suggest that the complexes with maltolato ligands favor *cis*-configuration, as shown by EPR and NMR experiments [83].

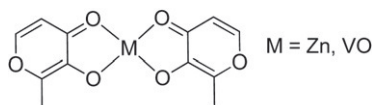


Fig. 6. Zn^{II} and vanadyl complexes exhibiting insulin-mimetic properties.

The mechanism of action appears to be related to the ability of vanadium ions to mimic insulin in the oxidation states +4 and +5 and was recognized already in the 1980ies for sodium and ammonium vanadate, but the bioavailability of these inorganic salts was found to be insufficient. Therefore, the focus shifted to coordination compounds of V^{IV}O²⁺ with the aim to increase the uptake, so that lower doses of vanadium can be applied without loss of efficacy. The therapeutic potential of BMOV and BEOV was found to be significantly higher and they passed clinical phase I and IIa trials with very promising results [84].

In addition to the well investigated pyronato complexes, a series of studies on analogous (thio)pyridones as ligands for V^{IV}O²⁺ were reported and are summarized in recent review papers by Sakurai and Kiss [85,86]. In addition, also other metal complexes of maltol with Mo^{VI}O₂²⁺ [87], Cu^{II} and Co^{II} [88] have been evaluated on their insulin mimicking properties, but were found less effective than BMOV and BEOV. Another promising metal ion for the treatment of diabetes type-2 is Zn^{II}. (Thio)pyr(id)onato complexes were investigated for their insulin mimicking potential *in vivo* [89] and in particular thiopyronato ligands led to increased activity [60,87].

2.3. Pyr(id)ones as chelators for Al^{III} in Alzheimer's disease

Although aluminum is one of the most common elements in the Earth's crust, it has no essential function in living organisms. High levels of aluminum have been discussed for several decades to be a health risk and especially accumulation in the brain is supposed to be responsible for the development of Alzheimer's disease [90,91]. The coordination chemistry of Fe^{III} and Al^{III} has many similarities and as a consequence, the influence of maltol on the aluminum level has been investigated. Unfortunately, the formed [tris(maltolato)-aluminum(III)] (see Fig. 5) was found to be neurotoxic [92]. However, this undesired effect led to the development of an animal

model for Al neurotoxicity by the application of 2-alkylpyronato complexes of aluminum. Current research is focused on mobilization of Al^{III} by applying 3-hydroxypyridones as stronger complexation agents instead of the analogous pyrones [59,92].

2.4. Pyrone complexes as diagnostic tools

MRI is a powerful tool in medicinal diagnostics and contrast agents are used to optimize imaging in approximately 30% of all medical examinations involving MRI. These agents are based on paramagnetic metal ions, normally Gd^{III}. The high toxicity of Gd^{III} has been overcome by binding to multi-dentate ligand systems, such as DOTA (1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid) or DTPA (diethylenetriaminepentaacetic acid), leaving at least one coordination site available for an aqua ligand, being crucial for contrast enhancement [93,94]. One approach to improve the contrast of the images is to use compounds with a higher number of coordination sites available for occupation by aqua ligands. This should provide complexes with high relaxivity without compromising stability. Based on this guiding principle, Raymond et al. have designed a library of trisbidentate hydroxypyridonato-based Gd^{III} complexes as contrast agents (for an example see Fig. 7 and for reviews see Refs. [95,96]) with two of the eight coordination sites of Gd^{III} available for coordination of aqua ligands [97,98].

The Gd complex of tris[(3-hydroxy-1-methyl-2(1*H*)-pyridone-4-carboxamido)ethyl]amine was among the first pyridone-containing coordination compounds evaluated as MRI contrast agents. This novel Gd complex was superior to commercial contrast agents in clinical use, both in terms of stability and relaxivity [99]. In order to optimize these parameters, the linker was varied [97] and the coordinating pyridonates were partly replaced with terephthalamide [100]. Compounds with two [101] or three [97] water molecules in the inner coordination sphere proved successful in *in vivo* assays [102].

2.5. The anticancer drug candidate tris(maltolato)gallium(III)

Gallium-67 citrate was used as an imaging agent for soft tumor tissue [103], and GaCl₃ and Ga(NO₃)₃ were found to possess anti-neoplastic properties. The coordination chemistry of Ga^{III} is similar to Fe^{III} and it is supposed to follow the same biochemical transport pathways [104,105]. The most significant difference between these metal ions is the redox inertness of Ga^{III}, which prevents the metal of participating in biologically important redox processes. The iron-containing enzyme ribonucleotide reductase was suggested as the

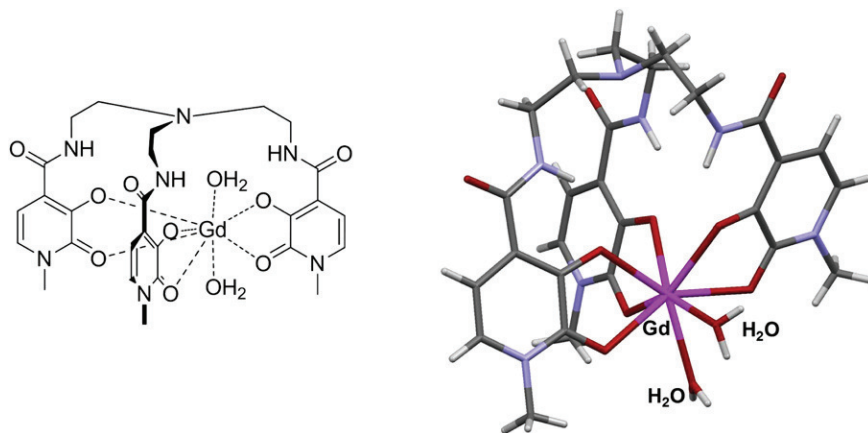


Fig. 7. Structures of a Gd complex with promising properties for application as MRI contrast agent (adapted from Ref. [99]).

molecular target, which catalyzes the reduction of ribonucleotides. The metal ion binds to the iron site, leading to the destabilization of the tyrosyl radical, which is essential for the enzyme activity [106].

Gallium nitrate was approved for the treatment of cancer-related hypercalcaemia and showed activity against lymphoma and bladder cancer in clinical phase II trials, but low bioavailability and severe side effects, such as nephrotoxicity and optical neurotoxicity, were observed [107]. As a consequence, the focus shifted towards coordination compounds to stabilize gallium against hydrolysis and to improve bioavailability by preparing neutral complexes. These efforts resulted in clinical trials on the 8-oxyquinolinato and maltolato compounds KP46 and gallium maltolate, *i.e.*, [tris(maltolato)gallium(III)] (Fig. 5), respectively. The latter pyrone compound can be administered orally and displays higher bioavailability than the earlier mentioned gallium salts, which is related to the lipophilicity of the neutral complex [108]. The compound was well tolerated in a phase I trial [108], however, clinical phase I/II trials were terminated [109] and to the best of our knowledge no results were reported so far. Also the question of the molecular target is yet to be answered [104], though, as for Ga^{III} salts, inhibition of ribonucleotide reductase has been discussed.

3. Pyr(id)one-based ruthenium and osmium complexes and their applications in bioinorganic chemistry

3.1. $\text{Ru}(\text{III})$ coordination compounds

Platinum-based anticancer agents have disadvantages that can potentially be overcome by replacing the platinum ion by other metal centers. The development of novel chemotherapeutics with different activity profiles, modes of action, lower toxicity and higher selectivity than observed for platinum compounds is essential to improve the treatment of cancer. Ruthenium complexes are promising alternatives and their tumor-inhibiting potential has been found more than three decades ago [110]. The activation by reduction hypothesis states that the active species of $\text{Ru}(\text{III})$ complexes (such as of clinically tested KP1019 and NAMI-A [111–113]) are the corresponding $\text{Ru}(\text{II})$ compounds. This activation step seems to be important for the selectivity towards tumorigenic tissue [114], as is the transport by proteins [115–117].

There are not many pyr(id)onato $\text{Ru}(\text{III})$ complexes with anticancer activity known [118–122]. James and co-workers synthesized tris[pyr(id)onato]ruthenium(III) compounds and the pyronato species with mal and ethylmaltolato ligands (for the structure of the mal compound, see Fig. 5) exhibited only low cytotoxicity in the human cancer cell line MDA-MB-435S (IC_{50} values of 140 and 90 μM , respectively), whereas the pyridonato analogues were not tested on their biological activity [119]. The slightly higher activity of the ethylmaltolato complex can be explained by its higher cellular accumulation [119]. In addition, bis (pyronato) compounds were prepared featuring both Ru^{II} and Ru^{III} centers. The remaining coordination sites were occupied by azole, sulfoxides or ethanol but the complexes were not significantly more active than their tris(pyronato) counterparts in the same cell line [121,122]. In an attempt to couple bioactive moieties to a Ru^{II} center, Prajapati and colleagues reported the antitumor activity of a flavone complex with high antiproliferative activity in Dalton lymphoma cells [120].

3.2. Organometallic $\text{M}^{\text{II}}(\text{arene})$ ($\text{M} = \text{Ru}, \text{Os}$) compounds

In recent years, organometallic Ru^{II} compounds, often developed for catalytic purposes, have attracted the interest of researchers (for examples see Fig. 8) [123,124]. Such compounds were shown to be reasonably stable for application as drug

compounds, stabilization that is achieved by η^6 -coordination of aromatic moieties. Furthermore, the arene moiety has important effects on the physicochemical and biological properties of the Ru complexes [125]. A multitude of derivatizations at the arene moiety and coordination of various mono- or bidentate ligands to Ru^{II} and Os^{II} are feasible and can be used to attach pharmacophores, targeting moieties, fluorophores, *etc.* (for recent examples see Refs. [123,124,126–131]). Most often leaving groups, such as halides or dicarboxylates, are coordinated to the ruthenium center, facilitating aquation and therefore reaction to biological targets, such as proteins and DNA, though also inert complexes with anticancer properties have been reported.

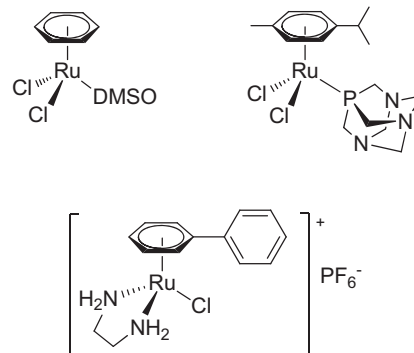


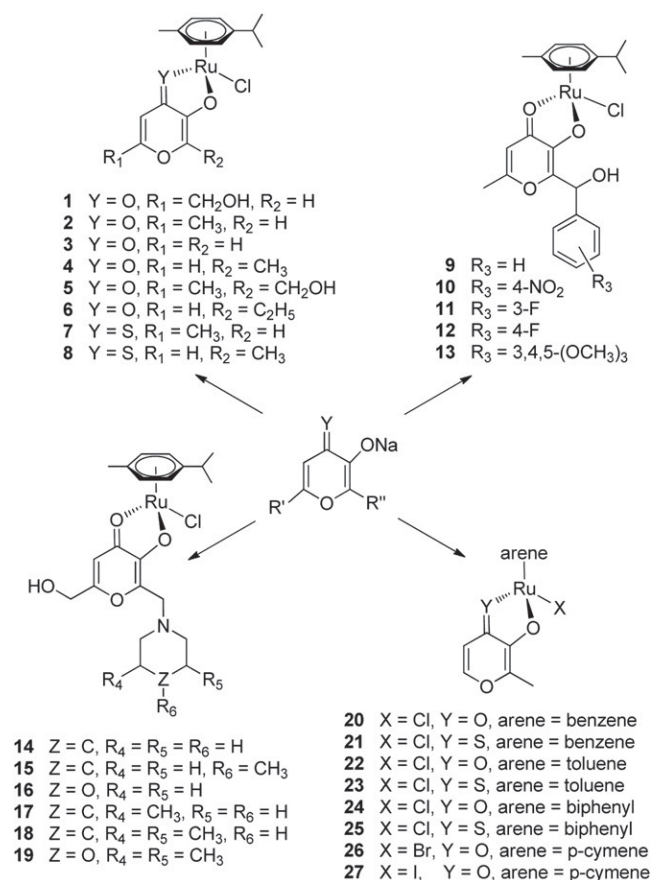
Fig. 8. Structures of mononuclear Ru^{II} complexes with tumor-inhibiting properties.

The first organometallic ruthenium compounds were developed more than twenty years ago and one of the first reported complexes was the topoisomerase II inhibitor $[\text{Ru}(\eta^6\text{-C}_6\text{H}_6)(\text{DMSO})\text{Cl}_2]$ (Fig. 8) [132]. However, $\text{Ru}(\text{arene})$ complexes bearing pta (1,3,5-triaza-7-phosphatricyclo[3.3.1]decane, “RAPTA”) or en (1,2-diaminoethane) ligands are the best studied representatives (Fig. 8) [133–135]. Dyson et al. have shown that the pta ligand leads to largely increased aqueous solubility, and antimetastatic activity with high selectivity next to low general toxicity, whereas methylation of one of the pta nitrogen atoms (ptn) leads to a slight increase in cytotoxicity [136]. The replacement of the chlorido ligands by oxalate or cyclobutane-1,1-dicarboxylate gives analogues of oxali- and carboplatin, with only minor influence on the biological activity compared to the parent compounds [137].

$[\text{Ru}(\eta^6\text{-arene})(\text{en})\text{X}]$ (arene = benzene or derivatives of benzene, en = 1,2-diaminoethane or derivatives, X = halide) complexes have been developed by Sadler et al. [138]. For this compound class, a strong influence of the arene ligands on the cytotoxic activity against the human ovarian cancer cells A2780 was shown with the benzene derivative exhibiting the lowest and tetrahydroanthracene the highest activity [138]. The type of chelating ligand plays an important role and has a strong influence on the chemical, physical and biological properties of Ru^{II} compounds. The exchange of ethylenediamine by acetylacetonate (acac) leads to neutral complexes (Fig. 8) with increased electron density at the Ru^{II} center, which facilitates ligand exchange and hydrolysis processes [139]. Furthermore, $[\text{Ru}(\text{cym})(\text{acac})\text{Cl}]$ shows higher affinity towards adenine than to guanine, whereas the en analogue prefers binding to guanine with least affinity to adenine.

3.2.1. Organometallic $\text{Ru}(\text{arene})$ complexes bearing pyrone-derived ligands and their potential as anticancer drugs

With the aim to develop $\text{M}(\text{arene})(\text{maltolato})$ complexes as anticancer agents, Sadler and co-workers compared the hydrolysis behavior of $\text{Ru}^{\text{II}}(\text{cym})$ (4 in Scheme 3) and $\text{Os}^{\text{II}}(\text{cym})$ compounds bearing a maltolato ligand to that of the respective en complexes.



Scheme 3. The complexation of hydroxy(thio)pyrones to Ru^{II} centers is achieved by reacting the deprotonated (thio)pyronate with various Ru^{II}(arene) dimers.

Table 2

Trend of dimer formation, and pK_a and IC₅₀ values of pyrone-based Ru^{II}(arene) complexes.

	Dimer formation	pK _a	IC ₅₀ [μM]			Ref.
			CH1	SW480	A549	
1	+	8.93	234 ± 21	429 ± 10	n.d.	[141]
2	+	9.01	239 ± 22	359 ± 119	518 ± 65	
3	+	8.91	112 ± 50	206 ± 94	490 ± 43	[143]
4	+	9.23 ^c	81 ± 14	159 ± 41	482 ± 20	
5	–	8.92	242 ± 39	457 ± 33	510 ± 29	[141]
6	–	9.12	81 ± 8	165 ± 31	389 ± 37	
7	–	>10 (12.8) ^d	35 ± 8	20 ± 7	n.d.	[143]
8	–	>10 (12.5) ^d	13 ± 4	5.1 ± 0.5	n.d.	
9	–	9.05	50 ± 9	67 ± 10	172 ± 5	[142]
10 ^a	–	9.80	–	–	–	
11	–	8.99	24 ± 4	44 ± 10	98 ± 4	
12	–	9.56	29 ± 2	57 ± 8	138 ± 6	
13	–	9.64	48 ± 6	84 ± 7	220 ± 14	
14–19 ^b	++	–	–	–	–	[144]
20	–	8.30	134 ± 19	445 ± 33	495 ± 33	[145]
21	–	–	4.0 ± 0.3	4.3 ± 1.0	–	
22	–	8.82	103 ± 12	276 ± 30	465 ± 18	
23	–	–	2.6 ± 0.3	2.7 ± 0.5	13 ± 3	
24	–	8.35	40 ± 1	66 ± 10	204 ± 7	
25	–	–	12 ± 5	14 ± 0.2	–	
26	–	– ^d	130 ± 2	298 ± 68	459 ± 16	
27	–	– ^d	68 ± 24	173 ± 49	426 ± 11	

Degrees of dimer formation after 18 h: –, 0–5% dimer formation observed; +, 5–10% dimer; ++, >10% dimer.

^a pK_a was determined in 10% DMSO due to low aqueous solubility.

^b Too low stability to warrant biological tests.

^c From Ref. [140].

^d Obtained by DFT calculations.

The aquation of both complexes, i.e., replacement of the chlorido by an aqua ligand, was too fast to be followed by NMR spectroscopy yielding aqua species with pK_a values of 9.23 and 7.60, respectively. Over time the complexes tend to lose the maltolato ligand and form to some extent, in dependence of concentration, reaction time and pH, very stable dimeric complexes with the general formula [M₂(cym)₂(OH)₃]⁺ (M = Ru, Os). Biological assays against human cancer cells *in vitro* revealed minor cytotoxicity of **4** which was correlated to the dimer formation (Table 2) [140].

In an attempt to improve the stability of Ru(arene)(pyronato) complexes, a series of complexes with modified pyronato ligands was reported (Scheme 3) [141–145]. The complexes were characterized with regard to their stability in aqueous solution. They undergo a rapid ligand exchange reaction to charged aqua compounds [Ru(cym)(L)(H₂O)]⁺, which is often followed by the formation of the dimeric side product (Fig. 9), also observed by others [140,141,143,144]. However, some of the complexes, such as

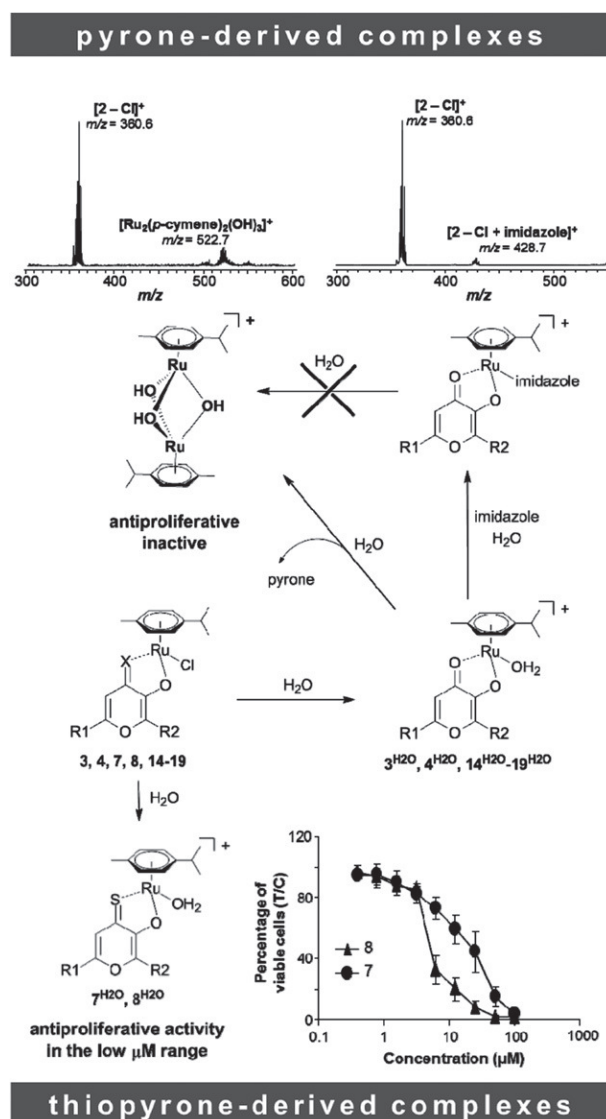


Fig. 9. Ru(cym)(pyronato) complexes hydrolyze in aqueous solution via [Ru(cym)(pyronato)(H₂O)]⁺ eventually to [Ru₂(cym)₂(OH)₃]⁺. The latter step can be prevented by addition of imidazole to replace the aqua ligand of the intermediates. In the case of thiopyronato compounds **7** and **8** no ligand cleavage and significant cytotoxic activity in human cancer cell lines was observed. Adapted from Ref. [143] with permission of the American Chemical Society.

those bearing ethylmaltol **6** and 2-hydroxymethyl-3-oxo-6-methyl-4(1*H*)-pyronato ligands **5** and **9–13** (see below), are more stable and no dimer formation was observed [141,142]. The same result was obtained when replacing the chlorido ligands by an imidazole molecule to form the corresponding positively charged compounds (stable in solution for 18 h; Fig. 9) [141,143,144]. The pK_a values (8.30–9.64) implicate that the complexes are predominantly present as reactive aqua species under physiological conditions. None of the $Ru^{II}(cym)(pyronato)$ complexes **1–6** are significantly active in *in vitro* anticancer assays, though **5** and **6** are not prone to dimer formation. These data suggest that the formation of dinuclear, hydroxido-bridged Ru compounds is at least not solely responsible for the limited anticancer activity.

In another approach, allomaltol was reacted with substituted benzaldehydes leading to the pyronato complexes **9–13** bearing an additional hydroxyl-methyl-aryl moiety in position 2 of the pyrone ring. The aryl substitution pattern was shown to dictate the *in vitro* anticancer activity of the respective $Ru(cym)$ complexes and also their aqueous solubility. In comparison to the unsubstituted pyrone complexes, significantly improved *in vitro* anticancer activity was determined in ovarian, colon and lung carcinoma cells (Table 2). Ovarian cancer CH1 cells were found to be most sensitive to this group of pyrone-derived $Ru^{II}(cym)$ complexes and compound **12** with the fluoro substituent in *para*-position of the aryl part was the most active representative. In general, compounds with electron withdrawing substituents at the aryl moiety exhibited higher cytotoxic activity than unsubstituted compounds or those bearing electron-donating moieties. The increased cytotoxic activity of the $Ru^{II}(cym)$ complexes with pyronato ligands bearing an additional hydroxyl-methyl-aryl moiety seems to be related to increased lipophilicity and therefore probably improved cellular uptake on the expense of aqueous solubility [142].

All the $Ru(arene)(pyronato)(halido)$ complexes hydrolyze to the respective aqua species under physiological conditions (Table 2). The aqua ligand can be rapidly replaced by biological targets and test reactions with small biomolecules, such as the DNA model 5'-GMP, were conducted [141–143,145]. For the latter selective binding to the N7 of guanine was shown and such adducts are stable in solution for more than 18 h, making DNA a possible, though probably not the exclusive, target for these and related organometallic Ru^{II} compounds [146–152]. Incubation with amino acids showed that Cys leads to fast decomposition of all examined complexes, whereas the reaction with Met and His leads to the replacement of the aqua ligand with the amino acid. In case of His, the ruthenium center was found coordinated to both the N1 and N3 atoms of the imidazole moiety. In both cases the pyronato ligand is cleaved within 18 h. The reaction with Gly is significantly slower, yet leads to stable chelate complexes, where Gly is bound *via* the amino and carboxylic group to the ruthenium center. Notably, the formation of the dimeric hydrolysis side products is prevented through coordination to amino acids. This is another indication that dimerization upon aquation may not be the only reason for the *in vitro* inactivity of the maltol- and the related pyrone-derived complexes [140,141].

In order to improve the solubility of the compound class, kojic acid was reacted with formaldehyde and piperidine derivatives, leading to ligands with additional hydroxy and amine functionalities, and the respective $Ru^{II}(cym)$ complexes **14–19** with higher hydrophilicity. However, the low stability of the complexes in aqueous solution did not warrant further development as anticancer agents [144].

Besides the introduction of substituents at the pyrone scaffold, the replacement of the carbonyl oxygen by a sulfur atom resulted in improved stability of the Ru complexes **7, 8, 21, 23** and **25** [143]. In addition, such substitution yields more lipophilic complexes,

a property that often facilitates intracellular accumulation [153]. In contrast to the $Ru^{II}(cym)$ complexes of maltol **4** and allomaltol **2**, *in vitro* anticancer activity assays revealed potent growth inhibition for their thiopyrone analogues **7** and **8**, respectively [143]. Notably, the improved anticancer activity was also accompanied by a reversal in sensitivity from CH1 ovarian cancer to SW480 colon carcinoma cells. It is obvious that the substitution pattern of the pyronato ligand has an influence on the activity, with the thiomaltolato complex **8** being ~3–4 times more active than the thioallomaltolato analogue **7** (Table 2). The aquation of the thio complexes results in the formation of aqua complexes with pK_a values of 12.8 for **7** and 12.5 for **8**, but does not proceed to $[Ru_2(cym)_2(\mu-OH)_3]^+$. DFT calculations revealed that the $Ru-S_{C=S}$ bond energies of the used model complexes are by 7.8–8.0 kcal/mol higher than the $Ru-O_{C=O}$ bond energies. Thus the stronger binding of thiopyrones to the ruthenium center explains the higher stability in aqueous solution compared to the pyrone analogues [143]. As observed for their pyrone counterparts, the complexes react rapidly with 5'-GMP to form monoadducts. However, they show a significantly different behavior in presence of the amino acids Met, His and Gly, forming stable complexes with Met and His, and the thiopyronato ligand remaining bound to the Ru center, whereas with Gly no adducts are formed. This demonstrates again the higher stability of the thiopyronato complexes which might allow them to enter cells to a higher degree in their unmodified form [141].

In order to extend the structure-activity relationships, the influence of the arene ligand and of the leaving group on the stability in aqueous solution, the reactivity with biomolecules and the cytotoxicity against cancer cells were investigated. $Ru^{II}(\eta^6\text{-arene})$ complexes of maltol and thiomaltol **20–25** were synthesized with different arenes such as benzene, toluene, *p*-cymene and biphenyl [145]. The complexes behave similarly to their cym analogues in aqueous solution and form predominantly $[Ru(\eta^6\text{-arene})(L)(H_2O)]^+$ as aquation products, which are stable for more than 48 h without formation of dimeric side products. The different arenes have an impact on the pK_a values of the Ru-maltolato complexes (benzene **20** < biphenyl **24** < toluene **22** < cym **4**) [145]. The compounds with the *S,O*-chelating thiomaltolato ligand exhibit promising cytotoxic activity with IC_{50} values in the low μM range (Table 2), whereas the corresponding maltolato compounds were not active. In contrast to the arene ligands, the influence of the leaving group on the tumor-inhibiting properties is only minor. By switching from the chloride (**4**) leaving group to bromide (**26**) and iodide (**27**) the solubility decreased, without significantly improving their *in vitro* anticancer activity. However, this result was expectable since the leaving group only influences the hydrolysis kinetics but the compounds form identical hydrolysis products [145].

3.2.2. From pyridone-based supramolecular biosensors to polynuclear anticancer agents

The stability constants of complexes formed with pyridones are in general higher than for pyrones (Table 1) and therefore they offer advantages over their pyrone analogues for drug development. In addition, the nitrogen-containing heterocycle can be further functionalized. Severin and co-workers exploited the tendency to form coordination compounds and were the first to synthesize $Ru^{II}(\text{arene})$ complexes with 4-pyridonato ligands [154] yielding triangular supramolecular metallocycles [155]. A similar self-assembly was observed for 2-pyridones featuring the structural motif of 12-crown-3 with selectivity for lithium ions (Fig. 10) [156], even in the presence of a large excess of other metal ions [157]. These trinuclear complexes equipped with fluorescent moieties at the 2-pyridone are capable of detecting Li^+ at physiologically relevant

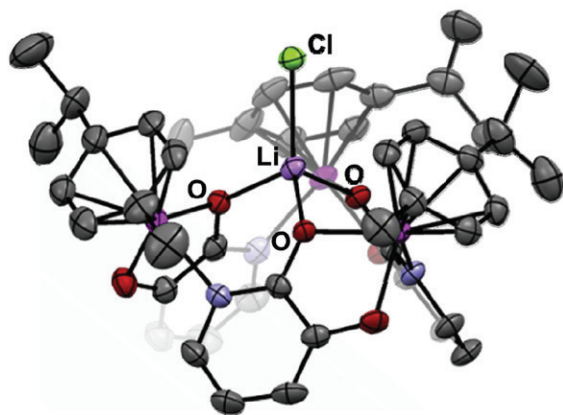


Fig. 10. Molecular structure of a trinuclear macrocycle with encapsulated Li^+ ; adapted from Ref. [156].

concentrations by fluorescence spectroscopy in water and human blood serum [158]. Therefore, they can be used as Li^+ sensors, being of medicinal relevance for the control of plasma levels because of toxic side effects occurring during treatment of, e.g., bipolar disorders.

Recently, larger structures featuring hexanuclear macrocyclic cylinders were obtained by self-assembly of bis- or trispyridonates and Ru^{II} (arenes) [159]. These cylinders have a length of ~ 3 nm and were able to encapsulate acetate and phosphate anions [160]. The synthesis of large molecular cages was recently described by connecting free aldehyde functionalities of macrocyclic triangles with triamines [161], which can be possibly used as carriers for encapsulated compounds, as small reactors for chemical reactions or they can stabilize highly reactive guest molecules [162].

Besides the applicability as biosensors, such self-assembling metallamacrocycles were also investigated on their potential as anticancer agents [163]. Interestingly, such compounds can be switched reversibly from mononuclear ($\text{pH} < 7$) to trinuclear complexes ($\text{pH} > 8.5$) by changing the pH value in aqueous solutions (Fig. 11). This observation can be explained by elevated pH causing deprotonation of the pyridone nitrogen, which allows the pyridone to act as bridging ligand to another ruthenium center to form eventually a trinuclear metallacycle (Fig. 11). Administration of the trinuclear metallamacrocycles and subsequent activation by the lower pH in the tumor tissue was thought to be a promising targeting strategy, but the compounds are inactive in *in vitro* anticancer assays [163]. However, as seen in the development of NAMI-A and RAPTA *in vitro* activity or inactivity is not necessarily an indicator for a compound to exhibit tumor-inhibiting properties *in vivo* or in clinical trials [164,165].

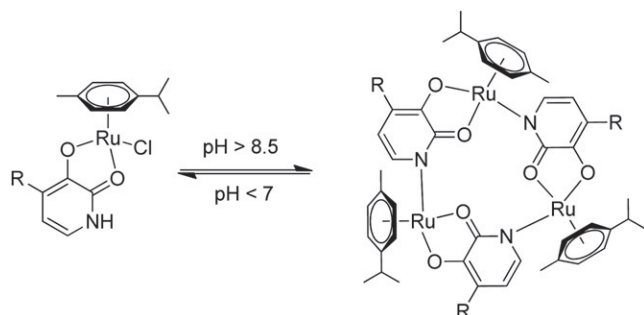


Fig. 11. pH induced self-assembly of a $\text{Ru}(\text{cym})(\text{pyridonato})$ complex to a trimeric structure [163].

By switching from pyridones to *N*-functionalized derivatives, a series of ruthenium and osmium compounds bearing *O,O*-coordinated alkoxy-carbonylmethyl-3-hydroxy-2(1*H*)-pyridones was reported (Fig. 12) [130]. In contrast to the macrocyclic complexes discussed before, the pyridone nitrogen cannot act as a donor atom for other $\text{Ru}(\text{arene})$ moieties and therefore they are not capable of forming supramolecular structures. The compounds were investigated with regard to chemical stability, biological activity and interaction with DNA model nucleobases, such as 5'-GMP and 9-ethylguanine, and with proteins, in order to correlate their affinity to biomolecules with anticancer activity. The complexes exhibit low cytotoxicity, notably the osmium compound being slightly more active than the ruthenium analogue [130]. Both Os and Ru complexes hydrolyze rapidly in aqueous solution by exchange of the chlorido by an aqua ligand and exist predominantly as the charged monoaqua species. When comparing the hydrolysis behavior of Ru and Os analogues, the Os compound hydrolyzes at a significantly lower rate than the ruthenium analogue and the aqua species are stable for more than five days. In contrast to analogous pyronato complexes [140], no dimeric species of $[\text{Ru}_2(\text{cym})_2(\mu\text{-OH})_3]^+$ are formed [141,142]. The pK_a values for these complexes are in a similar range as those of structurally related hydroxypyridone compounds [38,140,141], and the aqua ligands coordinated to osmium are significantly more acidic than at ruthenium centers [140,166].

$[\text{Ru}(\text{cym})(2\text{-pyridonato})\text{Cl}]$ **28** (Fig. 12) and its osmium analogue react rapidly with amino acids, such as Cys, Met and His, accompanied by quantitative replacement of the pyridonato ligand [130], as also demonstrated for related pyrone-derived complexes [141]. This property is probably related to the ability of amino acids to act as bidentate or tridentate chelating ligands (e.g., with the thioether of Met and N1 or N3 atoms of the imidazole moiety of His as third donors in addition to NH_2 and COOH). Os has an even greater affinity for softer ligands compared to Ru but ligand exchange rates are lower and replacement of the pyridone is thus slower [130]. Similar behavior was observed in the reactions with the model proteins ubiquitin (Ub) and cytochrome *c*, as studied by electrospray ionization mass spectrometry. The Ru complex reacts quickly with Ub – within 1 h the peak assigned to unreacted ubiquitin had vanished and mono- and doubly-ruthenated $[\text{Ub} + \text{Ru}_n(\text{cym})_n]$ ($n = 1, 2$) were detected. The Os analogue reacts with ubiquitin in a similar manner and forms the same adducts, though at lower rate, as also observed in the reaction with cytochrome *c* [130].

Similar to the 2-pyridonato compounds, complexes based on 4-pyridonato ligands were reported and by linking pyridones the corresponding di- and trinuclear $\text{Ru}(\text{arene})$ and $\text{Os}(\text{arene})$ derivatives were obtained (Fig. 12) [38–40,130]. Out of the series of compounds, the dinuclear species proved to be most efficient in *in vitro* anticancer activity assays, being in some cell lines superior to platinum-based anticancer drugs. The most potent dinuclear compound was 1,12-bis{chlorido[3-(oxo- κO)-2-methyl-4-pyridinonato- κO4](cym)ruthenium(II)]dodecane (Fig. 12, $n = 12$, $\text{M} = \text{Ru}$, arene = cym, $\text{X} = \text{Cl}$) ($\text{IC}_{50} = 0.29 \mu\text{M}$) which is an order of magnitude more active than cisplatin ($\text{IC}_{50} = 4.5 \mu\text{M}$) and equally active as oxaliplatin ($\text{IC}_{50} = 0.30 \mu\text{M}$) in SW480 cells [37,130] (Table 3). Interestingly, two cell lines resistant to the $\text{Pt}(\text{IV})$ -based cisplatin prodrug oxaliplatin are more sensitive to dinuclear Ru^{II} (arene) organometallics than the native cell lines.

As observed for $\text{Ru}(\text{arene})$ complexes of other hydroxypyridone and related *O,O*-chelating ligands, rapid aquation was observed for the dinuclear compounds with pK_a values of ~ 9.70 for the aqua species [38]. They show strong affinity for transferrin, but surprisingly no interaction with the smaller cellular proteins ubiquitin and cytochrome *c* was detected. The complexes react

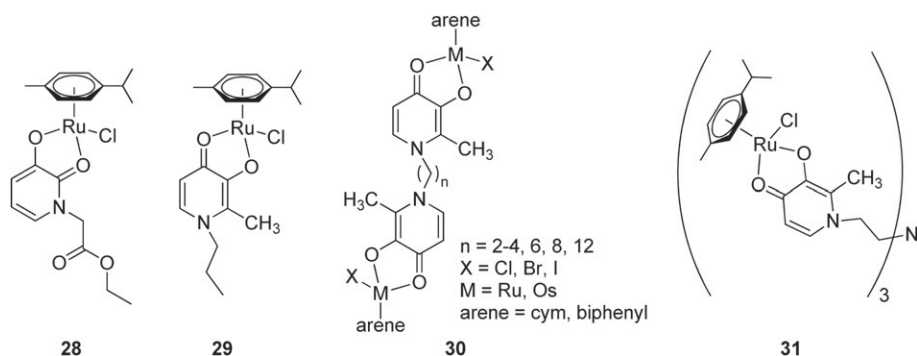


Fig. 12. Structures of mono- (2-pyridone **28**, 4-pyridone **29**), di- and trinuclear $\text{Ru}^{\text{II}}(\text{pyridonato})$ complexes with tumor-inhibiting properties.

rapidly with DNA and model nucleotides, as observed by NMR spectroscopy, DNA precipitation, mass spectrometry, and gel electrophoresis. The anticancer activity of this compound class appears to be at least partly determined by their lipophilicity [37–40], which is an important factor for cellular uptake of anticancer drugs, since hydrophobic molecules can cross cell membranes easier than hydrophilic ones, resulting often in enhanced antiproliferative activity.

In order to establish structure-activity relationships, structural modifications, such as change of spacer length, arene moiety, number and nature of metal center and leaving group, were evaluated on their influence on the *in vitro* antiproliferative activity. Variation of the spacer between the pyridone moieties has a profound effect on the cytotoxicity and the properties of both Ru and Os complexes, the compound with the highest spacer length being the most lipophilic was also the most cytotoxic. Notably, changing the nuclearity causes a less pronounced effect than modifying the spacer length or switching the metal center from Ru to Os [39]. Similarly, the nature of the leaving group and the arene ligand, e.g., with a more extended π -electron system, have only minor effects on the *in vitro* activity. Importantly, replacing the *p*-cymene by a biphenyl group causes a significant change in lipophilicity but does not alter the cytotoxicity against cancer cells to a meaningful extent [39]. These results implicate that factors other than lipophilicity play a role in the mode of action. In addition, DNA and protein interaction studies revealed significant potential for DNA-protein and interduplex cross-linking, which might be an option to design compounds as specific cross-linkers of biomolecules [167].

In an attempt to alter the aquation kinetics and thereby the anticancer activity of dinuclear $\text{Ru}^{\text{II}}(\text{arene})$ complexes, the bromido

and iodo compounds were prepared and compared to their chlorido analogue [39]. No spectral changes were observed for an aqueous solution of the chlorido complex over 24 h by UV/vis spectroscopy, a result that was confirmed by studies using a chloride selective electrode. This suggests that the fast aquation results in the formation of a single product $\{[\text{Ru}(\text{cym})(\text{H}_2\text{O})_2\text{L}]^2\}^+$, which is independent of the leaving group and not surprisingly, mostly small differences in their anticancer activity were measured [39]. Further modifications of the dinuclear complexes focused on the extension of the spacer, paralleled by efforts to improve water solubility [40]. However, compounds with longer alkyl spacers were not sufficiently soluble to perform *in vitro* anticancer assays, and introduction of polyether spacers resulted in low stability.

4. Conclusions

(Thio)pyr(id)ones have been an area of intensive research and their fields of application are constantly expanding. This can be explained by often low toxicity and high affinity towards a large number of metal ions in various oxidation states. Important chemical and biological properties can be tuned by specific modification of the heterocyclic backbone, making them versatile scaffolds. An increasing number of applications is related to their *O,O*- and *S,O*-chelating ability to form thermodynamically stable and water soluble metal complexes. These are ideal characteristics to use such compounds for the removal of metal ions against metal overload disorders, or administration of metal ions in form of pyrone-based complexes, such as in the case of iron deficiency, in the self-assembly of metallamacrocycles as biosensors for Li^+ , or as drug carriers, e.g., as ligands for vanadium with potential in the treatment of type-2 diabetes or for Gd^{III} in imaging agents. Among the newest developments are (thio)pyr(id)one-based metal complexes with tumor-inhibiting properties. These ligand systems offer potential to incorporate non-classical modes of action and tunable anticancer activity into metal complexes.

Acknowledgments

We thank the “Johanna Mahlke geb. Obermann-Stiftung”, the Higher Education Commission of Pakistan, the Austrian Exchange Service (ÖAD), the Hochschuljubiläumsstiftung Vienna, the FFG – Austrian Research Promotion Agency (811591), the Austrian Council for Research and Technology Development (IS526001), COST D39 and CM0902 and the Austrian Science Fund for financial support.

References

- [1] E.J. Gralla, R.B. Stebbins, G.L. Coleman, C.S. Delahunt, *Toxicol. Appl. Pharmacol.* 15 (1969) 604–613.

Table 3

Anticancer activity of mono-, di- and trinuclear 4-pyridonato complexes in SW480 and A2780 human cancer cell lines (for structures compare Fig. 12).

M	Nuclearity	Arene	X	n	$\text{IC}_{50}/\mu\text{M}$		Ref.
					SW480	A2780	
Ru	2	Cym	Cl	2	76 ± 4	84 ± 3	[38]
Ru	2	Cym	Cl	3	62 ± 14	25 ± 2	[37,38]
Ru	2	Cym	Cl	4	28 ± 1	41 ± 2	[38]
Ru	2	Cym	Cl	6	26 ± 8	30 ± 6	[37,38]
Ru	2	Cym	Cl	8	2.5 ± 0.2	5.7 ± 0.5	[38]
Ru	2	Cym	Cl	12	0.29 ± 0.05	1.5 ± 0.3	[37,38]
Ru	1	Cym	Cl	1-Propyl	42 ± 1	88 ± 12	[39]
Os	2	Cym	Cl	6	88 ± 4	–	
Ru	2	Cym	Cl	6	26 ± 8	30 ± 6	
Os	2	Cym	Cl	8	15 ± 5	29 ± 13	
Ru	2	Cym	Cl	8	2.5 ± 0.2	5.7 ± 0.5	
Ru	2	Cym	Br	6	54 ± 4	52 ± 15	
Ru	2	Cym	I	6	33 ± 4	36 ± 2	
Ru	2	Biphenyl	Cl	6	25.7 ± 0.3	43 ± 1	
Ru	3	Cym	Cl	$\text{N}(\text{C}_2\text{H}_4)_3$	59 ± 18	80 ± 7	

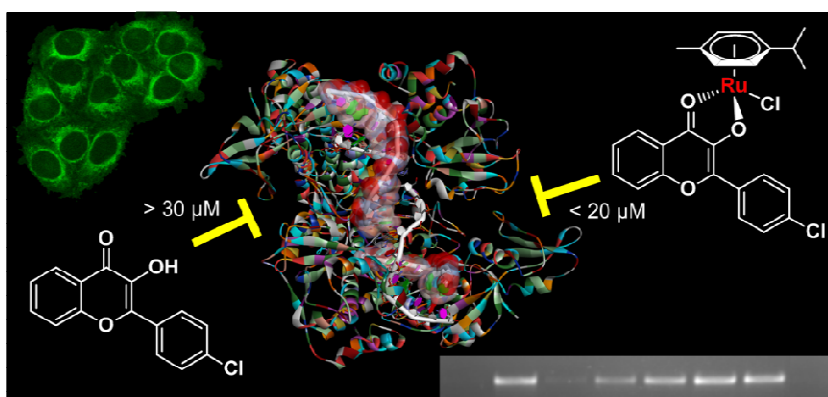
- [2] R. Bentley, Nat. Prod. Rep. 23 (2006) 1046–1062.
- [3] M.A. Spielmann, M. Freierlender, J. Am. Chem. Soc. 69 (1947) 2908.
- [4] S. Torii, H. Tanaka, T. Anoda, Y. Simizu, Chem. Lett. (1976) 495–498.
- [5] I. Ichimoto, C. Tatsumi, Agric. Biol. Chem. 34 (1970) 961.
- [6] T. Yabuta, Chem. Abstr. 7 (1913) 2191.
- [7] T. Yabuta, Nippon Kagaku Kaishi 37 (1916) 1185.
- [8] J. Cabanes, S. Chazarra, F. Garcia-Carmona, J. Pharm. Pharmacol. 46 (1994) 982–985.
- [9] M.A. Santos, Coord. Chem. Rev. 252 (2008) 1213–1224.
- [10] T. Yabuta, J. Chem. Soc. 125 (1924) 575–587.
- [11] A. Beelik, C.B. Purves, Can. J. Chem. 33 (1955) 1361–1374.
- [12] S. Fakhri, M. Podinovskaia, X. Kong, H.L. Collins, U.E. Schaible, R.C. Hider, J. Med. Chem. 51 (2008) 4539–4552.
- [13] B.E. Tate, R.L. Miller, US Patent 3130204 (1964).
- [14] B.L. Ellis, A.K. Duhme, R.C. Hider, M.B. Hossain, S. Rizvi, D. van der Helm, J. Med. Chem. 39 (1996) 3659–3670.
- [15] Z.D. Liu, H.H. Khodr, D.Y. Liu, S.L. Lu, R.C. Hider, J. Med. Chem. 42 (1999) 4814–4823.
- [16] M.D. Aytemir, B. Ozcelik, Eur. J. Med. Chem. 45 (2010) 4089–4095.
- [17] K. Kawase, K. Hayashi, J. Agr. Chem. Soc. Jpn. 46 (1972) 335–339.
- [18] H. Takao, Y. Endo, T. Horie, Heterocycles 36 (1993) 1803–1808.
- [19] E. Shimmmin, F. Challenger, J. Chem. Soc. (1949) 1185.
- [20] L.L. Woods, J. Am. Chem. Soc. 74 (1952) 1107.
- [21] Y. Ma, W. Luo, P.J. Quinn, Z. Liu, R.C. Hider, J. Med. Chem. 47 (2004) 6349–6362.
- [22] P. Pace, E. Nizi, B. Pacini, S. Pesci, V. Matassa, R. De Francesco, S. Altamura, V. Summa, Bioorg. Med. Chem. Lett. 14 (2004) 3257–3261.
- [23] Y.L. Yan, M.T. Miller, Y.C. Cao, S.M. Cohen, Bioorg. Med. Chem. Lett. 19 (2009) 1970–1976.
- [24] J.G. Atkinson, Y. Girard, J. Rokach, C.S. Rooney, C.S. McFarlane, A. Rackham, N.N. Share, J. Med. Chem. 22 (1979) 99–106.
- [25] L.S. Dehkordi, Z.D. Liu, R.C. Hider, Eur. J. Med. Chem. 43 (2008) 1035–1047.
- [26] T. Storr, D. Mitchell, P. Buglyo, K.H. Thompson, V.G. Yuen, J.H. McNeill, C. Orvig, Bioconjug. Chem. 14 (2003) 212–221.
- [27] H.S. Rho, H.S. Baek, S.M. Ahn, D.H. Kim, I.S. Chang, Bull. Kor. Chem. Soc. 29 (2008) 1569–1571.
- [28] Y.L. Chen, P.H. Chen, C.H. Chung, K.C. Li, H.Y. Jeng, C.C. Tzeng, Helv. Chim. Acta 86 (2003) 778–786.
- [29] J. Kadokawa, T. Nishikura, R. Muraoka, H. Tagaya, N. Fukuoka, Synth. Commun. 33 (2003) 1081–1086.
- [30] G. O'Brien, J.M. Patterson, J.R. Meadow, J. Org. Chem. 25 (1960) 86–89.
- [31] S. Lovell, P. Subramony, B. Kahr, J. Am. Chem. Soc. 121 (1999) 7020–7025.
- [32] M.D. Aytemir, U. Calis, Arch. Pharm. 343 (2010) 173–181.
- [33] B. Tamhina, K. Jakopcic, F. Zorko, M.J. Herak, J. Inorg. Nucl. Chem. 36 (1974) 1855–1857.
- [34] K. Jakopcic, B. Tamhina, F. Zorko, M.J. Herak, J. Inorg. Nucl. Chem. 39 (1977) 1201–1203.
- [35] R.L.N. Harris, Aust. J. Chem. 29 (1976) 1329–1334.
- [36] P.S. Dobbin, R.C. Hider, A.D. Hall, P.D. Taylor, P. Sarpong, J.B. Porter, G. Xiao, D. van der Helm, J. Med. Chem. 36 (1993) 2448–2458.
- [37] M.G. Mendoza-Ferri, C.G. Hartinger, R.E. Eichinger, N. Stolyarova, M.A. Jakupcic, A.A. Nazarov, K. Severin, B.K. Keppler, Organometallics 27 (2008) 2405–2407.
- [38] M.G. Mendoza-Ferri, C.G. Hartinger, M.A. Mendoza, M. Groessl, A.E. Egger, R.E. Eichinger, J.B. Mangrum, N.P. Farrell, M. Maruszak, P.J. Bednarski, F. Klein, M.A. Jakupcic, A.A. Nazarov, K. Severin, B.K. Keppler, J. Med. Chem. 52 (2009) 916–925.
- [39] M.G. Mendoza-Ferri, C.G. Hartinger, A.A. Nazarov, R.E. Eichinger, M.A. Jakupcic, K. Severin, B.K. Keppler, Organometallics 28 (2009) 6260–6265.
- [40] M.G. Mendoza-Ferri, C.G. Hartinger, A.A. Nazarov, W. Kandioller, K. Severin, B.K. Keppler, Appl. Organomet. Chem. 22 (2008) 326–332.
- [41] A.V. Hoffbrand, A. Cohen, C. Hershko, Blood 102 (2003) 17–24.
- [42] S. Chaves, P.I. Dron, F.A. Danalache, D. Sacoto, L. Gano, M.A. Santos, J. Inorg. Biochem. 103 (2009) 1521–1529.
- [43] T.P.A. Kruck, T.E. Burrow, J. Inorg. Biochem. 88 (2002) 19–24.
- [44] M.A. Santos, M. Gil, L. Gano, S. Chaves, J. Biol. Inorg. Chem. 10 (2005) 564–580.
- [45] M.A. Santos, S. Gama, L. Gano, G. Cantinho, E. Farkas, J. Chem. Soc. Dalton Trans. (2004) 3772–3781.
- [46] M.A. Santos, S. Gama, L. Gano, E. Farkas, J. Inorg. Biochem. 99 (2005) 1845–1852.
- [47] R. Grazina, L. Gano, J. Sebestik, M.A. Santos, J. Inorg. Biochem. 103 (2009) 262–273.
- [48] J. Xu, P.W. Durbin, B. Kullgren, S.N. Ebbe, L.C. Uhler, K.N. Raymond, J. Med. Chem. 45 (2002) 3963–3971.
- [49] S. Chaves, M. Gil, S. Canario, R. Jelic, M.J. Romao, J. Trincao, E. Herdtweck, J. Sousa, C. Diniz, P. Fresco, M.A. Santos, Dalton Trans. (2008) 1773–1782.
- [50] V. Monga, B.O. Patrick, C. Orvig, Inorg. Chem. 44 (2005) 2666–2677.
- [51] J.A. Lewis, D.T. Puerta, S.M. Cohen, Inorg. Chem. 42 (2003) 7455–7459.
- [52] D. Brayton, F.E. Jacobsen, S.M. Cohen, P.J. Farmer, Chem. Commun. (2006) 206–208.
- [53] W. Tejchman, K. Zborowski, W. Lasocha, L.M. Proniewicz, Heterocycles 75 (2008) 1931–1942.
- [54] R.D. Hancock, A.E. Martell, Chem. Rev. 89 (1989) 1875.
- [55] K.H. Thompson, C.A. Barta, C. Orvig, Chem. Soc. Rev. 35 (2006) 545–556.
- [56] R.E. Scarrow, P.E. Riley, K. Abu-Dari, D.L. White, K.N. Raymond, J. Inorg. Chem. 24 (1985) 954–967.
- [57] J. Xu, D.W. Whisenhunt Jr., A.C. Veeck, L.C. Uhler, K.N. Raymond, Inorg. Chem. 42 (2003) 2665–2674.
- [58] S.A. Mukha, I.A. Antipova, S.A. Medvedeva, V.V. Saraev, L.I. Larina, A.V. Tsyrenzhapov, B.G. Sukhov, Chem. Sustainable Dev. (2007) 457–466.
- [59] M.A. Santos, M. Gil, S. Marques, L. Gano, G. Cantinho, S. Chaves, J. Inorg. Biochem. 92 (2002) 43–54.
- [60] C. Orvig, P. Caravan, L. Gelmini, N. Glover, F.G. Herring, H. Li, J.H. McNeill, S.J. Rettig, I.A. Setyawati, J. Am. Chem. Soc. 117 (1995) 12759–12770.
- [61] M. Rangel, Transit. Met. Chem. 26 (2001) 219.
- [62] L. Biró, E. Farkas, P. Buglyó, Dalton Trans. (2010) 10272–10278.
- [63] Y. Ma, W. Luo, M. Camplo, Z. Liu, R.C. Hider, Bioorg. Med. Chem. Lett. 15 (2005) 3450–3452.
- [64] C.T. Yang, S.G. Sreerama, W.Y. Hsieh, S. Liu, Inorg. Chem. 47 (2008) 2719–2727.
- [65] A. Katoh, T. Tsukahara, R. Saito, K.K. Ghosh, Y. Yoshikawa, Y. Kojima, A. Tamura, H. Sakurai, Chem. Lett. (2002) 114–115.
- [66] D.T. Puerta, M.O. Griffin, J.A. Lewis, D. Romero-Perez, R. Garcia, F.J. Villarreal, S.M. Cohen, J. Biol. Inorg. Chem. 11 (2006) 131–138.
- [67] A. Agrawal, S.L. Johnson, J.A. Jacobsen, M.T. Miller, L.H. Chen, M. Pellecchia, S.M. Cohen, ChemMedChem 5 (2010) 195–199.
- [68] J.L. Major Jourden, S.M. Cohen, Chem. Commun. 46 (2010) 1241–1243.
- [69] M.J. Cairns, M. Carland, W.D. McFadyen, W.A. Denny, V. Murray, J. Inorg. Biochem. 103 (2009) 1151–1155.
- [70] G. Papanikolaou, K. Pantopoulos, Toxicol. Appl. Pharmacol. 202 (2005) 199–211.
- [71] W.C. Tsai, K.H. Ling, J. Chin. Biochem. Soc. 2 (1973) 70.
- [72] W. Schlindwein, E. Waltham, J. Burgess, N. Binsted, A. Nunes, A. Leite, M. Rangel, Dalton Trans. (2006) 1313–1321.
- [73] G.J. Kontoghiorghes, L. Sheppard, Inorg. Chim. Acta 136 (1987) L11–L12.
- [74] D. Cappellini, P. Patteroneri, Annu. Rev. Med. 43 (2009) 1035.
- [75] S. Singh, R.O. Epemolu, P.S. Dobbin, G.S. Tilbrook, B.L. Ellis, L.A. Damani, R.C. Hider, Drug Metab. Dispos. 20 (1992) 256–261.
- [76] M.A. Barrand, R.C. Hider, B.A. Callingham, J. Pharm. Pharmacol. 42 (1990) 279–282.
- [77] B. Morgan, O. Lahav, Chemosphere 68 (2007) 2080–2084.
- [78] T. Hedlund, L.O. Öhmann, Acta Chem. Scand. Ser. A 42 (1988) 702.
- [79] D.G. Maxton, R.P. Thompson, R.C. Hider, Br. J. Nutr. 71 (1994) 203–207.
- [80] K.H. Thompson, J.H. McNeill, C. Orvig, Chem. Rev. 99 (1999) 2561–2572.
- [81] Y. Shechter, Diabetes 39 (1990) 1–5.
- [82] A. Sheela, S.M. Roopan, R. Vijayaraghavan, Eur. J. Med. Chem. 43 (2008) 2206–2210.
- [83] P. Buglyo, E. Kiss, I. Fabian, T. Kiss, D. Sanna, E. Garribba, G. Micera, Inorg. Chim. Acta 306 (2000) 174–183.
- [84] K.H. Thompson, J. Lichter, C. LeBel, M.C. Scaife, J.H. McNeill, C. Orvig, J. Inorg. Biochem. 103 (2009) 554–558.
- [85] H. Sakurai, A. Tamura, J. Fugono, H. Yasui, T. Kiss, Coord. Chem. Rev. 245 (2003) 31–37.
- [86] T. Kiss, T. Jakusch, D. Hollender, A. Doernyei, E.A. Enyedy, J.C. Pessoa, H. Sakurai, A. Sanz-Medel, Coord. Chem. Rev. 252 (2008) 1153–1162.
- [87] S. Chaves, R. Jelic, C. Mendonca, M. Carrasco, Y. Yoshikawa, H. Sakurai, M.A. Santos, Metallomics 2 (2010) 220–227.
- [88] K.H. Thompson, J. Chiles, V.G. Yuen, J. Tse, J.H. McNeill, C. Orvig, J. Inorg. Biochem. 98 (2004) 683–690.
- [89] A. Katoh, Y. Matsumura, Y. Yoshikawa, H. Yasui, H. Sakurai, J. Inorg. Biochem. 103 (2009) 567–574.
- [90] D.R. Crapper, S.S. Krishnan, S. Quittkat, Brain 99 (1976) 67–80.
- [91] G. Berthon, Coord. Chem. Rev. 228 (2002) 319–341.
- [92] M.M. Finnegan, T.G. Lutz, W.O. Nelson, A. Smith, C. Orvig, Inorg. Chem. 26 (1987) 2171–2176.
- [93] S. Aime, M. Botta, E. Terreno, Adv. Inorg. Chem. 57 (2005) 173–237.
- [94] K.W.Y. Chan, W.T. Wong, Coord. Chem. Rev. 251 (2007) 2428–2451.
- [95] A. Datta, K.N. Raymond, Acc. Chem. Res. 42 (2009) 938–947.
- [96] E.J. Werner, A. Datta, C.J. Jocher, K.N. Raymond, Angew. Chem. Int. Ed. Engl. 47 (2008) 8568–8580.
- [97] E.J. Werner, S. Avedano, M. Botta, B.P. Hay, E.G. Moore, S. Aime, K.N. Raymond, J. Am. Chem. Soc. 129 (2007) 1870–1871.
- [98] V.C. Pierre, M. Botta, S. Aime, K.N. Raymond, J. Am. Chem. Soc. 128 (2006) 5344–5345.
- [99] J. Xu, S.J. Franklin, D.W. Whisenhunt, K.N. Raymond, J. Am. Chem. Soc. 117 (1995) 7245–7246.
- [100] E.J. Werner, J. Kozhukh, M. Botta, E.G. Moore, S. Avedano, S. Aime, K.N. Raymond, Inorg. Chem. 48 (2009) 277–286.
- [101] K.N. Raymond, V.C. Pierre, Bioconjug. Chem. 16 (2005) 3–8.
- [102] M.K. Thompson, B. Misselwitz, L.S. Tso, D.M.J. Doble, H. Schmitt-Willich, K.N. Raymond, J. Med. Chem. 48 (2005) 3874–3877.
- [103] R.E. Coleman, Cancer 67 (1991) 1261–1270.
- [104] M.A. Jakupcic, M. Galanski, V.B. Arion, C.G. Hartinger, B.K. Keppler, Dalton Trans. (2008) 183–194.
- [105] M. Groessl, A. Bytzeck, C.G. Hartinger, Electrophoresis 30 (2009) 2720–2727.
- [106] J. Narasimhan, W.E. Antholine, C.R. Chitambar, Biochem. Pharmacol. 44 (1992) 2403–2408.
- [107] C.R. Chitambar, Curr. Opin. Oncol. 16 (2004) 547–552.
- [108] L.R. Bernstein, T. Tanner, C. Godfrey, B. Noll, Met. Based Drugs 7 (2000) 33–47.

- [109] <http://clinicaltrials.gov> which was accessed on 2010-09-02.
- [110] R. Margalit, H.B. Gray, M.J. Clarke, L. Podbielski, *Chem. Biol. Interact.* 59 (1986) 231–245.
- [111] C.G. Hartinger, S. Zorbas-Seifried, M.A. Jakupiec, B. Kynast, H. Zorbas, B.K. Keppler, *J. Inorg. Biochem.* 100 (2006) 891–904.
- [112] J.M. Rademaker-Lakhai, D. Van Den Bongard, D. Pluim, J.H. Beijnen, J.H.M. Schellens, *Clin. Cancer Res.* 10 (2004) 3717–3727.
- [113] C.G. Hartinger, M.A. Jakupiec, S. Zorbas-Seifried, M. Groessl, A. Egger, W. Berger, H. Zorbas, P.J. Dyson, B.K. Keppler, *Chem. Biodivers.* 5 (2008) 2140–2155.
- [114] M.J. Clarke, in: H. Sigel (Ed.), *Metal Ions in Biological Systems*, vol. 11, Marcel Dekker, New York, 1980, pp. 231–283.
- [115] C.G. Hartinger, B.K. Keppler, *Electrophoresis* 28 (2007) 3436–3446.
- [116] A.R. Timerbaev, C.G. Hartinger, S.S. Alekseenko, B.K. Keppler, *Chem. Rev.* 106 (2006) 2224–2248.
- [117] M. Suljok, S. Hann, C.G. Hartinger, B.K. Keppler, G. Stinger, G. Koellensperger, *J. Anal. At. Spectrom.* 20 (2005) 856–863.
- [118] J. Burgess, S.A. Parsons, K. Singh, E. Waltham, P. Lopez, F. Sanchez, M. Rangel, W. Schlindwein, *Transit. Met. Chem.* 33 (2008) 553–561.
- [119] D.C. Kennedy, A. Wu, B.O. Patrick, B.R. James, *Inorg. Chem.* 44 (2005) 6529–6535.
- [120] R. Prajapati, S.K. Dubey, R. Gaur, R.K. Koiri, B.K. Maurya, S.K. Trigun, L. Mishra, *Polyhedron* 29 (2010) 1055–1061.
- [121] D.C. Kennedy, A. Wu, B.O. Patrick, B.R. James, *J. Inorg. Biochem.* 100 (2006) 1974–1982.
- [122] A. Wu, D.C. Kennedy, B.O. Patrick, B.R. James, *Inorg. Chem.* 42 (2003) 7579–7586.
- [123] C.G. Hartinger, P.J. Dyson, *Chem. Soc. Rev.* 38 (2009) 391–401.
- [124] G. Süss-Fink, *Dalton Trans.* 39 (2010) 1673–1688.
- [125] M.J. Clarke, *Coord. Chem. Rev.* 236 (2003) 209–233.
- [126] J.É. Debreczeni, A.N. Bullock, G.E. Atilla, D.S. Williams, H. Bregman, S. Knapp, E. Meggers, *Angew. Chem.* 45 (2006) 1580–1585.
- [127] C.A. Vock, C. Scolaro, A.D. Phillips, R. Scopelliti, G. Sava, P.J. Dyson, *J. Med. Chem.* 49 (2006) 5552–5561.
- [128] C.A. Vock, W.H. Ang, C. Scolaro, A.D. Phillips, L. Lagopoulos, L. Juillerat-Jeanneret, G. Sava, R. Scopelliti, P.J. Dyson, *J. Med. Chem.* 50 (2007) 2166–2175.
- [129] F. Zobi, B.B. Mood, P.A. Wood, F.P.A. Fabbiani, S. Parsons, P.J. Sadler, *Eur. J. Inorg. Chem.* (2007) 2783–2796.
- [130] M. Hanif, H. Henke, S.M. Meier, S. Martic, M. Labib, W. Kandioller, M.A. Jakupiec, V.B. Arion, H.B. Kraatz, B.K. Keppler, C.G. Hartinger, *Inorg. Chem.* 49 (2010) 7953–7963.
- [131] I. Berger, M. Hanif, A.A. Nazarov, C.G. Hartinger, R.O. John, M.L. Kuznetsov, M. Groessl, F. Schmitt, O. Zava, F. Biba, V.B. Arion, M. Galanski, M.A. Jakupiec, L. Juillerat-Jeanneret, P.J. Dyson, B.K. Keppler, *Chem. Eur. J.* 14 (2008) 9046–9057.
- [132] Y.N.V. Gopal, D. Jayaraju, A.K. Kondapi, *Biochemistry* 38 (1999) 4382–4388.
- [133] W.H. Ang, P.J. Dyson, *Eur. J. Inorg. Chem.* (2006) 4003–4018.
- [134] P.J. Dyson, *Chimia* 61 (2007) 698–703.
- [135] A.F.A. Peacock, P.J. Sadler, *Chem. Asian J.* 3 (2008) 1890–1899.
- [136] C. Scolaro, A. Bergamo, L. Brescacin, R. Delfino, M. Cocchietto, G. Laurenczy, T.J. Geldbach, G. Sava, P.J. Dyson, *J. Med. Chem.* 48 (2005) 4161–4171.
- [137] A. Casini, G. Mastrobuoni, W.H. Ang, C. Gabbiani, G. Pieraccini, G. Moneti, P.J. Dyson, L. Messori, *ChemMedChem* 2 (2007) 631–635.
- [138] R.E. Aird, J. Cummings, A.A. Ritchie, M. Muir, R.E. Morris, H. Chen, P.J. Sadler, D.I. Jodrell, *Br. J. Cancer* 86 (2002) 1652–1657.
- [139] R. Fernandez, M. Melchart, A. Habtemariam, S. Parsons, P.J. Sadler, *Chem. Eur. J.* 10 (2004) 5173–5179.
- [140] A.F.A. Peacock, M. Melchart, R.J. Deeth, A. Habtemariam, S. Parsons, P.J. Sadler, *Chem. Eur. J.* 13 (2007) 2601–2613.
- [141] W. Kandioller, C.G. Hartinger, A.A. Nazarov, C. Bartel, M. Skocic, M.A. Jakupiec, V.B. Arion, B.K. Keppler, *Chem. Eur. J.* 15 (2009) 12283–12291.
- [142] W. Kandioller, C.G. Hartinger, A.A. Nazarov, J. Kasser, R. John, M.A. Jakupiec, V.B. Arion, P.J. Dyson, B.K. Keppler, *J. Organomet. Chem.* 694 (2009) 922–929.
- [143] W. Kandioller, C.G. Hartinger, A.A. Nazarov, M.L. Kuznetsov, R.O. John, C. Bartel, M.A. Jakupiec, V.B. Arion, B.K. Keppler, *Organometallics* 28 (2009) 4249–4251.
- [144] J.H. Kasser, W. Kandioller, C.G. Hartinger, A.A. Nazarov, V.B. Arion, P.J. Dyson, B.K. Keppler, *J. Organomet. Chem.* 695 (2010) 875–881.
- [145] M. Hanif, W. Schaaf, M. Kandioller, M.A. Hejl, A. Jakupiec, B.K. Roller, C.G. Keppler, Hartinger, *Aust. J. Chem.* 63 (2010) 1521–1528.
- [146] A.K. Renfrew, A.D. Phillips, A.E. Egger, C.G. Hartinger, S.S. Bosquain, A.A. Nazarov, B.K. Keppler, L. Gonsalvi, M. Peruzzini, P.J. Dyson, *Organometallics* 28 (2009) 1165–1172.
- [147] A. Dorcier, C.G. Hartinger, R. Scopelliti, R.H. Fish, B.K. Keppler, P.J. Dyson, *J. Inorg. Biochem.* 102 (2008) 1066–1076.
- [148] A. Dorcier, P.J. Dyson, C. Gossens, U. Rothlisberger, R. Scopelliti, I. Tavernelli, *Organometallics* 24 (2005) 2114–2123.
- [149] A.E. Egger, C.G. Hartinger, A.K. Renfrew, P.J. Dyson, *J. Biol. Inorg. Chem.* 15 (2010) 919–927.
- [150] M. Groessl, Y.O. Tsybin, C.G. Hartinger, B.K. Keppler, P.J. Dyson, *J. Biol. Inorg. Chem.* 15 (2010) 677–688.
- [151] I. Khalaila, A. Bergamo, F. Bussy, G. Sava, P.J. Dyson, *Int. J. Oncol.* 29 (2006) 261–268.
- [152] C. Scolaro, A.B. Chaplin, C.G. Hartinger, A. Bergamo, M. Cocchietto, B.K. Keppler, G. Sava, P.J. Dyson, *Dalton Trans.* (2007) 5065–5072.
- [153] A.E. Egger, C. Rappel, M.A. Jakupiec, C.G. Hartinger, P. Heffeter, B.K. Keppler, *J. Anal. At. Spectrom.* 24 (2009) 51–61.
- [154] R. Lang, K. Polborn, T. Severin, K. Severin, *Inorg. Chim. Acta* 294 (1999) 62–67.
- [155] T. Haberer, M. Warchhold, H. Noth, K. Severin, *Angew. Chem. Int. Ed.* 38 (1999) 3225–3228.
- [156] H. Piotrowski, K. Polborn, G. Hilt, K. Severin, *J. Am. Chem. Soc.* 123 (2001) 2699–2700.
- [157] H. Piotrowski, K. Severin, *Proc. Natl. Acad. Sci.* 99 (2002) 4997–5000.
- [158] S. Rochat, Z. Grote, K. Severin, *Org. Biomol. Chem.* 7 (2009) 1147–1153.
- [159] C. Olivier, R. Scopelliti, K. Severin, *Eur. J. Inorg. Chem.* (2009) 207–210.
- [160] C. Olivier, Z. Grote, E. Solari, R. Scopelliti, K. Severin, *Chem. Commun.* (2007) 4000–4002.
- [161] A. Granzhan, T. Riis-Johannessen, R. Scopelliti, K. Severin, *Angew. Chem. Int. Ed. Engl.* 49 (2010) 5515–5518.
- [162] B. Therrien, *Eur. J. Inorg. Chem.* (2009) 2445–2453.
- [163] W.H. Ang, Z. Grote, R. Scopelliti, L. Juillerat-Jeanneret, K. Severin, P.J. Dyson, *J. Organomet. Chem.* 694 (2009) 968–972.
- [164] S. Chatterjee, S. Kundu, A. Bhattacharyya, C.G. Hartinger, P.J. Dyson, *J. Biol. Inorg. Chem.* 13 (2008) 1149–1155.
- [165] A. Bergamo, R. Gagliardi, V. Scarica, A. Furlani, E. Alessio, G. Mestroni, G. Sava, *J. Pharmacol. Exp. Ther.* 289 (1999) 559–564.
- [166] A.F.A. Peacock, A. Habtemariam, R. Fernandez, V. Walland, F.P.A. Fabbiani, S. Parsons, R.E. Aird, D.I. Jodrell, P.J. Sadler, *J. Am. Chem. Soc.* 128 (2006) 1739–1748.
- [167] O. Nováková, A.A. Nazarov, C.G. Hartinger, B.K. Keppler, V. Brabec, *Biochem. Pharmacol.* 77 (2009) 364–374.

3.2. Targeting the DNA-topoisomerase Complex in a Double-strike Approach with a Topoisomerase Inhibiting Moiety and Covalent DNA Binder

Chemical Communications **2012**, 48, 4839–4841.

Graphical abstract



Ru^{II}(arene)-flavonoids with high *in vitro* antitumour activity were synthesised. These compounds are capable of inhibiting human topoisomerase II α and binding covalently to DNA.

Cite this: *Chem. Commun.*, 2012, **48**, 4839–4841

www.rsc.org/chemcomm

COMMUNICATION

Targeting the DNA-topoisomerase complex in a double-strike approach with a topoisomerase inhibiting moiety and covalent DNA binder†

Andrea Kurzwernhart,^{ab} Wolfgang Kandoller,^a Caroline Bartel,^a Simone Bächler,^c Robert Trondl,^a Gerhard Mühlgassner,^a Michael A. Jakupiec,^{ab} Vladimir B. Arion,^{ab} Doris Marko,^c Bernhard K. Keppler^{ab} and Christian G. Hartinger^{*abd}

Received 13th February 2012, Accepted 20th March 2012

DOI: 10.1039/c2cc31040f

Ru^{II}(arene)–flavonoids with high *in vitro* antitumour activity were synthesised. These compounds are capable of inhibiting human topoisomerase II α and binding covalently to DNA.

Tumourigenic diseases are one of the major burdens of mankind and many patients are still not treatable or do not respond to standard drugs. This is often related to acquired or intrinsic resistance which hampers the success of chemotherapy with organic and inorganic anticancer agents, such as cisplatin. In order to overcome this drawback, several approaches have been used. The concept of multi-targeted anticancer agents (Fig. 1), *i.e.*, components of a molecule impact multiple separate targets,¹ has been shown to offer several advantages over “classic” chemotherapeutics, *e.g.*, altered pharmacological properties, metabolism and resistance development, tuneable antitumour activity, “intramolecular” combination therapy, and also selective targeted properties.¹

Among the metal complexes developed as anticancer agents, Ru(III) compounds are considered the most promising drug candidates, and KP1019 and NAMI-A are currently undergoing clinical trials. More recently, Ru^{II}(arene) organometallics have attracted considerable interest, and especially the RAPTA family and ethylene-1,2-diamine complexes are at an advanced preclinical development stage.²

One way to prepare biologically active molecules with multi-targeted properties is to link metal fragments to bioactive ligand systems. This strategy has already resulted in promising approaches with compounds exhibiting novel modes of action.^{3–5} Especially the use of ligand systems derived from natural compounds appears attractive due to the often

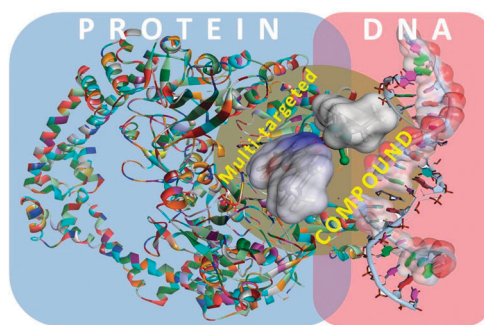


Fig. 1 The concept of a multi-targeted small molecule. We aimed to prepare a compound which is capable of binding *via* its ligand system into the active site of a protein, whereas the metal fragment can form a covalent bond to DNA.

advantageous toxicity profile.⁶ Flavonoids as secondary metabolites of plants are such a compound class with a rich variety of functions.⁷ Importantly, flavonoids are known to exhibit properties such as antiradical and antioxidant, anti-inflammatory, estrogenic, antimicrobial and also anticancer activity.⁸

We decided to link flavonoids to Ru^{II}(arene) moieties (Scheme 1), since a few examples of metal–flavonoid complexes are known to exhibit promising biological properties.⁹ However, these studies often did not aim to correlate the biological activity with the inhibition of particular targets.^{10,11} Flavonoids act primarily through the inhibition of several enzymes, and they have also been shown to interact with human topoisomerases.^{7,12}

The flavonol ligands **2a–d** were prepared in two steps by a Claisen–Schmidt condensation and subsequent Algar–Flynn–Oyamada reaction (Scheme 1, ESI†).^{13–15} Ligands **2a–d** were converted in good yields into **3a–d** with bis[dichlorido(η^6 -*p*-cymene)-ruthenium(II)] under alkaline conditions (Scheme 1).¹⁶ The complexes only show minor signs of hydrolysis in aqueous solution within 6 days.

In addition to characterisation by standard analytical methods (Supporting Information†), single crystals of **3b**·CH₃OH were analysed by X-ray diffraction (Fig. 2).† **3b** features a pseudo-octahedral “piano-stool” configuration.¹⁷ The 3-hydroxyflavone upon coordination to Ru acts as a bidentate ligand, forming an envelope-like five-membered cycle, and the two Ru–O bonds

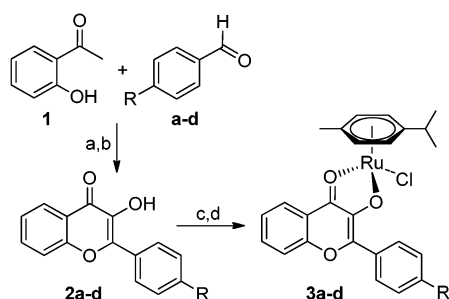
^a University of Vienna, Institute of Inorganic Chemistry, Währinger Str. 42, 1090 Vienna, Austria.
E-mail: christian.hartinger@univie.ac.at; Fax: 43 1 4277 9526;
Tel: 43 1 4277 52609

^b University of Vienna, Research Platform “Translational Cancer Therapy Research”, Währinger Str. 42, A-1090 Vienna, Austria

^c University of Vienna, Institute of Food Chemistry and Toxicology, Währinger Str. 38, 1090 Vienna, Austria

^d The University of Auckland, School of Chemical Sciences, Private Bag 92019, Auckland 1142, New Zealand

† Electronic supplementary information (ESI) available: Materials and methods, synthetic procedures, experimental setup. CCDC 826085. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c2cc31040f



Scheme 1 Synthesis of ligands (**2a–d**) and $\text{Ru}^{\text{II}}(\eta^6\text{-}p\text{-cymene})$ complexes (**3a–d**): (a) NaOH; (b) H_2O_2 ; (c) NaOMe; (d) $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$. **2a/3a**: R = H, **2b/3b**: R = CH_3 , **2c/3c**: R = F, **2d/3d**: R = Cl.

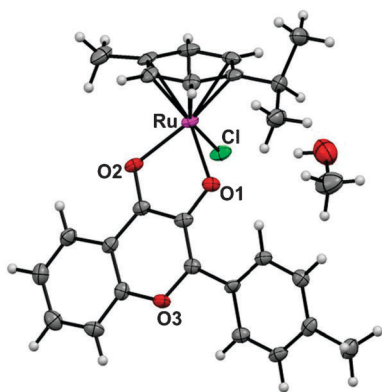


Fig. 2 Molecular structure of the $\text{Ru}^{\text{II}}(\eta^6\text{-}p\text{-cymene})$ complex **3b-CH₃OH**.

are slightly different with 2.076(2) and 2.099(3) Å, as observed in structurally related compounds.¹⁷ The phenyl substituent of the ligand is twisted with a torsion angle of 15.11°.

The stability of the complexes in aqueous solution has been studied by ^1H NMR spectroscopy.

The *in vitro* anticancer activity of ligands **2a–d** and complexes **3a–d** was determined in the human cancer cell lines CH1 (ovarian carcinoma), SW480 (colon carcinoma) and A549 (non-small cell lung carcinoma) by means of the colorimetric MTT assay (Table 1). Notably, the IC_{50} values of **3a–d** were found to be in the low μM range, and only a few examples with similar *in vitro* anticancer activity have been reported.^{1,18–20} The substituent in *para* position of the phenyl ring has a significant influence on the *in vitro* activity, with IC_{50} values of the unsubstituted compound **3a** being 2–3 times higher than those of the most active chloro derivative **3d**. Compared to cisplatin, **3a–c** are only 2–3 times less active and **3d** even exhibits the same activity in the SW480 cell line. In the CH1 and A549 cell lines **3a–d** are about one order of magnitude less active than cisplatin (Table 1), but significantly more active than the majority of known tumour-inhibiting organoruthenium compounds.

In order to demonstrate the multi-targeted character of $\text{Ru}^{\text{II}}(\text{flavone})$ complexes, we studied the inhibition of human topoisomerase II α activity and the binding ability to DNA models. Topoisomerase II α is over-expressed in many types of cancer and inhibitors, such as doxorubicin, etoposide and mitoxantrone, are routinely used in the clinic,²¹ but only a small number of Ru complexes with topoisomerase inhibitory

Table 1 *In vitro* anticancer activity^a (IC_{50} values in μM) of **2a–d** and **3a–d** in ovarian (CH1), colon (SW480) and non-small cell lung carcinoma (A549) compared to cisplatin and topoisomerase II α inhibition of **2a–d** and **3a–d**^b

	Topoisomerase inhibition ^b	$\text{IC}_{50}/\mu\text{M}$		
		CH1	SW480	A549
2a	+	1.9 ± 0.2	11 ± 3	25 ± 10
3a	++	2.1 ± 0.2	9.6 ± 1.5	20 ± 2
2b	+	1.1 ± 0.1	6.3 ± 1.1	81 ± 9
3b	++	1.8 ± 0.2	7.2 ± 0.5	17 ± 2
2c	+	1.56 ± 0.04	7.0 ± 0.9	37 ± 10
3c	++	1.7 ± 0.4	7.9 ± 2.1	18 ± 1
2d	++	0.60 ± 0.10	3.7 ± 0.4	7.9 ± 1.2
3d	+++	0.86 ± 0.06	3.8 ± 0.5	9.5 ± 0.5
cisplatin ^c	—	0.14 ± 0.03	3.3 ± 0.4	1.3 ± 0.4

^a 96 h exposure. ^b Estimated 50% inhibitory activity: + >40 μM , ++ $\approx$ 20–40 μM , +++ <20 μM inhibitor. ^c Taken from ref. 25.

activity have been reported. The major part of them are polypyridyl- and related Ru^{II} complexes with DNA intercalating ligands (for a review see ref. 22) and only a few $\text{Ru}^{\text{II}}(\text{arene})$ complexes are known which inhibit topoisomerases.^{23,24} In this study, human topoisomerase II α catalytic activity was determined by means of the decatenation assay (Fig. 3; Supporting information†).

Catenated kinetoplast DNA (kDNA) was incubated with topoisomerase II α in the presence of different concentrations of the flavone complexes **3a–d** and their ligands **2a–d**. Depending on the substituent in *para* position of the phenyl ring, differing potential to inhibit topoisomerase II α was observed. The chloro compound **3d** is the most potent inhibitor, and in general the extent of inhibition correlates well with the *in vitro* anticancer activity (Table 1). The complexes were generally more active than the ligands, which could however at least be related to a partial release of the ligand. The role of the metal centre in topoisomerase inhibition was recently shown for Cu-thiosemicarbazonato complexes, where the Cu compounds were about an order of magnitude more potent than the respective ligands.²⁶ However, $\text{Ru}^{\text{II}}(\text{arene})$ complexes *per se* inhibit the enzyme only to a minor extent, as demonstrated for $[\text{Ru}(\eta^6\text{-benzene})(\text{DMSO})\text{Cl}_2]$.²⁷ To the best of our knowledge, this is the first example of metal compounds that show topoisomerase inhibitory potency correlating to their antiproliferative activity.

The altered topoisomerase II α inhibitory activity of the complexes as compared to the ligands may be explained by the multi-targeted character of the complexes. In order to demonstrate the potential of the compounds to interact covalently with DNA as the second target molecule, the reactions of **3a–d**

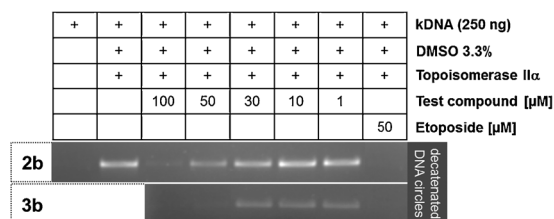


Fig. 3 Effect of complex **3b** and ligand **2b** on the catalytic activity of topoisomerase II α , as determined by the decatenation assay.

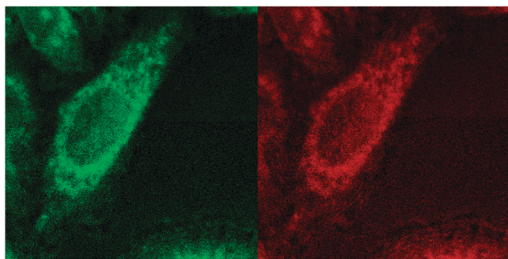


Fig. 4 Live cell imaging with confocal fluorescence microscopy in SW480 cells of **3c** (left) and co-stained with ER-Tracker™ Red (right).

with the DNA model compound 5'-GMP were studied by ^1H NMR spectroscopy. Complexes **3a–d** reacted quickly with the N7 atom of 5'-GMP (H8 shift from $\delta = 8.1$ to approximately 7.6 ppm). Notably, the flavone ligand remains attached to the Ru centre to interact with topoisomerase II α . However, the simultaneous interaction of the compounds with DNA and the protein is difficult to prove and will be subject of a separate study.

The flavonoids and their $\text{Ru}^{\text{II}}(\eta^6\text{-}p\text{-cymene})$ complexes are fluorescent, with an emission maximum at *ca.* 520 nm (**3c**; $\lambda_{\text{ex}} = 458$ nm). This intrinsic property was used to localise **3c** in SW480 cells in co-staining experiments with fluorescence confocal laser scanning microscopy (Fig. 4). **3c** and the endoplasmic reticulum (ER) marker ER-Tracker™ Red ($\lambda_{\text{ex}} = 587$ nm, $\lambda_{\text{em}} = 615$ nm) give largely overlapping signals, and therefore we conclude that the ER is the primarily targeted organelle. This observation is common for lipophilic compounds,²⁸ and the ER might act as a reservoir for the cytotoxic species.

In conclusion, the $\text{Ru}^{\text{II}}(\text{arene})\text{-flavonoid}$ system offers access to multi-targeted anticancer drugs consisting of a DNA binding metal centre and a biologically active ligand system inhibiting topoisomerase II α . With the accumulation in the endoplasmic reticulum as a reservoir for the anticancer active moiety and the covalent binding to DNA accompanied by increased topoisomerase II α inhibitory activity as compared to its ligand **2d** and the high *in vitro* antitumour activity, the $\text{Ru}^{\text{II}}(\eta^6\text{-}p\text{-cymene})$ complex **3d** is a promising development candidate for an anticancer drug following a double-strike approach.

We thank the University of Vienna, the Austrian Science Fund (FWF), the Johanna Mahlke geb. Obermann Foundation, and COST D39 for financial support. We gratefully acknowledge Alexander Roller for collecting the X-ray diffraction data.

References

† Crystallographic details: **3b**·CH₃OH: C₂₇H₂₉ClO₄Ru, $M_r = 554.02$, $0.12 \times 0.05 \times 0.01$ mm, triclinic, $P\bar{1}$, $a = 7.8882(5)$ Å, $b = 11.9690(9)$ Å, $c = 13.5794(11)$ Å, $\alpha = 74.879(5)^\circ$, $\beta = 73.366(4)^\circ$, $\gamma = 89.101(4)^\circ$, $V = 1183.49(15)$ Å³, $Z = 2$, $\rho_{\text{calcd}} = 1.555$ mg m⁻³, $\mu = 0.807$ mm⁻¹, Mo-K α , $\lambda = 0.71073$ Å, $T = 100(2)$ K, $2\theta_{\text{max}} = 27.50^\circ$, 5420 measured independent reflections, $R_{\text{int}} = 0.0972$, $R_1 = 0.0466$, $wR_2 = 0.1020$; description of data collection and refinement see supporting information;

CCDC 826085 contains the supplementary crystallographic data for this paper (The Cambridge Crystallographic Data Centre, www.ccdc.cam.ac.uk/data_request/cif).

- G. R. Zimmermann, J. Lehar and C. T. Keith, *Drug Discovery Today*, 2007, **12**, 34.
- W. H. Ang, A. Casini, G. Sava and P. J. Dyson, *J. Organomet. Chem.*, 2011, **696**, 989.
- D. Hamels, P. M. Dansette, E. A. Hillard, S. Top, A. Vessieres, P. Herson, G. Jaouen and D. Mansuy, *Angew. Chem., Int. Ed.*, 2009, **48**, 9124.
- W. H. Ang, L. J. Parker, A. De Luca, L. Juillerat-Jeanneret, C. J. Morton, M. Lo Bello, M. W. Parker and P. J. Dyson, *Angew. Chem., Int. Ed.*, 2009, **48**, 3854.
- I. Ott, B. Kircher, C. P. Bagowski, D. H. W. Vlecken, E. B. Ott, J. Will, K. Bensdorf, W. S. Sheldrick and R. Gust, *Angew. Chem., Int. Ed.*, 2009, **48**, 1160.
- J. E. Debreczeni, A. N. Bullock, G. E. Atilla, D. S. Williams, H. Bregman, S. Knapp and E. Meggers, *Angew. Chem., Int. Ed.*, 2006, **45**, 1580.
- R. J. Nijveldt, E. van Nood, D. E. van Hoorn, P. G. Boelens, K. van Norren and P. A. van Leeuwen, *Am. J. Clin. Nutr.*, 2001, **74**, 418.
- E. Middleton, Jr., C. Kandaswami and T. C. Theoharides, *Pharmacol. Rev.*, 2000, **52**, 673.
- M. Grazul and E. Budzisz, *Coord. Chem. Rev.*, 2009, **253**, 2588.
- X. Chen, L.-J. Tang, Y.-N. Sun, P.-H. Qiu and G. Liang, *J. Inorg. Biochem.*, 2010, **104**, 379.
- J. Ochocki, M. Kasprzak, L. Checinska, A. Erxleben, E. Zyner, L. Szmigiero, A. Garza-Ortiz and J. Reedijk, *Dalton Trans.*, 2010, **39**, 9711.
- O. J. Bandle and N. Osherooff, *Biochemistry*, 2007, **46**, 6097.
- T. A. Geissman and D. K. Fukushima, *J. Am. Chem. Soc.*, 1948, **70**, 1686.
- M. Bennett, A. J. Burke and W. I. O'Sullivan, *Tetrahedron*, 1996, **52**, 7163.
- C. X. Qin, X. Chen, R. A. Hughes, S. J. Williams and O. L. Woodman, *J. Med. Chem.*, 2008, **51**, 1874.
- W. Kandioller, C. G. Hartinger, A. A. Nazarov, J. Kasser, R. John, M. A. Jakupiec, V. B. Arion, P. J. Dyson and B. K. Keppler, *J. Organomet. Chem.*, 2009, **694**, 922.
- W. Kandioller, C. G. Hartinger, A. A. Nazarov, M. L. Kuznetsov, R. O. John, C. Bartel, M. A. Jakupiec, V. B. Arion and B. K. Keppler, *Organometallics*, 2009, **28**, 4249.
- M. G. Mendoza-Ferri, C. G. Hartinger, R. E. Eichinger, N. Stolyarova, K. Severin, M. A. Jakupiec, A. A. Nazarov and B. K. Keppler, *Organometallics*, 2008, **27**, 2405.
- R. E. Aird, J. Cummings, A. A. Ritchie, M. Muir, R. E. Morris, H. Chen, P. J. Sadler and D. I. Jodrell, *Br. J. Cancer*, 2002, **86**, 1652.
- M. G. Mendoza-Ferri, C. G. Hartinger, A. A. Nazarov, R. E. Eichinger, M. A. Jakupiec, K. Severin and B. K. Keppler, *Organometallics*, 2009, **28**, 6260.
- A. K. Larsen, A. E. Escargueil and A. Skladanowski, *Pharmacol. Ther.*, 2003, **99**, 167.
- F. Gao, H. Chao and L.-N. Ji, *Chem. Biodiversity*, 2008, **5**, 1962.
- S. K. Singh, S. Joshi, A. R. Singh, J. K. Saxena and D. S. Pandey, *Inorg. Chem.*, 2007, **46**, 10869.
- F. Beckford, D. Dourth, M. Shaloski, J. Didion, J. Thessing, J. Woods, V. Crowell, N. Gerasimchuk, A. Gonzalez-Sarrias and N. P. Seeram, *J. Inorg. Biochem.*, 2011, **105**, 1019.
- Y. Y. Scaffidi-Domianello, A. A. Legin, M. A. Jakupiec, V. B. Arion, V. Y. Kukushkin, M. Galanski and B. K. Keppler, *Inorg. Chem.*, 2011, **50**, 10673.
- B. M. Zeglis, V. Divilov and J. S. Lewis, *J. Med. Chem.*, 2011, **54**, 2391.
- Y. N. V. Gopal, D. Jayaraju and A. K. Kondapi, *Biochemistry*, 1999, **38**, 4382.
- D. Marko, K.-H. Merz, C. Kunz, A. Müller, N. Tarasova and G. Eisenbrand, *Biochem. Pharmacol.*, 2002, **63**, 669.

SUPPORTING INFORMATION

Targeting the DNA-topoisomerase complex in a double-strike approach with a topoisomerase inhibiting moiety and covalent DNA binder

Andrea Kurzwernhart, Wolfgang Kandioller, Caroline Bartel, Simone Bächler, Robert Trondl, Gerhard Mühlgassner, Michael A. Jakupec, Vladimir B. Arion, Doris Marko, Bernhard K. Keppler, and Christian G. Hartinger

Table of Contents

1. Materials and methods.....	2
2. Synthetic procedures	2
2.1. Synthesis of 3-hydroxyflavones	2
2.2. Synthesis of ruthenium(II)(η^6 - <i>p</i> -cymene) complexes	4
3. Biological and biophysical experiments	6
3.1. Cytotoxicity in cancer cell lines	6
3.1.1. Cell lines and culture conditions	6
3.1.2. MTT assay	6
3.2. Interaction with the DNA model compound 5'-GMP	7
3.3. Determination of topoisomerase II activity	7
3.4. Live cell imaging	7
4. Acknowledgement.....	7
5. References.....	8

1. Materials and methods

All solvents were dried and distilled prior to use. 2-Hydroxyacetophenone **1** (Fluka, Acros Organics), benzaldehyde **a** (Fluka), 4-tolualdehyde **b** (Acros Organics), 4-fluorobenzaldehyde **c** (Fluka), 4-chlorobenzaldehyde **d** (Acros Organics), ruthenium(III) chloride (Johnson Matthey), α -terpinene (Acros Organics), sodium methoxide (Aldrich) were purchased and used without further purification. Bis[(η^6 -*p*-cymene)dichloridoruthenium(II)] was synthesized as described elsewhere.^[1]

Melting points were determined with a Büchi *Melting Point* B-540 apparatus. Elemental analyses were carried out with a Perkin Elmer 2400 CHN Elemental Analyser at the Microanalytical Laboratory of the University of Vienna. NMR spectra were recorded at 25 °C using a Bruker FT-NMR spectrometer Avance III™ 500 MHz. ¹H NMR spectra were measured in *d*₆-DMSO or CDCl₃ at 500.10 MHz and ¹³C{¹H} NMR spectra at 125.75 MHz. The 2D NMR spectra were recorded in a gradient-enhanced mode. The X-ray diffraction measurements were performed with single crystals of **3b**·CH₃OH using a Bruker X8 APEXII CCD diffractometer at 100 K. The single crystal was positioned at 40 mm from the detector, and 1541 frames were measured, each for 80 s over 1° scan width. The data were processed using the SAINT software package.^[2] 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. The following computer programs were used: structure solution, SHELXS-97; refinement, SHELXL-97;^[3] molecular diagrams, ORTEP-3;^[4] scattering factors.^[5]

2. Synthetic procedures

2.1. Synthesis of 3-hydroxyflavones

General procedure: NaOH (5 M, 4.3 eq) was added to a solution of 2'-hydroxyacetophenone **1** and aldehyde **a-d** in ethanol and the mixture was stirred for 18 h at room temperature. The reaction mixture was acidified by addition of acetic acid (30%) and the 2'-hydroxychalcone was isolated by filtration. The 2'-hydroxychalcone was suspended in ethanol, and NaOH (5 M, 2 eq) and H₂O₂ (30%, 2.2 eq) were added at 4 °C. The mixture was stirred for 18 h at room temperature, afterwards acidified with HCl (2 M) and poured into water (400 mL). The precipitate was collected by filtration and the pure product was obtained by recrystallization from methanol.

3-Hydroxy-2-phenyl-4H-chromen-4(1H)-one (2a): The synthesis was performed according to the general procedure using **1** (2.00 g, 14.7 mmol, 1 eq) and **a** (1.56 g, 14.7 mmol, 1 eq) to

afford **2a** as a yellow powder (2.63 g, 75%). Mp: 165–169 °C; ^1H NMR (500.10 MHz, d_6 -DMSO): δ = 7.49–7.54 (m, 2H, H4'/H7), 7.59 (dd, $^3J(\text{H,H})$ = 7 Hz, $^3J(\text{H,H})$ = 7 Hz, 2H, H3'/H5'), 7.79 (d, $^3J(\text{H,H})$ = 7 Hz, 1H, H8), 7.83–7.81 (m, 1H, H6), 8.14 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.24 (d, $^3J(\text{H,H})$ = 7 Hz, 2H, H2'/H6'), 9.64 (br s, 1H, OH) ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, d_6 -DMSO): δ = 118.9 (C8), 121.8 (C8a), 125.1 (C7), 125.3 (C5), 128.1 (C2'/C6'), 129.0 (C3'/C5'), 130.4 (C4'), 131.8 (C2), 134.2 (C6), 139.6 (C1'), 145.7 (C3), 155.1 (C4a), 173.5 (C4) ppm; elemental analysis calcd for $\text{C}_{15}\text{H}_{10}\text{O}_3$: C 75.62, H 4.23%; found: C 75.62, H 4.09%.

3-Hydroxy-2-(4-methylphenyl)-4H-chromen-4(1H)-one (2b): The synthesis was performed according to the general procedure using **1** (2.00 g, 14.7 mmol, 1 eq) and **b** (1.76 g, 14.7 mmol, 1 eq) to afford **2b** as yellow crystals (1.64 g, 44%). Mp: 191–195 °C; ^1H NMR (500.10 MHz, d_6 -DMSO): δ = 2.41 (s, 3H, CH₃), 7.40 (d, $^3J(\text{H,H})$ = 8 Hz, 2H, H3'/H5'), 7.47–7.50 (m, 1H, H7), 7.79 (d, $^3J(\text{H,H})$ = 7 Hz, 1H, H8), 7.79–7.82 (m, 1H, H6), 8.14 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.16 (d, $^3J(\text{H,H})$ = 8 Hz, 2H, H2'/H6'), 9.56 (br s, 1H, OH) ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, d_6 -DMSO): δ = 21.5 (CH₃), 118.9 (C8), 121.7 (C8a), 125.0 (C7), 125.3 (C5), 128.1 (C2'/C6'), 129.1 (C2), 129.6 (C3'/C5'), 134.1 (s, C6), 139.4 (C1'), 140.3 (C4'), 145.9 (C3), 155.1 (C4a), 173.4 (C4) ppm; elemental analysis calcd for $\text{C}_{16}\text{H}_{12}\text{O}_3 \cdot 0.15\text{H}_2\text{O}$: C 75.37, H 4.86%; found: C 75.38, H 4.47%.

3-Hydroxy-2-(4-fluorophenyl)-4H-chromen-4(1H)-one (2c): The synthesis was performed according to the general procedure using **1** (2.00 g, 14.7 mmol, 1 eq) and **c** (1.17 g, 14.7 mmol, 1 eq) to afford **2c** as yellow needles (1.65 g, 44%). Mp: 151–152 °C; ^1H NMR (500.10 MHz, d_6 -DMSO): δ = 7.42–7.45 (m, 2H, H3'/H5'), 7.47–7.50 (m, 1H, H7), 7.79 (d, $^3J(\text{H,H})$ = 7 Hz, 1H, H8), 7.81–7.83 (m, 1H, H6), 8.13 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.29–8.32 (m, 2H, H2'/H6'), 9.72 (br s, 1H, OH) ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, d_6 -DMSO): δ = 116.1 (d, $^2J(\text{H,H})$ = 22 Hz, C3'/C5'), 118.9 (C8), 121.8 (C8a), 125.1 (C7), 125.3 (C5), 128.3 (d, $^4J(\text{C,F})$ = 3 Hz, C1'), 130.6 (d, $^3J(\text{C,F})$ = 8 Hz, C2'/C6'), 134.2 (C6), 139.3 (C2), 144.9 (C3), 155.0 (C4a), 163.0 (d, $^1J(\text{C,F})$ = 249 Hz, C4'), 173.4 (C4) ppm; elemental analysis calcd for $\text{C}_{15}\text{H}_9\text{O}_3\text{F} \cdot 0.15\text{H}_2\text{O}$: C 69.58, H 3.62%; found: C 69.66, H 3.47%.

3-Hydroxy-2-(4-chlorophenyl)-4H-chromen-4(1H)-one (2d): The synthesis was performed according to the general procedure using **1** (2.00 g, 14.7 mmol, 1 eq) and **d** (2.10 g, 14.7 mmol, 1 eq) to afford **2d** as yellow powder (2.31 g, 58%). Mp: 202–204 °C; ^1H NMR (500.10 MHz, d_6 -DMSO): δ = 7.49 (m, 1H, H7), 7.65 (d, $^3J(\text{H,H})$ = 8 Hz, 2H, H3'/H5'), 7.78 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.83–7.85 (m, 1H, H6), 8.13 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.26 (d, $^3J(\text{H,H})$ = 9 Hz, 2H, H2'/H6'), 9.84 (br s, 1H, OH) ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, d_6 -DMSO): δ = 118.9 (C8), 121.7 (C8a), 125.1 (C7), 125.3 (C5), 129.2 (C2'/C6'), 129.8 (C3'/C5'), 130.7 (C2), 134.4 (C6), 139.9 (C4'), 140.3 (C1'), 144.5 (C3), 155.0 (C4a), 173.5 (C4) ppm; elemental analysis calcd for $\text{C}_{15}\text{H}_9\text{O}_3\text{Cl} \cdot 0.05\text{H}_2\text{O}$: C 65.85, H 3.35%; found: C 65.80, H 3.17%.

2.2. Synthesis of ruthenium(II)(η^6 -*p*-cymene) complexes

General procedure: A solution of $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ in CH_2Cl_2 (15 mL) was added to a solution of the 3-hydroxyflavone ligands **2a–d** and sodium methoxide in methanol (15 mL). The reaction mixture was stirred at room temperature and under argon atmosphere for 18 h. The solvent was evaporated in vacuum; the residue was extracted with dichloromethane, filtered and concentrated. Pure complex was obtained by recrystallization from methanol.

Chlorido[3-(oxo- κO)-2-phenyl-chromen-4(1*H*)-onato- κO](η^6 -*p*-cymene)ruthenium(II) (3a**):**

The reaction was performed according to the general complexation procedure using **2a** (164 mg, 0.73 mmol, 1 eq), NaOMe (43 mg, 0.8 mmol, 1.1 eq) and $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol, 0.45 eq) to afford **3a** as a deep red powder (170 mg, 51%). Mp: 229–230 °C (decomp.); ^1H NMR (500.10 MHz, CDCl_3): δ = 1.42 (m, 6H, $\text{CH}_{3,\text{Cym}}$), 2.43 (s, 3H, $\text{CH}_{3,\text{Cym}}$), 3.02 (m, 1H, CH_{Cym}), 5.39 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H3/H5 $_{\text{Cym}}$), 5.67 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H2/H6 $_{\text{Cym}}$), 7.33–7.35 (m, 1H, H7), 7.41 (dd, $^3J(\text{H,H})$ = 7 Hz, $^3J(\text{H,H})$ = 7 Hz, 1H, H4'), 7.48 (dd, $^3J(\text{H,H})$ = 7 Hz, $^3J(\text{H,H})$ = 7 Hz, 2H, H3'/H5'), 7.57 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.59–7.61 (m, 1H, H6), 8.22 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.61 (d, $^3J(\text{H,H})$ = 7 Hz, 2H, H2'/H6') ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 18.7 ($\text{CH}_{3,\text{Cym}}$), 22.5 ($\text{CH}_{3,\text{Cym}}$), 31.3 (CH_{Cym}), 78.0 (C3/C5 $_{\text{Cym}}$), 81.0 (C2/C6 $_{\text{Cym}}$), 95.9 (C4 $_{\text{Cym}}$), 98.9 (C1 $_{\text{Cym}}$), 117.9 (C8), 120.0 (C8a), 124.0 (C7), 124.6 (C5), 127.3 (C2'/C6'), 128.2 (C3'/C5'), 129.3 (C4'), 132.5 (C2), 132.6 (C6), 149.2 (C1'), 153.9 (C4a), 154.0 (C3), 183.3 (C4) ppm; elemental analysis calcd for $\text{C}_{25}\text{H}_{23}\text{O}_3\text{ClRu}$: C 59.11, H 4.56%; found: C 59.04, H 4.39%.

Chlorido[3-(oxo- κO)-2-(4-methylphenyl)-chromen-4(1*H*)-onato- κO](η^6 -*p*-cymene)ruthenium(II) (3b**):**

The reaction was performed according to the general complexation procedure using **2b** (184 mg, 0.73 mmol, 1 eq), NaOMe (43 mg, 0.8 mmol, 1.1 eq) and $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol, 0.45 eq) to afford **3b** as a red powder (240 mg, 68%). Single crystals were grown from CHCl_3/n -hexane, suitable for X-ray diffraction analysis. Mp: 235–236 °C (decomp.); ^1H NMR (500.10 MHz, CDCl_3): δ = 1.41 (m, 6H, $\text{CH}_{3,\text{Cym}}$), 2.42 (s, 3H, $\text{CH}_{3,\text{Cym}}$), 2.44 (s, 3H, CH_3), 3.01 (m, 1H, CH_{Cym}), 5.38 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H3/H5 $_{\text{Cym}}$), 5.65 (dd, $^3J(\text{H,H})$ = 6 Hz, $^3J(\text{H,H})$ = 6 Hz, 2H, H2/H6 $_{\text{Cym}}$), 7.28 (d, $^3J(\text{H,H})$ = 9 Hz, 2H, H3'/H5'), 7.32–7.35 (m, 1H, H7), 7.55 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.58–7.61 (m, 1H, H6), 8.21 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.50 (d, $^3J(\text{H,H})$ = 8 Hz, 2H, H2'/H6') ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 18.7 ($\text{CH}_{3,\text{Cym}}$), 21.6 (CH_3), 22.5 ($\text{CH}_{3,\text{Cym}}$), 31.2 (CH_{Cym}), 77.9 (C3/C5 $_{\text{Cym}}$), 80.9 (C2/C6 $_{\text{Cym}}$), 95.9 (C4 $_{\text{Cym}}$), 98.9 (C1 $_{\text{Cym}}$), 117.8 (C8), 120.4 (C8a), 124.0 (C7), 124.5 (C5), 127.3 (C2'/C6'), 129.0 (C3'/C5'), 130.0 (C2), 132.6 (C6), 139.8 (C4'), 149.9 (C1'), 153.7 (C4a), 154.2 (C3), 183.1 (C4) ppm; elemental analysis calcd for $\text{C}_{26}\text{H}_{25}\text{O}_3\text{ClRu}$: C 59.82, H 4.83%; found: C 59.82, H 4.57%.

Chlorido[3-(oxo- κO)-2-(4-fluorophenyl)-chromen-4(1*H*)-onato- κO](η^6 -*p*-cymene)ruthenium(II) (3c**):**

The reaction was performed according to the general

complexation procedure using **2c** (187 mg, 0.73 mmol, 1 eq), NaOMe (43 mg, 0.8 mmol, 1.1 eq) and $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol, 0.45 eq) to afford **3c** as red needles (230 mg, 66%). Mp: 235–236 °C (decomp.); ^1H NMR (500.10 MHz, CDCl_3): δ = 1.42 (m, 6H, $\text{CH}_{3,\text{Cym}}$), 2.42 (s, 3H, $\text{CH}_{3,\text{Cym}}$), 3.00 (m, 1H, CH_{Cym}), 5.40 (dd, $^3J(\text{H,H}) = 5$ Hz, $^3J(\text{H,H}) = 5$ Hz, 2H, $\text{H3/H5}_{\text{Cym}}$), 5.66 (dd, $^3J(\text{H,H}) = 5$ Hz, $^3J(\text{H,H}) = 5$ Hz, 2H, $\text{H2/H6}_{\text{Cym}}$), 7.15–7.18 (m, 2H, H3'/H5'), 7.33–7.36 (m, 1H, H7), 7.54 (d, $^3J(\text{H,H}) = 8$ Hz, 1H, H8), 7.60–7.62 (m, 1H, H6), 8.22 (dd, $^4J(\text{H,H}) = 1$ Hz, $^3J(\text{H,H}) = 8$ Hz, 1H, H5), 8.61–8.64 (m, 2H, H2'/H6') ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 18.7 ($\text{CH}_{3,\text{Cym}}$), 22.5 ($\text{CH}_{3,\text{Cym}}$), 31.3 (CH_{Cym}), 78.0 ($\text{C3/C5}_{\text{Cym}}$), 81.0 ($\text{C2/C6}_{\text{Cym}}$), 95.9 (C4_{Cym}), 98.9 (C1_{Cym}), 115.2 (d, $^2J(\text{C,F}) = 21$ Hz, C3'/C5'), 117.7 (C8), 120.1 (C8a), 124.1 (C7), 124.6 (C5), 128.8 (C2), 129.4 (d, $^3J(\text{C,F}) = 8$ Hz, C2'/C6'), 132.6 (C6), 148.4 (d, $^4J(\text{C,F}) = 1$ Hz, C1'), 153.8 (C4a), 154.1 (C3), 163.0 (d, $^1J(\text{C,F}) = 251$ Hz, C4'), 183.3 (C4) ppm; elemental analysis calcd for $\text{C}_{25}\text{H}_{22}\text{O}_3\text{FCIRu}$: C 57.09, H 4.22%; found: C 56.98, H 4.06%.

Chlorido[3-(oxo- κO)-2-(4-chlorophenyl)-chromen-4(1H)-onato- κO](η^6 -*p*-

cymene)ruthenium(II) (3d): The reaction was performed according to the general complexation procedure using **2d** (199 mg, 0.73 mmol, 1 eq), NaOMe (43 mg, 0.8 mmol, 1.1 eq) and $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol, 0.45 eq) to afford **3d** as a deep red powder (179 mg, 100%). Mp: 214–217 °C (decomp.); ^1H NMR (500.10 MHz, CDCl_3): δ = 1.42 (m, 6H, $\text{CH}_{3,\text{Cym}}$), 2.42 (s, 3H, $\text{CH}_{3,\text{Cym}}$), 3.01 (m, 1H, CH_{Cym}), 5.39 (dd, $^3J(\text{H,H}) = 5$ Hz, $^3J(\text{H,H}) = 5$ Hz, 2H, $\text{H3/H5}_{\text{Cym}}$), 5.66 (dd, $^3J(\text{H,H}) = 5$ Hz, $^3J(\text{H,H}) = 5$ Hz, 2H, $\text{H2/H6}_{\text{Cym}}$), 7.32–7.35 (m, 1H, H7), 7.44 (d, $^3J(\text{H,H}) = 9$ Hz, 2H, H3'/H5'), 7.54 (d, $^3J(\text{H,H}) = 8$ Hz, 1H, H8), 7.62–7.63 (m, 1H, H6), 8.21 (dd, $^4J(\text{H,H}) = 1$ Hz, $^3J(\text{H,H}) = 8$ Hz, 1H, H5), 8.55 (d, $^3J(\text{H,H}) = 9$ Hz, 2H, H2'/H6') ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 18.7 ($\text{CH}_{3,\text{Cym}}$), 22.4 ($\text{CH}_{3,\text{Cym}}$), 30.0 (CH_{Cym}), 77.9 ($\text{C3/C5}_{\text{Cym}}$), 81.0 ($\text{C2/C6}_{\text{Cym}}$), 95.9 (C4_{Cym}), 99.0 (C1_{Cym}), 117.8 (C8), 120.0 (C8a), 124.1 (C7), 124.7 (C5), 125.5 (C2'/C6'/C3'/C5'), 131.0 (C2), 132.8 (C6), 134.9 (C4'), 143.5 (C1'), 153.9 (C4a), 154.6 (C3), 183.5 (C4); elemental analysis calcd for $\text{C}_{25}\text{H}_{22}\text{O}_3\text{Cl}_2\text{Ru}$: C 55.36, H 4.09%; found: C 55.28, H 3.90%.

3. Biological and biophysical experiments

3.1. Cytotoxicity in cancer cell lines

3.1.1. Cell lines and culture conditions

CH1 cells originate from an ascites sample of a patient with a papillary cystadenocarcinoma of the ovary and were a generous gift from Lloyd R. Kelland, CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK. SW480 (human adenocarcinoma of the colon) and A549 (human non-small cell lung cancer) cells were kindly provided by Brigitte Marian (Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Austria). All cell culture reagents were obtained from Sigma-Aldrich Austria. Cells were grown in 75 cm² culture flasks (Iwaki) as adherent monolayer cultures in Minimum Essential Medium (MEM) supplemented with 10% heat inactivated fetal calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine and 1% nonessential amino acids (100x). Cultures were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂.

3.1.2. MTT assay

Cytotoxicity was determined by the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Fluka] microculture assay. For this purpose, cells were harvested from culture flasks by trypsinization and seeded in 100 µL aliquots into 96-well microculture plates (Iwaki). Cell densities of 1.5×10^3 cells/well (CH1), 2.5×10^3 cells/well (SW480) and 4×10^3 cells/well (A549) were chosen in order to ensure exponential growth of untreated controls throughout the experiment. Cells were allowed to settle and resume exponential growth in drug-free complete culture medium for 24 h. Stock solutions of the test compounds in DMSO were appropriately diluted in complete culture medium so that the maximum DMSO content did not exceed 1%. These dilutions were added in 100 µL aliquots to the microcultures and cells were exposed to the test compounds for 96 hours. At the end of exposure, all media were replaced by 100 µL/well RPMI1640 culture medium (supplemented with 10% heat-inactivated fetal bovine serum) plus 20 µL/well MTT solution in phosphate-buffered saline (5 mg/ml). After incubation for 4 h, the supernatants were removed, and the formazan crystals formed by viable cells were dissolved in 150 µL DMSO per well. Optical densities at 550 nm were measured with a microplate reader (Tecan Spectra Classic), using a reference wavelength of 690 nm to correct for unspecific absorption. The quantity of viable cells was expressed in terms of T/C values by comparison to untreated controls, and 50% inhibitory concentrations (IC₅₀) were calculated from concentration-effect curves by interpolation. Evaluation is based on means from at least three independent experiments, each comprising at least three replicates per concentration level.

3.2. Interaction with the DNA model compound 5'-GMP

Complexes **3a–d** (~0,1 mg/mL) were dissolved in D₂O (containing 10% *d*₆-DMSO due to the low solubility in water), yielding the corresponding highly reactive aqua species. The aqua complexes were converted *in situ* by addition of 100 µL of 5'-GMP solution (10 mg/mL) into the respective 5'-GMP adduct and the reaction was monitored by ¹H NMR.

3.3. Determination of topoisomerase II activity

Effects on the catalytic activity of topoisomerase II were determined using a decatenation assay. Catenated kinetoplast DNA (kDNA) was used as a substrate. kDNA is an aggregate of interlocked DNA minicircles (mostly 2.5 kb), which can be released by topoisomerase II. kDNA (200 ng, TopoGen, OH, USA) was incubated in a final volume of 30 µL (containing 40 ng of topoisomerase IIα; 50 mM Tris, pH 7.9; 120 mM KCl; 10 mM MgCl₂; 1 mM ATP; 0.5 mM DTT; 0.5 mM EDTA; and 0.03 mg/mL BSA) at 37 °C for 60 min. The reaction was stopped by the addition of 1/10 volume of 1 mg/mL proteinase K in 10% (w/v) SDS and incubation at 37 °C for further 30 min. Gel electrophoresis was performed in the absence of ethidium bromide at 60 V for 3 h in 1% (w/v) agarose gels with Tris acetate/EDTA buffer (40 mM Tris; 1 mM EDTA, pH 8.5; and 20 mM acetic acid). Subsequently, the gel was stained in 10 µg/mL ethidium bromide solution for 20 min. The fluorescence of ethidium bromide was detected with the LAS-4000 system (Fujifilm, Raytest, Germany).

3.4. Live cell imaging

Live cell images of SW480 colon carcinoma cells were obtained with a confocal laser scanning microscope (CSLM, Leica) at the Institution of Cell Imaging and Ultrastructure Research, University of Vienna, Austria. Cells were pretreated with 600 µM of **3c** for 5 min at 37 °C, and pictures were taken after excitation with a 458 nm laser. Co-staining experiments were performed with ER-TrackerTM Red (Invitrogen, Paisely, UK) at a concentration of 1 µM according to the protocol given by the manufacturer. ER-TrackerTM Red was chosen, because the excitation spectrum of this dye is not interfering with spectra of compound **3c**.

4. Acknowledgement

We are grateful to Prof. Irene Lichtscheidl (Institution of Cell Imaging and Ultrastructure Research, University of Vienna, Austria) for providing access to the confocal laser scanning microscopic equipment.

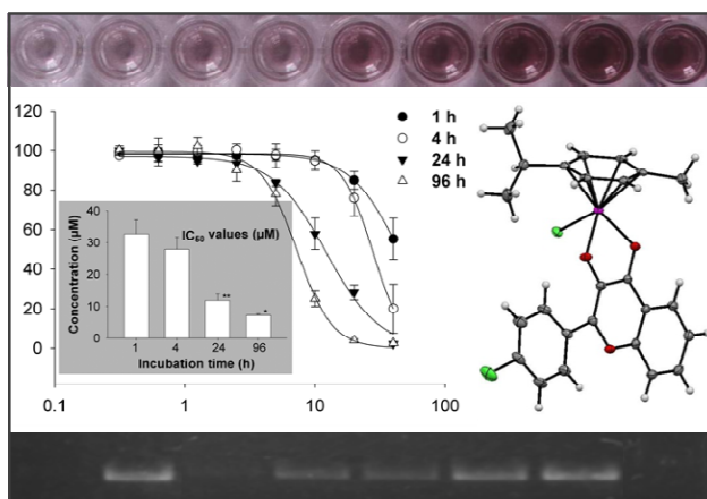
5. References

- [1] M. A. Bennett, A. K. Smith, *J. Chem. Soc., Dalton Trans.* **1974**, 233-241.
- [2] M. R. Pressprich, J. Chambers, Bruker Analytical X-ray systems, Madison, **2004**.
- [3] G. M. Sheldrick, *Acta Crystallogr., Sect. A: Found. Crystallogr.* **2008**, A64, 112-122.
- [4] L. J. Farrugia, *J. Appl. Crystallogr.* **1997**, 30, 565.
- [5] *International Tables for X-ray Crystallography, Vol. C*, Kluwer Academic Press, Dordrecht, The Netherlands, **1992**.

3.3. Structure–Activity Relationships of Targeted Ru^{II}(η^6 -*p*-Cymene) Anticancer Complexes with Flavonol-Derived Ligands

Journal of Medicinal Chemistry **2012**, 55, 10512–10522.

Graphical abstract



Ru^{II}(arene) complexes have been shown to be promising anticancer agents, capable of overcoming major drawbacks of currently used chemotherapeutics. We have synthesized Ru^{II}(η^6 -arene) compounds carrying bioactive flavonol ligands with the aim to obtain multi-targeted anticancer agents. To validate this concept, studies on the mode of action of the complexes were conducted which indicated that they form covalent bonds to DNA, have only minor impact on the cell cycle, but inhibit CDK2 and topoisomerase II α in vitro. The cytotoxic activity was determined in human cancer cell lines, resulting in very low IC₅₀ values as compared to other Ru^{II}(arene) complexes and showing a structure–activity relationship dependent on the substitution pattern of the flavonol ligand. Furthermore, the inhibition of cell growth correlates well with the topoisomerase inhibitory activity. Compared to the flavonol ligands, the Ru^{II}(η^6 -*p*-cymene) complexes are more potent antiproliferative agents, which can be explained by potential multitargeted properties.

Structure–Activity Relationships of Targeted Ru^{II}(η^6 -*p*-Cymene) Anticancer Complexes with Flavonol-Derived Ligands

Andrea Kurzwernhart,[†] Wolfgang Kandioller,^{†,‡} Simone Bächler,[§] Caroline Bartel,[†] Sanela Martić,^{||} Magdalena Buczkowska,[⊥] Gerhard Mühlgassner,[†] Michael A. Jakupec,^{†,‡} Heinz-Bernhard Kraatz,^{||} Patrick J. Bednarski,[⊥] Vladimir B. Arion,[†] Doris Marko,[§] Bernhard K. Keppler,^{†,‡} and Christian G. Hartinger^{*,†,‡,‡,‡}

[†]Institute of Inorganic Chemistry, University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria

[‡]Research Platform “Translational Cancer Therapy Research”, University of Vienna, Waehringer Strasse 42, 1090 Vienna, Austria

[§]Institute of Food Chemistry and Toxicology, University of Vienna, Waehringer Strasse 38, 1090 Vienna, Austria

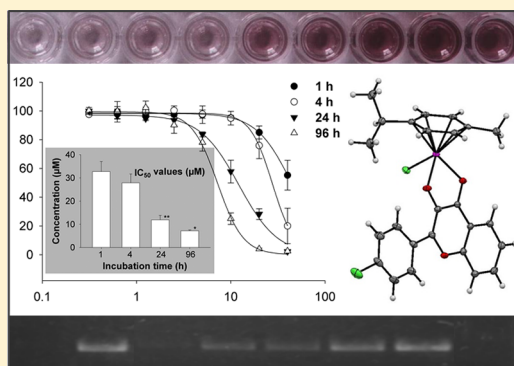
^{||}Department of Physical and Environmental Sciences, University of Toronto, Scarborough, Canada

[⊥]Department of Pharmaceutical and Medicinal Chemistry, University of Greifswald, 17487 Greifswald, Germany

[#]The University of Auckland, School of Chemical Sciences, Private Bag 92019, Auckland 1142, New Zealand

Supporting Information

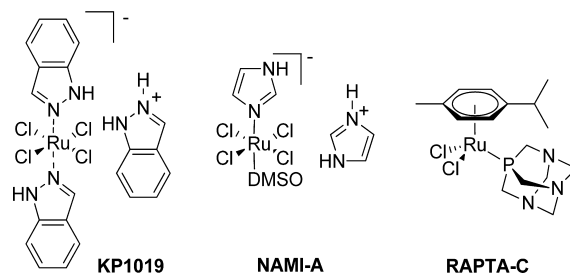
ABSTRACT: Ru^{II}(arene) complexes have been shown to be promising anticancer agents, capable of overcoming major drawbacks of currently used chemotherapeutics. We have synthesized Ru^{II}(η^6 -arene) compounds carrying bioactive flavonol ligands with the aim to obtain multitargeted anticancer agents. To validate this concept, studies on the mode of action of the complexes were conducted which indicated that they form covalent bonds to DNA, have only minor impact on the cell cycle, but inhibit CDK2 and topoisomerase II α in vitro. The cytotoxic activity was determined in human cancer cell lines, resulting in very low IC₅₀ values as compared to other Ru^{II}(arene) complexes and showing a structure–activity relationship dependent on the substitution pattern of the flavonol ligand. Furthermore, the inhibition of cell growth correlates well with the topoisomerase inhibitory activity. Compared to the flavonol ligands, the Ru^{II}(η^6 -*p*-cymene) complexes are more potent antiproliferative agents, which can be explained by potential multitargeted properties.



INTRODUCTION

Metallodrugs have become important compounds in cancer therapy, and, in particular, platinum complexes are used worldwide against many tumor types.¹ To overcome severe side effects and drug resistance during treatment, which are their major drawbacks,² complexes with metal ions other than platinum have become the focus of research.¹ Especially, ruthenium complexes offer a number of interesting properties such as lower toxicity and a range of physiologically accessible oxidation states.³ The Ru^{III} complexes NAMI-A and KP1019 have shown the most promising results in preclinical and clinical studies (Chart 1).^{4–6} The more selective activity of these compounds due to an efficient uptake as protein adducts and activation by reduction inside the tumor is thought to be responsible for the low general toxicity.⁴ During the last years, organometallic Ru^{II} complexes, especially half-sandwich Ru^{II}(arene) compounds, moved into the focus of interest because biological activity and pharmacological properties can easily be modulated by ligand selection. Important examples of this compound type comprise the RAPTA family⁷ (Chart 1) containing the pta ligand (1,3,5-triaza-7-phosphatricyclo-

Chart 1. Chemical Structures of KP1019, NAMI-A, and RAPTA-C



[3.3.1.1]decane) and Ru^{II}(arene) complexes of bidentate ethylenediamine, which are at an advanced preclinical development stage.⁸

The RAPTA complexes are a good example of organometallics which can be equipped with ligands to obtain desired

Received: July 30, 2012

Published: November 7, 2012



properties. Whereas the parent compound RAPTA-C is a metastasis inhibitor, tethering the organometallic fragment to ethacrynic acid resulted in compounds with glutathione-S-transferase inhibitory activity, accompanied by a cleavage of the enzyme inhibiting moiety from the metal fragment which can target a second biomolecule, e.g., DNA. This concept of multitargeted anticancer agents, i.e., drugs designed to act against several individual molecular targets, offers a number of advantages over classic chemotherapeutics, including tunable pharmacological properties and anticancer activity and altered metabolism and resistance development.⁹ Such drugs can be prepared by linking metal fragments to biologically active ligand systems and, as shown in the RAPTA case, by functionalization of an arene ligand or, as previously reported by us, by direct coordination of 3-hydroxyflavones to a Ru^{II}(cym) moiety (cym = η^6 -*p*-cymene).¹⁰

Flavonoids are known as natural components of plants, fruits, and vegetables and exhibit interesting biological properties such as antioxidant, anti-inflammatory, estrogenic, antimicrobial, and anticarcinogenic activity.^{11–13} The 3-hydroxy-4-keto motif of 3-hydroxyflavones offers facile coordination to many metal ions. The resulting metal complexes exhibit high stability constants, high molar absorptances, and fluorescence properties and are biocompatible.^{14,15} Therefore, such compounds have been investigated as new fluorochromic indicators for ion chelation and biomembrane structure studies in the human body.¹⁶ In addition, a few examples of tumor-inhibiting metal–flavonoid complexes are known.¹⁷

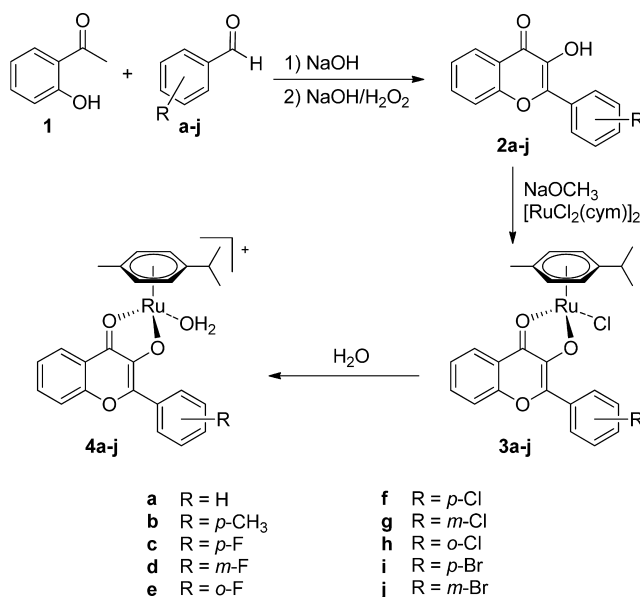
Herein, we report a series of Ru^{II}(cym) complexes with 3-hydroxyflavone ligands with the aim to study the influence of the substituent on the phenyl ring of the ligand on the anticancer activity. To support the hypothesis of multitargeted compounds, their behavior in aqueous solution and in the presence of nucleotides was studied. Furthermore, the inhibition of cyclin-dependent kinases (CDKs) and their effect on the cell cycle has been assayed, as well as their human topoisomerase II α inhibitory activity.

RESULTS AND DISCUSSION

We have extensively studied the application of *O,O*-chelating hydroxypyrrone complexes as potential anticancer agents and recently extended these investigations to 3-hydroxyflavones with the same binding motif.¹⁰ To establish structure–activity relationships (SAR), a series of derivatives was synthesized in two steps, starting with a Claisen–Schmidt condensation of 2-hydroxyacetophenone **1** and benzaldehydes **a–j** in alkaline solution. In the second step, the obtained 2'-hydroxychalcones were converted into the respective 3-hydroxyflavones **2a–j** under Algar–Flynn–Oyamada reaction conditions with hydrogen peroxide and NaOH (Scheme 1).^{18–20} The Ru^{II}(cym) complexes **3a–j** were synthesized by deprotonation of **2a–j** with sodium methoxide and subsequent reaction with bis-[dichlorido(η^6 -*p*-cymene)ruthenium(II)] (Scheme 1).²¹ All complexes were obtained in moderate to good yields (30–68%) and are stable for more than one year, even if exposed to sunlight and air, which was confirmed by NMR spectroscopy and elemental analysis.

All synthesized compounds were characterized by standard methods (see Experimental Section), and single crystals of **3d**, **3f**, and **3h** were analyzed by X-ray diffraction methods (Figure 1) and are compared to **3b**.¹⁰ Complex **3d** crystallizes in the centrosymmetric space group $P\bar{1}$ and **3f** and **3h** in the monoclinic space group $P2_1/n$. The compounds feature the

Scheme 1. Synthesis of 3-Hydroxyflavone Ligands and their Ru^{II}(η^6 -*p*-Cymene) Complexes



pseudo-octahedral “piano-stool” configuration similar to related Ru^{II}(cym) complexes.^{10,22} The 3-hydroxyflavone acts as a bidentate ligand, forming a nonplanar, envelope-like five-membered metallocycle. The phenyl substituent of the ligand is twisted with a torsion angle of 48.4(4)° in **3h**, but only 5.1(3)° in **3f**, 0.2(3)° in **3d**, and 15.3(6)° in **3b**. The two Ru–O bonds were found to be slightly different as in **3b** with 2.0664(14) and 2.1154(14) Å in **3d**, 2.0747(11) and 2.1098(11) in **3f**, and 2.0870(16) and 2.1339(16) Å in **3h**, which is in accordance to Ru–O bonds in similar compounds.^{23,24} The Ru–Cl bonds [2.4105(5) Å (**3d**), 2.4132(4) Å (**3f**), and 2.4100(7) Å (**3h**)] are in the range of recently published Ru^{II}(cym) complexes [2.4200(11)–2.4273(8) Å]^{4–7,10} but shorter than in **3b** with 2.4326(10) Å. The Ru–cym_{centroid} distances were found at 1.638 Å for both **3d** and **3f** and at 1.647 Å for **3h**.

The aqueous stability of the complexes was studied by ¹H NMR spectroscopy (Figure 2). Dissolution of organometallic Ru(arene) compounds featuring an *O,O*-chelating motif and a halido ligand often results in rapid exchange of the chlorido ligand by an aqua moiety.²⁵ The same behavior was demonstrated for such complexes which aquated within seconds, leading to charged **4a–c**, **4f**, and **4i** (Scheme 1).^{26,27} The hydrolyzed compounds are relatively stable in aqueous solution for about 24 h, however, after 6 days, the formation of the dimeric hydrolysis side product [Ru₂(cym)₂(OH)₃]⁺ was clearly visible in ESI-mass and NMR spectra. This species does not affect the cytotoxic activity as it is thermodynamically stable and unreactive toward nucleophiles.^{25,28} Notably, **3a–j** exhibit approximately 10-fold better solubility in water than the respective ligands, e.g., 0.03 mg/mL for **2a** and 0.3 mg/mL for **3a** in 1% DMSO/H₂O.

Cytotoxic Activity. The *in vitro* anticancer activity was determined in the human cancer cell lines CH1 (ovarian carcinoma), SW480 (colon carcinoma), and A549 (non-small cell lung carcinoma) by means of the colorimetric MTT assay and in human urinary bladder (5637), human large cell lung (LCLC-103H), and human pancreatic carcinoma cell lines (DAN-G) with the crystal violet assay, both after 96 h.

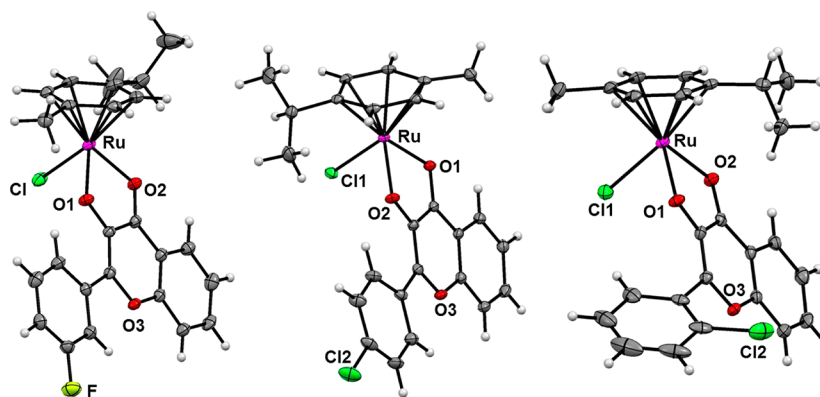


Figure 1. Molecular structures of the Ru^{II}(cym) complexes **3d** (left), **3f** (center), and **3h** (right).

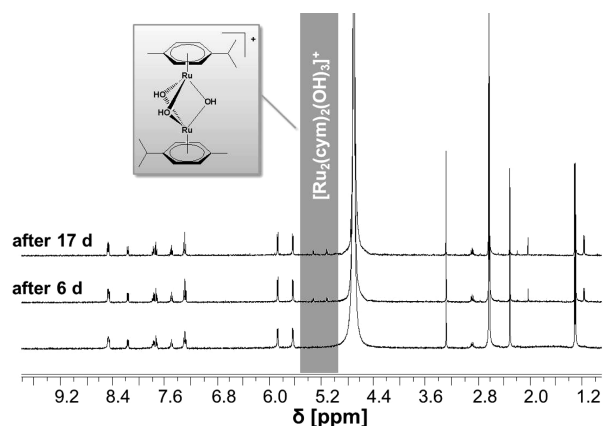


Figure 2. Hydrolysis of **3c** in 10% DMSO-*d*₆/D₂O studied by ¹H NMR spectroscopy. After 6 d, the formation of the dimeric hydrolysis side product [Ru₂(cym)₂(OH)₃]⁺ was observed giving signals at 5.1 and 5.3 ppm.

In general, the biological activity as determined by the MTT assay and given as the IC₅₀ values (50% inhibitory concentration; Table 1) was in the low micromolar range, which is very unusual for Ru^{II}(arene) compounds, and only a few more examples with similar cytotoxicity are known.^{4,29,30} The chemosensitive CH1 cell line was most sensitive to the test compounds with IC₅₀ values lower than 7.9 μM, followed by SW480 with IC₅₀ values ranging from 3.4–26 μM. The lowest cytotoxic potency was found for the generally more chemo-

resistant A549 cells. In comparison to compound **3a** with an unsubstituted ligand structure, *ortho* substitution of the phenyl ring (**3e** and **3h**) appears unfavorable, whereas *meta* and *para* substitution (**3f**, **3g**, **3i**) increases the anticancer activity. This may be due to a structural effect, as the phenyl ring is considerably more twisted in the *ortho* derivative than in the *meta*- and *para*-substituted compounds (Figure 1), which may influence the interaction with biological targets.³¹ However, the type of substituent, whether electron-withdrawing or electron-donating, seems to be of minor importance, whereas their position appears to be crucial for the cytotoxic activity. We have shown earlier that the ligands are the bioactive moiety, which is mainly responsible for the antiproliferative activity.¹⁰ However, they are poorly soluble in aqueous solutions and therefore hardly suitable for pharmaceutical applications.

The crystal violet assay for antiproliferative activity in the 5637, the LCLC-103H, and the DAN-G cell lines (Table 1) revealed slightly higher IC₅₀ values than that for the MTT assay in the CH1 cell line but still notable for Ru^{II}(arene) complexes. Compounds **3d** and **3f** exhibit the lowest IC₅₀ values at around 5 μM in these three cell lines and also show the highest potency in the MTT assays. Their antiproliferative activity is in the same range as that of related dinuclear Ru(arene)(pyridone) compounds.³² Compounds **3b** and **3d** show the best overall activity in the used cell lines. However, some compounds are more specific for one of the three cell lines used in the crystal violet assays, e.g., **3i** for DAN-G, **3f** and **3j** for 5637, and **3c** and **3g** for LCLC-103H, which is a highly desirable characteristic for anticancer agents.^{32,33}

Table 1. In Vitro Anticancer Activity of **3a–j** (96 h Exposure) in Human Ovarian, Colon, Non-Small Cell Lung, Urinary Bladder, Large Cell Lung, and Pancreatic Carcinoma Cell Lines (Mean Values ± SD of Three Independent Determinations unless Otherwise Noted)

compd	IC ₅₀ values/μM					
	CH1	SW480	A549	5637	LCLC-103H	DAN-G
3a	2.1 ± 0.2	9.6 ± 1.5	20 ± 2	11 ± 5	13 ± 6	12 ± 2
3b	1.8 ± 0.2	7.2 ± 0.5	17 ± 2	5.7 ± 3.2	5.2 ± 0.8	6.6 ± 2.5
3c	1.7 ± 0.4	7.9 ± 2.0	18 ± 1	33 ± 5	5.5 ± 5.2	12 ± 2
3d	1.5 ± 0.1	7.0 ± 1.0	15 ± 1	4.3 ± 2.5	4.3 ± 1.1	5.3 ± 1.6
3e	4.0 ± 0.8	24 ± 3	30 ± 1	nd	nd	nd
3f	0.86 ± 0.06	3.8 ± 0.5	9.5 ± 0.5	3.3 ± 1.1	13 ± 1	19 ± 7
3g	1.0 ± 0.1	7.0 ± 0.7	12 ± 2	30 ± 2	5.0 ± 3.5	19 ± 5
3h	7.9 ± 0.6	26 ± 1	51 ± 5	nd	nd	nd
3i	1.2 ± 0.2	3.4 ± 0.1	8.6 ± 0.7	12 ± 1	19 ± 6	5.7 ± 1.9
3j	2.3 ± 0.7	7.2 ± 0.4	17 ± 3	5.9 ± 1.2	16 ± 4	20 ± 5

Furthermore, the time dependence of the cytotoxicity of **3j** was investigated and the IC_{50} values of **3j** were determined after 1, 4, 24, and 96 h in SW480 cells (Figure 3). The calculated IC_{50} values for **3j** decreased from about $>40 \mu M$ after 1 h to $12 \mu M$ after 24 h and finally to $7 \mu M$ after 96 h.

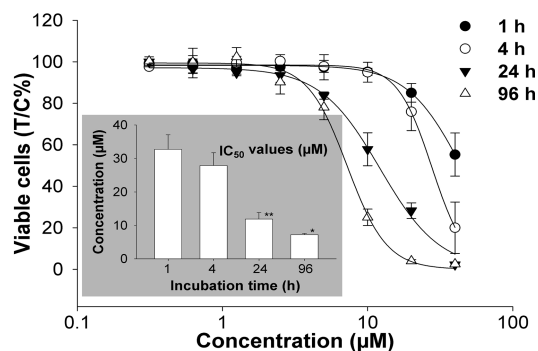


Figure 3. Time-dependent anticancer activity of **3j** in SW480 cells. Significances indicated refer to the next shorter incubation time calculated by Student's *t* test (* $p < 0.1$, ** $p < 0.05$).

Inhibition of CDK2. The anticancer activity of the flavonoid flavopiridol is linked to its inhibition of cyclin-dependent kinases (CDKs),³⁴ and therefore this group of proteins is also a possible target for Ru–flavonoid complexes. To estimate the compounds' potential to inhibit kinases, the inhibition of CDK2 was studied by an electrochemical assay employing Fc-ATP as a cosubstrate in the kinase phosphorylation reaction.^{35,36} The peptide HHASPRK was used as a substrate and immobilized onto the Au surface for the study of CDK2/cyclin A protein kinase activity. Following the kinase-catalyzed Fc-phosphorylation reaction, the Fc group is transferred from Fc-ATP to the immobilized peptide and results in an electrochemical signal. Hence, the electrochemical readout is directly related to the extent of Fc-phosphorylation by a protein kinase. In general, the Fc-phosphorylations are monitored by square-wave voltammetry wherein the oxidation peak at $\sim 440 \pm 5$ mV signals the successful Fc-phosphorylation. The dependence of the integrated current density in CDK2/cyclin A kinase assays as a function of inhibitors **3a–h** ($10 \mu M$) is shown in Figure 4. With the exception of **3c** and **3f**, all compounds inhibit CDK2 in about the same range as structurally related HOPO complexes³⁷ and are nearly as active as the well-known CDK2 inhibitor roscovitine (**R**), which was included for comparison (Figure 4). It appears that *para* substituents at the phenyl ring of the flavone ligands are less favorable than *ortho* and *meta* substitution. However, no direct structure–activity relationships can be derived when compared to the *in vitro* anticancer activity data set. Therefore, considering also the minor influence on the cell cycle (see below), CDK2 and CDKs in general are not considered as the main target of this type of compounds.

Impact on the Cell Cycle. The cell cycle distribution of A549 cells was studied by treating exponentially growing A549 cells with **3a–c** and **3e** in various concentrations for 48 h. Then cells were stained with propidium iodide and analyzed for their DNA content by flow cytometry. The four Ru compounds have comparable effects on the cell cycle, most probably due to their similar structures. The effects vary in dependence of the concentration (Figure 5). Low concentrations ($<32 \mu M$ of **3a**, **3b**, and **3c** and $<16 \mu M$ of **3e**) induce a slight G_2/M arrest,

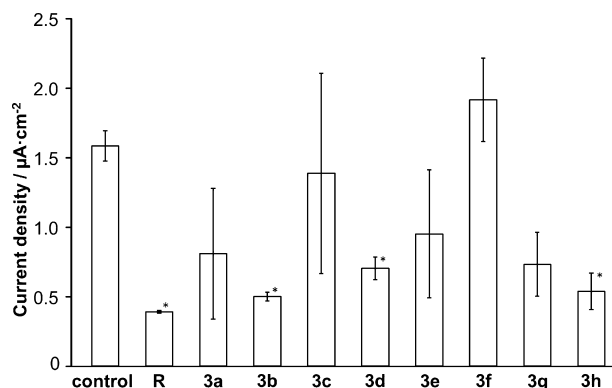


Figure 4. Integrated current densities (estimated from square-wave voltammograms) observed in CDK2/cyclin A kinase-catalyzed Fc-phosphorylation of the surface bound peptide in the presence of the organometallic compounds (**3a–3h**) and compared to roscovitine (**R**) (inhibitor concentration = $10 \mu M$, 100 mV/s scan rate, 0.1 M phosphate buffer pH 7.4 electrolyte, triplicate measurements). Significances indicated refer to the control calculated by Student's *t* test (* $p < 0.1$, ** $p < 0.05$).

accompanied by a decrease of the G_0/G_1 -phase fraction, whereas at higher concentrations these effects tend to level out again. The impact on the S phase is less pronounced, but a slight increase can be observed at increasing concentrations of **3b**, **3c**, and especially **3f** ($>16 \mu M$, Figure 5). These results indicate that CDK2 is not very likely to be an intracellular target because this kinase is involved in the G_1/S transition of the cell cycle, which is not influenced in a dose-dependent manner by the Ru complexes studied.

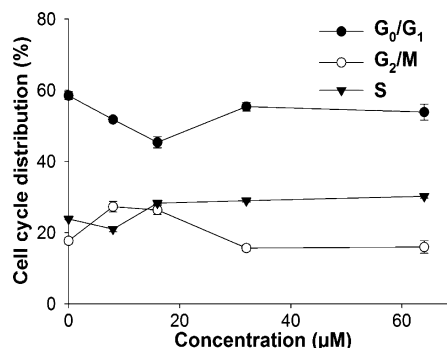


Figure 5. Concentration-dependent impact of **3f** on the cell cycle distribution of A549 cells after exposure for 48 h (values are means $\pm$ standard deviations of two independent experiments).

Topoisomerase Inhibition. Topoisomerases are overexpressed in many types of cancer and thus are major targets for antineoplastic agents such as doxorubicin, etoposide, and mitoxantrone.^{38,39} Flavonoids are also known to inhibit human topoisomerases,^{40,41} which are enzymes that change the topology of DNA by introducing a transient break in the DNA strand, allowing a second DNA region from either the same molecule (relaxation, knotting, or unknotting) or a different molecule (catenation or decatenation) to pass through. During this process, the enzymes are covalently bound to the DNA via an active tyrosine residue, termed “cleavable complex”. After the DNA is untangled or unwound, the strands are reannealed by the enzyme so that the overall composition of the DNA strand does not change. DNA

topoisomerases are participating in nearly all biological processes involving DNA including replication, transcription, recombination, and chromatin remodeling.^{42,43} They are classified as type I and type II depending on if they induce single- or double-strand breaks, respectively.

In the case of topoisomerase inhibitors, an increase of the proportion of cells in the S-phase and at higher concentrations a G2/M arrest are expected. In the case of the Ru complexes studied here, a slight increase in the S-phase fraction was observed (Figure 5) and therefore topoisomerase inhibition studies were conducted. The catalytic activity of topoisomerase II α is determined by means of the decatenation assay. Catenated kinetoplast DNA (kDNA) consists of interlocked DNA minicircles (mostly 2.5 kb), which can be released to single DNA circles by catalytically active topoisomerase II. If topoisomerase II is inhibited, kDNA stays in its catenated form, which is not able to enter the agarose gel, whereas the single DNA circles, released by topoisomerase II, are able to migrate into the gel (Figure 6, compare lane 1 with lane 2). Thus,

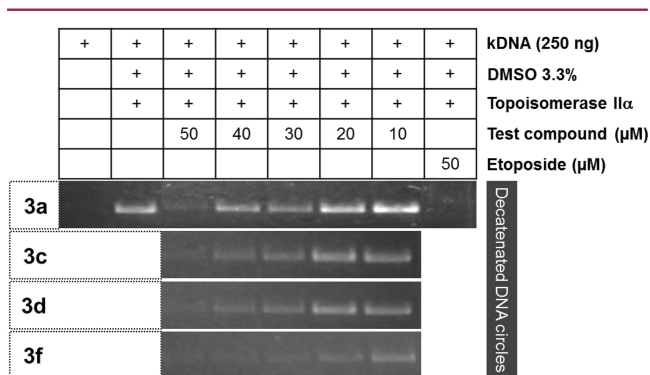


Figure 6. Concentration-dependent effect of the Ru complexes **3a**, **3c**, **3d**, and **3f** on the catalytic activity of topoisomerase II α , as determined by the decatenation assay. The topoisomerase poison etoposide was used as a control.

kDNA was incubated with topoisomerase II α in the presence of different concentrations of the Ru^{II}(cym) complexes **3a–3d**, **3f**, **3g**, **3i**, and **3j** and the flavonols **2a–c**, **2f**, and **2i**. All complexes inhibit the catalytic activity of human topoisomerase II α at concentrations $\geq 10 \mu$ M. The extent of inhibition appears to be well correlated to their in vitro anticancer potency, with **3f** being the most potent inhibitor followed by **3d** (Figure 6), and with **3a** as the least cytotoxic and weakest inhibitor of topoisomerase II α . Compared to the flavonols, their Ru^{II}(cym) complexes are more potent in all studied cases, demonstrating that the inhibition of topoisomerase II α is enhanced by the linkage of the topoisomerase-inhibiting flavonoid scaffold to the Ru^{II}(cym) moiety. We explain this observation by multitargeted properties of the complexes, as they are also able to interact with the DNA.¹⁰

Nucleotide Binding. To help estimate the reactivity to DNA, the interaction of the aqua species **4a–c**, **4f**, and **4i** with the DNA model compound 5'-GMP was investigated by ¹H NMR spectroscopy. The 5'-GMP N7-adducts were formed immediately, as indicated by an upfield shift of the H8 signal of 5'-GMP from approximately $\delta = 8.1$ to 7.6 ppm.^{10,44} Therefore, DNA represents a suitable binding partner for this class of compounds.

CONCLUSIONS

Flavonoids exhibit interesting biological properties, and members of this compound class have been investigated in clinical trials as anticancer agents. By combining such moieties with metal fragments, we aimed to obtain anticancer agents that can interact with more than one target, facilitating single-molecular combination therapy. A series of light- and air-stable Ru^{II}(η^6 -*p*-cymene) complexes with flavonol ligands were prepared in good yields. The flavonol ligands **2a–j** are hardly soluble in aqueous solutions, which prevents their potential use as anticancer agents. In contrast, the Ru^{II}(cym) complexes **3a–j** exhibit approximately 10-fold better solubility in water than the ligands. However, they aquate within seconds by an exchange of the chlorido ligand with an aqua moiety, leading to **4a–j**, which are then able to bind to DNA, as shown for the DNA model compound 5'-GMP. The flavonol-derived compounds exhibit IC₅₀ values in the low μ M range, which is unusual for Ru^{II}(cym) complexes, but this indicates the central role of the ligand system as the anticancer activity determining factor. This is also highlighted by comparison of the IC₅₀ values of the coordination compounds and the respective ligand systems.¹⁰ Complexes **3f**, **3g**, and **3i** with *para*- and *meta*-substituted ligands exhibit lower IC₅₀ values than their unsubstituted analogue **3a** or the *ortho*-substituted derivatives **3e** and **3h**.

To gain information on the mode of action of the complexes and in light of the biological properties of the ligands, the inhibitory activity of the complexes on topoisomerase II α and CDK2 was assayed and flow cytometry analyses of the cell cycle were conducted. These studies revealed that **3a–c** and **3e** have an influence on the cell cycle distribution, and especially at concentrations around the IC₅₀ values of the compounds, an increase in the cell fraction in G₀/G₁ phase was observed. Furthermore, most of the complexes were shown to inhibit CDK2 to an extent approaching that of roscovitine, a well-known CDK2 inhibitor. However, these results did not resemble the activity pattern observed in the in vitro anticancer assays. Notably, the inhibition of topoisomerase II α correlated well with the in vitro anticancer activity data, with the compounds exhibiting the lowest IC₅₀ values in the MTT assay being also the most potent topoisomerase II α inhibitors. Compared to the unsubstituted flavonol ligands **2a–j**, which are considered as the topoisomerase-inhibiting moiety in the coordination compound, the respective Ru^{II}(cym) complexes **3a–j** are shown to inhibit topoisomerase II α to a greater extent. This may be explained by their additional ability to form bonds to the DNA base guanine and thereby acting in a bifunctional manner, which could be beneficial in tumor therapy.

EXPERIMENTAL SECTION

All solvents were dried and distilled prior to use. 2-Hydroxyacetophenone **1** (Fluka, Acros Organics), benzaldehyde **a** (Fluka), 4-tolualdehyde (Acros Organics), 4-fluorobenzaldehyde **c** (Fluka), 3-fluorobenzaldehyde **d** (Fluka), 2-fluorobenzaldehyde **e** (Fluka), 4-chlorobenzaldehyde **f** (Acros Organics), 3-chlorobenzaldehyde **g** (Aldrich), 2-chlorobenzaldehyde **h** (Aldrich), 4-bromobenzaldehyde **i** (Aldrich), 3-bromobenzaldehyde **j** (Aldrich), ruthenium(III) chloride (Johnson Matthey), α -terpinene (Acros Organics), and sodium methoxide (Aldrich) were purchased and used without further purification. Bis[(η^6 -*p*-cymene)dichloridoruthenium(II)] was synthesized as described elsewhere.⁴⁵

Melting points were determined with a Büchi melting point B-540 apparatus. Elemental analyses were carried out with a Perkin-Elmer 2400 CHN elemental analyzer by the Microanalytical Laboratory of the University of Vienna. NMR spectra were recorded at 25 °C using a

Bruker FT-NMR spectrometer Avance III 500 MHz. ^1H NMR spectra were measured at 500.10 MHz and $^{13}\text{C}\{^1\text{H}\}$ -NMR spectra at 125.75 MHz in $\text{DMSO}-d_6$ or CDCl_3 . The 2D NMR spectra were measured in a gradient-enhanced mode.

The X-ray diffraction data for **3d**, **3f**, and **3h** were collected on a Bruker X8 APEX II CCD diffractometer at 100 K. The single crystals were positioned at 35, 35, and 40 mm from the detector, and 1746, 1367, and 1667 frames were measured, each for 20, 5, and 5 s over 1° scan width. The data were processed using the SAINT software package.⁴⁶ The structures were solved by direct methods and refined by full-matrix least-squares techniques. Non-hydrogen atoms were refined with anisotropic displacement parameters. H atoms were inserted at calculated positions and refined with a riding model. The following computer programs were used: structure solution, SHELXS-97; refinement, SHELXL-97;⁴⁷ molecular diagrams, ORTEP-3.⁴⁸ The crystallographic data files for **3d**, **3f**, and **3h** have been deposited with the Cambridge Crystallographic Database as CCDC 886667, 886666, and 886665, respectively.

General Procedure for the Synthesis of the 3-Hydroxy-flavone Ligands 2a–2j. NaOH (5 M, 4.3 equiv) was added to a solution of 2-hydroxyacetophenone **1** (1.0 equiv) and aldehydes **a–i** (1.0 equiv) in ethanol, and the solution was stirred for 18 h at room temperature. The reaction mixture was acidified to pH 6 by addition of acetic acid (30%), and the 2'-hydroxychalcone was isolated by filtration. The 2'-hydroxychalcone (1.0 equiv) was suspended in ethanol, NaOH (5 M, 2.0 equiv), and H_2O_2 (30%, 2.2 equiv) were added at 4°C . The mixture was stirred for 18 h at room temperature, afterward acidified to pH 1 with HCl (2 M) and poured onto water (400 mL). The precipitate was collected by filtration, and the pure product was obtained by recrystallization from methanol.

3-Hydroxy-2-phenyl-chromen-4(1H)-one (2a). The synthesis was performed according to the general procedure by using **1** (2.00 g, 14.7 mmol) and **a** (1.56 g, 14.7 mmol) to afford **2a** as a yellow powder (2.63 g, 75%); mp $165\text{--}168^\circ\text{C}$. ^1H NMR (500.10 MHz, $\text{DMSO}-d_6$): $\delta = 7.49\text{--}7.54$ (m, 2H, H4'/H7'), 7.59 (dd, $^3J(\text{H,H}) = 7$ Hz, $^3J(\text{H,H}) = 7$ Hz, 2H, H3'/H5'), 7.79 (d, $^3J(\text{H,H}) = 7$ Hz, 1H, H8), 7.83–7.81 (m, 1H, H6), 8.14 (dd, $^4J(\text{H,H}) = 1$ Hz, $^3J(\text{H,H}) = 8$ Hz, 1H, H5), 8.24 (d, $^3J(\text{H,H}) = 7$ Hz, 2H, H2'/H6'), 9.64 (br s, 1H, OH) ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, $\text{DMSO}-d_6$): $\delta = 118.9$ (C8), 121.8 (C8a), 125.1 (C7), 125.3 (C5), 128.1 (C2'/C6'), 129.0 (C3'/C5'), 130.4 (C4'), 131.8 (C2), 134.2 (C6), 139.6 (C1'), 145.7 (C3), 155.1 (C4a), 173.5 (C4) ppm. Elemental Anal. calcd for $\text{C}_{15}\text{H}_{10}\text{O}_3$: C 75.62, H 4.23%. Found: C 75.62, H 4.09%.

3-Hydroxy-2-(4-methylphenyl)-chromen-4(1H)-one (2b). The synthesis was performed according to the general procedure by using **1** (2.00 g, 14.7 mmol) and **b** (1.76 g, 14.7 mmol) to afford **2b** as yellow crystals (1.64 g, 44%); mp $191\text{--}194^\circ\text{C}$. ^1H NMR (500.10 MHz, $\text{DMSO}-d_6$): $\delta = 2.41$ (s, 3H, CH₃), 7.40 (d, $^3J(\text{H,H}) = 8$ Hz, 2H, H3'/H5'), 7.47–7.50 (m, 1H, H7), 7.79 (d, $^3J(\text{H,H}) = 7$ Hz, 1H, H8), 7.79–7.82 (m, 1H, H6), 8.14 (dd, $^4J(\text{H,H}) = 1$ Hz, $^3J(\text{H,H}) = 8$ Hz, 1H, H5), 8.16 (d, $^3J(\text{H,H}) = 8$ Hz, 2H, H2'/H6'), 9.56 (br s, 1H, OH) ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, $\text{DMSO}-d_6$): $\delta = 21.5$ (CH₃), 118.9 (C8), 121.7 (C8a), 125.0 (C7), 125.3 (C5), 128.1 (C2'/C6'), 129.1 (C2), 129.6 (C3'/C5'), 134.1 (s, C6), 139.4 (C1'), 140.3 (C4'), 145.9 (C3), 155.1 (C4a), 173.4 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{16}\text{H}_{12}\text{O}_3 \cdot 0.15\text{H}_2\text{O}$: C 75.37, H 4.86%. Found: C 75.38, H 4.47%.

3-Hydroxy-2-(4-fluorophenyl)-chromen-4(1H)-one (2c). The synthesis was performed according to the general procedure by using **1** (2.00 g, 14.7 mmol) and **c** (1.82 g, 14.7 mmol) to afford **2c** as yellow needles (1.65 g, 44%); mp $151\text{--}152^\circ\text{C}$. ^1H NMR (500.10 MHz, $\text{DMSO}-d_6$): $\delta = 7.42\text{--}7.45$ (m, 2H, H3'/H5'), 7.47–7.50 (m, 1H, H7), 7.79 (d, $^3J(\text{H,H}) = 7$ Hz, 1H, H8), 7.81–7.83 (m, 1H, H6), 8.13 (dd, $^4J(\text{H,H}) = 1$ Hz, $^3J(\text{H,H}) = 8$ Hz, 1H, H5), 8.29–8.32 (m, 2H, H2'/H6'), 9.72 (br s, 1H, OH) ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, $\text{DMSO}-d_6$): $\delta = 116.1$ (d, $^2J(\text{H,H}) = 22$ Hz, C3'/C5'), 118.9 (C8), 121.8 (C8a), 125.1 (C7), 125.3 (C5), 128.3 (d, $^4J(\text{C,F}) = 3$ Hz, C1'), 130.6 (d, $^3J(\text{C,F}) = 8$ Hz, C2'/C6'), 134.2 (C6), 139.3 (C2), 144.9 (C3), 155.0 (C4a), 163.0 (d, $^1J(\text{C,F}) = 249$ Hz, C4'), 173.4 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{15}\text{H}_9\text{FO}_3 \cdot 0.15\text{H}_2\text{O}$: C 69.58, H 3.62%. Found: C 69.66, H 3.47%.

3-Hydroxy-2-(3-fluorophenyl)-chromen-4(1H)-one (2d). The synthesis was performed according to the general procedure by using **1** (2.00 g, 14.7 mmol) and **d** (1.82 g, 14.7 mmol), affording **2d** as a yellow powder (0.89 g, 50%); mp $171\text{--}173^\circ\text{C}$. ^1H NMR (500.10 MHz, $\text{DMSO}-d_6$): $\delta = 7.37$ (ddd, $^4J(\text{H,F}) = 2$ Hz, $^3J(\text{H,H}) = 8$ Hz, $^3J(\text{H,H}) = 8$ Hz, 1H, H4'), 7.48–7.51 (m, 1H, H7), 7.64 (ddd, $^4J(\text{H,F}) = 2$ Hz, $^3J(\text{H,H}) = 8$ Hz, $^3J(\text{H,H}) = 8$ Hz, 1H, H5'), 7.81–7.86 (m, 2H, H6/H8), 8.04 (dd, $^4J(\text{H,H}) = 2$ Hz, $^3J(\text{H,H}) = 11$ Hz, 1H, H5), 8.10–8.14 (m, 2H, H2'/H6'), 9.93 (br s, 1H, OH) ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, $\text{DMSO}-d_6$): $\delta = 114.6$ (d, $^2J(\text{C,F}) = 24$ Hz, C2'), 117.1 (d, $^2J(\text{C,F}) = 21$ Hz, C4'), 119.0 (C8), 121.7 (C8a), 124.1 (d, $^4J(\text{C,F}) = 3$ Hz, C6'), 125.2 (C7), 125.3 (C5), 131.1 (d, $^3J(\text{C,F}) = 8$ Hz, C5'), 134.0 (d, $^3J(\text{C,F}) = 9$ Hz, C1'), 134.4 (C6), 140.0 (C2), 144.0 (C3), 155.0 (C4a), 162.0 (d, $^1J(\text{C,F}) = 243$ Hz, C3'), 173.6 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{15}\text{H}_9\text{FO}_3$: C 70.31, H 3.54%. Found: C 69.94, H 3.39%.

3-Hydroxy-2-(2-fluorophenyl)-chromen-4(1H)-one (2e). The synthesis was performed according to the general procedure by using **1** (2.00 g, 14.7 mmol) and **e** (1.82 g, 14.7 mmol), affording **2e** as a yellow powder (0.87 g, 49%); mp $181\text{--}182^\circ\text{C}$. ^1H NMR (500.10 MHz, $\text{DMSO}-d_6$): $\delta = 7.37\text{--}7.42$ (m, 2H, H3'/H6'), 7.48–7.51 (m, 1H, H7), 7.59–7.63 (m, 1H, H4'), 7.66 (d, $^3J(\text{H,H}) = 8$ Hz, 1H, H8), 7.76–7.82 (m, 2H, H5'/H6), 8.16 (dd, $^4J(\text{H,H}) = 1$ Hz, $^3J(\text{H,H}) = 8$ Hz, 1H, H5), 9.41 (br s, 1H, OH) ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, $\text{DMSO}-d_6$): $\delta = 116.6$ (d, $^2J(\text{C,F}) = 21$ Hz, C3'), 118.9 (C8), 119.5 (d, $^2J(\text{C,F}) = 14$ Hz, C1'), 122.3 (C8a), 124.9 (d, $^3J(\text{C,F}) = 3$ Hz, C6'), 125.2 (C7), 125.5 (C5), 131.7 (d, $^4J(\text{C,F}) = 2$ Hz, C5'), 133.0 (d, $^3J(\text{C,F}) = 8$ Hz, C4'), 134.3 (C6), 140.0 (C3), 143.9 (C2), 155.5 (C4a), 159.0 (d, $^1J(\text{C,F}) = 252$ Hz, C2'), 173.2 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{15}\text{H}_9\text{FO}_3 \cdot 0.05\text{H}_2\text{O}$: C 70.31, H 3.54%. Found: C 69.98, H 3.42%.

3-Hydroxy-2-(4-chlorophenyl)-chromen-4(1H)-one (2f). The synthesis was performed according to the general procedure by using **1** (2.00 g, 14.7 mmol) and **f** (2.10 g, 14.7 mmol) to afford **2f** as yellow powder (2.31 g, 58%); mp $202\text{--}204^\circ\text{C}$. ^1H NMR (500.10 MHz, $\text{DMSO}-d_6$): $\delta = 7.48\text{--}7.51$ (m, 1H, H7), 7.65 (d, $^3J(\text{H,H}) = 8$ Hz, 2H, H3'/H5'), 7.78 (d, $^3J(\text{H,H}) = 8$ Hz, 1H, H8), 7.83–7.85 (m, 1H, H6), 8.13 (dd, $^4J(\text{H,H}) = 1$ Hz, $^3J(\text{H,H}) = 8$ Hz, 1H, H5), 8.26 (d, $^3J(\text{H,H}) = 9$ Hz, 2H, H2'/H6'), 9.84 (br s, 1H, OH) ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, $\text{DMSO}-d_6$): $\delta = 118.9$ (C8), 121.7 (C8a), 125.1 (C7), 125.3 (C5), 129.2 (C2'/C6'), 129.8 (C3'/C5'), 130.7 (C2), 134.4 (C6), 139.9 (C4'), 140.3 (C1'), 144.5 (C3), 155.0 (C4a), 173.5 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{15}\text{H}_9\text{ClO}_3$: C 66.07, H 3.33%. Found: C 65.80, H 3.17%.

3-Hydroxy-2-(3-chlorophenyl)-chromen-4(1H)-one (2g). The synthesis was performed according to the general procedure by using **1** (2.00 g, 14.7 mmol) and **g** (2.10 g, 14.7 mmol), affording **2g** as a yellow powder (1.15 g, 29%); mp $157\text{--}159^\circ\text{C}$. ^1H NMR (500.10 MHz, $\text{DMSO}-d_6$): $\delta = 7.48\text{--}7.51$ (m, 1H, H4'), 7.58–7.64 (m, 2H, H5'/H7), 7.81–7.85 (m, 2H, H6/H8), 8.12 (d, $^3J(\text{H,H}) = 8$ Hz, 1H, H5), 8.20 (d, $^3J(\text{H,H}) = 8$ Hz, 1H, H6'), 8.28 (s, 1H, H2'), 9.93 (br s, 1H, OH) ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, $\text{DMSO}-d_6$): $\delta = 119.0$ (C8), 121.7 (C8a), 125.2 (C7), 125.3 (C5), 126.7 (C6'), 127.5 (C2'), 130.1 (C4'), 131.0 (C5'), 133.8 (C2/C3'), 134.4 (C6), 140.1 (C1'), 143.9 (C3), 155.1 (C4a), 173.6 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{15}\text{H}_9\text{ClO}_3 \cdot 0.1\text{H}_2\text{O}$: C 65.64, H 3.38%. Found: C 65.69, H 3.19%.

3-Hydroxy-2-(2-chlorophenyl)-chromen-4(1H)-one (2h). The synthesis was performed according to the general procedure by using **1** (2.00 g, 14.7 mmol) and **h** (2.10 g, 14.7 mmol), affording **2h** as deep-yellow needles (2.69 g, 67%); mp $177\text{--}179^\circ\text{C}$. ^1H NMR (500.10 MHz, $\text{DMSO}-d_6$): $\delta = 7.49\text{--}7.54$ (m, 2H, H5'/H7), 7.57–7.61 (m, 1H, H3'), 7.66–7.68 (m, 2H, H4'/H8), 7.72 (dd, $^4J(\text{H,H}) = 2$ Hz, $^3J(\text{H,H}) = 8$ Hz, 1H, H6'), 7.80–7.83 (m, 1H, H6), 8.17 (dd, $^4J(\text{H,H}) = 1$ Hz, $^3J(\text{H,H}) = 8$ Hz, 1H, H5), 9.34 (br s, 1H, OH) ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, $\text{DMSO}-d_6$): $\delta = 118.9$ (C8), 122.5 (C8a), 125.2 (C7), 125.5 (C5), 127.7 (C5'), 130.2 (C4'), 130.5 (C2'), 132.3 (C3'), 132.5 (C6'), 133.2 (C1'), 134.3 (C6), 139.7 (C2), 146.6 (C3), 155.5 (C4a), 173.4 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{15}\text{H}_9\text{ClO}_3 \cdot 0.1\text{H}_2\text{O}$: C 65.64, H 3.38%. Found: C 65.69, H 3.27%.

3-Hydroxy-2-(4-bromophenyl)-chromen-4(1H)-one (2i). The synthesis was performed according to the general procedure by using **1** (2.00 g, 14.7 mmol) and **i** (2.70 g, 14.7 mmol), affording **2i** as a yellow powder (2.31 g, 58%); mp 163–167 °C. ^1H NMR (500.10 MHz, DMSO- d_6): δ = 7.47–7.50 (m, 1H, H7), 7.77–7.87 (m, H3'/H5'/H6/H8), 8.12 (dd, $^3J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.19 (d, $^3J(\text{H,H})$ = 9 Hz, 2H, H2'/H6'), 9.85 (br s, 1H, OH) ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, DMSO- d_6): δ = 118.9 (C8), 121.8 (C8a), 123.8 (C4'), 125.1 (C7), 125.3 (C5), 130.0 (C2), 132.1 (C3'/C5'), 134.4 (C6), 139.9 (C1'), 144.5 (C3), 155.0 (C4a), 173.5 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{15}\text{H}_9\text{BrO}_3 \cdot 0.1\text{H}_2\text{O}$: C 56.49, H 2.91%. Found: C 56.51, H 2.68%.

3-Hydroxy-2-(3-bromophenyl)-chromen-4(1H)-one (2j). The synthesis was performed according to the general procedure by using **1** (2.00 g, 14.7 mmol) and **j** (2.70 g, 14.7 mmol), affording **2j** as orange crystals (0.82 g, 18%); mp 165–167 °C. ^1H NMR (500.10 MHz, DMSO- d_6): δ = 7.48–7.51 (m, 1H, H7), 7.56 (dd, $^3J(\text{H,H})$ = 8 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5'), 7.72–7.74 (m, 1H, H4'), 7.82–7.86 (m, 2H, H6/H8), 8.13 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.24–8.25 (m, 1H, H6'), 8.41–8.43 (m, 1H, H2'), 9.92 (br s, 1H, OH) ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, DMSO- d_6): δ = 119.0 (C8), 121.8 (C8a), 122.3 (C3'), 125.2 (C7), 125.3 (C5), 126.9 (C6'), 127.5 (C2'), 130.4 (C4'), 131.2 (C5'), 132.9 (C4'), 134.1 (C2), 134.4 (C6), 140.1 (C1'), 143.8 (C3), 155.1 (C4a), 173.6 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{15}\text{H}_9\text{BrO}_3$: C 56.81, H 2.86%. Found: C 56.65, H 2.76%.

General Procedure for the Synthesis of the $\text{Ru}(\eta^6\text{-p-cymene})\text{Cl}_2$ Complexes 3a–3j. A solution of $[\text{Ru}(\eta^6\text{-p-cymene})\text{Cl}_2]_2$ (0.45 equiv) in dichloromethane (15 mL) was added to a solution of 3-hydroxyflavones **2a–j** (1.00 equiv) and sodium methoxide (1.10 equiv) in methanol (15 mL). The reaction mixture was stirred at room temperature and under argon atmosphere for 18 h. The solvent was evaporated in vacuo, and the residue was extracted with dichloromethane, filtered, and concentrated. The pure product was obtained by recrystallization from methanol.

[Chlorido{3-(oxo- κO)-2-phenyl-chromen-4(1H)-onato- κO }($\eta^6\text{-p-cymene}$)ruthenium(II)] (3a). The reaction was performed according to the general complexation procedure by using **2a** (164 mg, 0.73 mmol), NaOMe (43 mg, 0.8 mmol), and $[\text{Ru}(\eta^6\text{-p-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol) affording **3a** as a deep-red powder (170 mg, 51%); mp 229–230 °C (decomp). ^1H NMR (500.10 MHz, CDCl_3): δ = 1.41–1.44 (m, 6H, CH_3Cym), 2.43 (s, 3H, CH_3Cym), 2.99–3.05 (m, 1H, CH_3Cym), 5.39 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H3/H5 Cym), 5.67 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H2/H6 Cym), 7.33–7.35 (m, 1H, H7), 7.41 (dd, $^3J(\text{H,H})$ = 7 Hz, $^3J(\text{H,H})$ = 7 Hz, 1H, H4'), 7.48 (dd, $^3J(\text{H,H})$ = 7 Hz, $^3J(\text{H,H})$ = 7 Hz, 2H, H3'/H5'), 7.57 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.59–7.61 (m, 1H, H6), 8.22 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.61 (d, $^3J(\text{H,H})$ = 7 Hz, 2H, H2'/H6') ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 18.7 (CH_3Cym), 22.5 (CH_3Cym), 31.3 (CH_3Cym), 78.0 (C3/C5 Cym), 81.0 (C2/C6 Cym), 95.9 (C4 Cym), 98.9 (C1 Cym), 117.9 (C8), 120.0 (C8a), 124.0 (C7), 124.6 (C5), 127.3 (C2'/C6'), 128.2 (C3'/C5'), 129.3 (C4'), 132.5 (C2), 132.6 (C6), 149.2 (C1'), 153.9 (C4a), 154.0 (C3), 183.3 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{23}\text{H}_{23}\text{ClO}_3\text{Ru}$: C 59.11, H 4.56%. Found: C 59.04, H 4.39%.

[Chlorido{3-(oxo- κO)-2-(4-methylphenyl)-chromen-4(1H)-onato- κO }($\eta^6\text{-p-cymene}$)ruthenium(II)] (3b). The reaction was performed according to the general complexation procedure by using **2b** (184 mg, 0.73 mmol), NaOMe (43 mg, 0.8 mmol), and $[\text{Ru}(\eta^6\text{-p-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol), affording **3b** as a red powder (240 mg, 68%). Single crystals suitable for X-ray diffraction analysis were grown from $\text{CHCl}_3/n\text{-hexane}$; mp 235–236 °C (decomp). ^1H NMR (500.10 MHz, CDCl_3): δ = 1.40–1.43 (m, 6H, CH_3Cym), 2.42 (s, 3H, CH_3Cym), 2.44 (s, 3H, CH_3), 2.98–3.04 (m, 1H, CH_3Cym), 5.38 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H3/H5 Cym), 5.65 (dd, $^3J(\text{H,H})$ = 6 Hz, $^3J(\text{H,H})$ = 6 Hz, 2H, H2/H6 Cym), 7.28 (d, $^3J(\text{H,H})$ = 9 Hz, 2H, H3'/H5'), 7.32–7.35 (m, 1H, H7), 7.55 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.58–7.61 (m, 1H, H6), 8.21 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.50 (d, $^3J(\text{H,H})$ = 8 Hz, 2H, H2'/H6') ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 18.7 (CH_3Cym), 21.6 (CH_3), 22.5

(CH_3Cym), 31.2 (CH_3Cym), 77.9 (C3/C5 Cym), 80.9 (C2/C6 Cym), 95.9 (C4 Cym), 98.9 (C1 Cym), 117.8 (C8), 120.4 (C8a), 124.0 (C7), 124.5 (C5), 127.3 (C2'/C6'), 129.0 (C3'/C5'), 130.0 (C2), 132.6 (C6), 139.8 (C4'), 149.9 (C1'), 153.7 (C4a), 154.2 (C3), 183.1 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{26}\text{H}_{25}\text{ClO}_3\text{Ru}$: C 59.82, H 4.83%. Found: C 59.82, H 4.57%.

[Chlorido{3-(oxo- κO)-2-(4-fluorophenyl)-chromen-4(1H)-onato- κO }($\eta^6\text{-p-cymene}$)ruthenium(II)] (3c). The reaction was performed according to the general complexation procedure by using **2c** (187 mg, 0.73 mmol), NaOMe (43 mg, 0.8 mmol), and $[\text{Ru}(\eta^6\text{-p-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol), affording **3c** as red needles (230 mg, 66%); mp 235–236 °C (decomp). ^1H NMR (500.10 MHz, CDCl_3): δ = 1.40–1.44 (m, 6H, CH_3Cym), 2.42 (s, 3H, CH_3Cym), 2.97–3.04 (m, 1H, CH_3Cym), 5.40 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H3/H5 Cym), 5.66 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H2/H6 Cym), 7.15–7.18 (m, 2H, H3'/H5'), 7.33–7.36 (m, 1H, H7), 7.54 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.60–7.62 (m, 1H, H6), 8.22 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.61–8.64 (m, 2H, H2'/H6') ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 18.7 (CH_3Cym), 22.5 (CH_3Cym), 31.3 (CH_3Cym), 78.0 (C3/C5 Cym), 81.0 (C2/C6 Cym), 95.9 (C4 Cym), 98.9 (C1 Cym), 115.2 (d, $^2J(\text{C,F})$ = 21 Hz, C3'/C5'), 117.7 (C8), 120.1 (C8a), 124.1 (C7), 124.6 (C5), 128.8 (C2), 129.4 (d, $^3J(\text{C,F})$ = 8 Hz, C2'/C6'), 132.6 (C6), 148.4 (d, $^4J(\text{C,F})$ = 1 Hz, C1'), 153.8 (C4a), 154.1 (C3), 163.0 (d, $^1J(\text{C,F})$ = 251 Hz, C4'), 183.3 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{25}\text{H}_{22}\text{ClFO}_3\text{Ru}$: C 57.09, H 4.22%. Found: C 56.98, H 4.06%.

[Chlorido{3-(oxo- κO)-2-(3-fluorophenyl)-chromen-4-onato- κO }($\eta^6\text{-p-cymene}$)ruthenium(II)] (3d). The reaction was performed according to the general complexation procedure by using **2d** (187 mg, 0.73 mmol), NaOMe (43 mg, 0.8 mmol), and $[\text{Ru}(\eta^6\text{-p-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol), affording **3d** as deep-red powder (180 mg, 51%); mp 210–212 °C (decomp). Single crystals suitable for X-ray diffraction analysis were grown from $\text{CHCl}_3/n\text{-hexane}$. ^1H NMR (500.10 MHz, CDCl_3): δ = 1.40–1.46 (m, 6H, CH_3Cym), 2.42 (s, 3H, CH_3Cym), 2.99–3.06 (m, 1H, CH_3Cym), 5.40 (d, $^3J(\text{H,H})$ = 6 Hz, 2H, H3/H5 Cym), 5.68 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H2/H6 Cym), 7.08 (ddd, $^4J(\text{H,F})$ = 2 Hz, $^3J(\text{H,H})$ = 8 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H4'), 7.33–7.36 (m, 1H, H7), 7.44 (ddd, $^3J(\text{H,F})$ = 6 Hz, $^3J(\text{H,H})$ = 8 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5'), 7.56 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.61–7.65 (m, 1H, H6), 8.22 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.31 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H6'), 8.44–8.48 (m, 1H, H2') ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 18.7 (CH_3Cym), 22.5 (CH_3Cym), 31.3 (CH_3Cym), 78.0 (C3/C5 Cym), 80.0 (C2/C6 Cym), 95.9 (C4 Cym), 99.0 (C1 Cym), 114.1 (d, $^2J(\text{C,F})$ = 25 Hz, C2'), 115.8 (d, $^2J(\text{C,F})$ = 22 Hz, C4'), 117.9 (C8), 119.9 (C8a), 122.5 (d, $^4J(\text{C,F})$ = 3 Hz, C6'), 124.2 (C7), 124.7 (C5), 127.2 (C2), 129.6 (d, $^3J(\text{C,F})$ = 8 Hz, C5'), 132.9 (C6), 125.5 (d, $^3J(\text{C,F})$ = 9 Hz, C1'), 153.9 (C4a), 154.9 (C3), 162.0 (d, $^1J(\text{C,F})$ = 243 Hz, C3'), 183.8 (C4) ppm. Elemental Analysis Calcd for $\text{C}_{25}\text{H}_{22}\text{ClFO}_3\text{Ru} \cdot 0.25\text{H}_2\text{O}$: C 56.61, H 4.28%. Found: C 56.51, H 4.28%.

[Chlorido{3-(oxo- κO)-2-(2-fluorophenyl)-chromen-4-onato- κO }($\eta^6\text{-p-cymene}$)ruthenium(II)] (3e). The reaction was performed according to the general complexation procedure, by using **2e** (187 mg, 0.73 mmol), NaOMe (43 mg, 0.8 mmol), and $[\text{Ru}(\eta^6\text{-p-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol), affording **3e** as red–brownish powder (220 mg, 63%); mp 199–202 °C (decomp). ^1H NMR (500.10 MHz, CDCl_3): δ = 1.37–1.42 (m, 6H, CH_3Cym), 2.39 (s, 3H, CH_3Cym), 2.97–3.02 (m, 1H, CH_3Cym), 5.36 (dd, $^3J(\text{H,H})$ = 6 Hz, $^3J(\text{H,H})$ = 6 Hz, 2H, H3/H5 Cym), 5.68 (d, $^3J(\text{H,H})$ = 6 Hz, 2H, H2/H6 Cym), 7.14–7.19 (m, 1H, H3'), 7.21 (ddd, $^4J(\text{H,F})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H6'), 7.33–7.36 (m, 1H, H7), 7.38–7.42 (m, 1H, H4'), 7.51 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.58–7.62 (m, 1H, H6), 8.23 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.31 (ddd, $^4J(\text{H,F})$ = 1 Hz, $^3J(\text{H,H})$ = 7 Hz, $^3J(\text{H,H})$ = 7 Hz, 1H, H5') ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 18.7 (CH_3Cym), 22.5 (CH_3Cym), 31.2 (CH_3Cym), 77.7 (C3/C5 Cym), 81.2 (C2/C6 Cym), 96.0 (C4 Cym), 98.9 (C1 Cym), 116.3 (d, $^2J(\text{C,F})$ = 22 Hz, C3'), 118.3 (C8), 120.1 (d, $^2J(\text{C,F})$ = 10 Hz, C1'), 120.1 (C8a), 123.6 (d, $^3J(\text{C,F})$ = 3 Hz, C6'), 124.1 (C7), 124.6 (C5), 131.1 (d, $^3J(\text{C,F})$ = 8 Hz, C4'),

131.6 (d, $^4J(\text{C},\text{F}) = 2 \text{ Hz}$, $\text{C}5'$), 132.7 (C6), 146.9 (C2/C3), 154.4 (C4a), 159.0 (d, $^1J(\text{C},\text{F}) = 256 \text{ Hz}$, $\text{C}2'$), 183.6 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{25}\text{H}_{22}\text{ClFO}_3\text{Ru}\cdot 0.5\text{H}_2\text{O}$: C 56.13, H 4.33%. Found: C 56.22, H 4.60%.

[Chlorido{3-(oxo- κO)-2-(4-chlorophenyl)-chromen-4(1H)-onato- κO }(η^6 -*p*-cymene)ruthenium(III)] (3f). The reaction was performed according to the general complexation procedure by using 2f (199 mg, 0.73 mmol), NaOMe (43 mg, 0.8 mmol), and $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol), affording 3f as deep-red powder (226 mg, 63%); mp 214–217 °C (decomp). ^1H NMR (500.10 MHz, CDCl_3): $\delta = 1.32\text{--}1.38$ (m, 6H, CH_3 , $\text{C}_{3\text{Cym}}$), 2.42 (s, 3H, CH_3 , $\text{C}_{3\text{Cym}}$), 2.85–2.91 (m, 1H, CH_{Cym}), 5.39 (dd, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, 2H, H3/H5 $_{\text{Cym}}$), 5.66 (dd, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, 2H, H2/H6 $_{\text{Cym}}$), 7.32–7.35 (m, 1H, H7), 7.44 (d, $^3J(\text{H},\text{H}) = 9 \text{ Hz}$, 2H, H3'/H5'), 7.54 (d, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, 1H, H8), 7.62–7.63 (m, 1H, H6), 8.21 (dd, $^4J(\text{H},\text{H}) = 1 \text{ Hz}$, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, 1H, H5), 8.55 (d, $^3J(\text{H},\text{H}) = 9 \text{ Hz}$, 2H, H2'/H6') ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): $\delta = 18.7$ (CH_3 , $\text{C}_{3\text{Cym}}$), 22.4 (CH_3 , $\text{C}_{3\text{Cym}}$), 30.0 (CH_{Cym}), 77.9 (C3/C5 $_{\text{Cym}}$), 81.0 (C2/C6 $_{\text{Cym}}$), 95.9 (C4 $_{\text{Cym}}$), 99.0 (C1 $_{\text{Cym}}$), 117.8 (C8), 120.0 (C8a), 124.1 (C7), 124.7 (C5), 125.5 (C2'/C6'/C3'/C5'), 131.0 (C2), 132.8 (C6), 134.9 (C4'), 143.5 (C1'), 153.9 (C4a), 154.6 (C3), 183.5 (C4). Elemental Anal. Calcd for $\text{C}_{25}\text{H}_{22}\text{Cl}_2\text{O}_3\text{Ru}$: C 55.36, H 4.09%. Found: C 55.28, H 3.90%.

[Chlorido{3-(oxo- κO)-2-(3-chlorophenyl)-chromen-4-onato- κO }(η^6 -*p*-cymene)ruthenium(III)] (3g). The reaction was performed according to the general complexation procedure by using 2g (199 mg, 0.73 mmol), NaOMe (43 mg, 0.8 mmol), and $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol), affording 3g as deep-red powder (244 mg, 68%); mp 213–220 °C (decomp). ^1H NMR (500.10 MHz, CDCl_3): $\delta = 1.43\text{--}1.48$ (m, 6H, CH_3 , $\text{C}_{3\text{Cym}}$), 2.44 (s, 3H, CH_3 , $\text{C}_{3\text{Cym}}$), 3.00–3.07 (m, 1H, CH_{Cym}), 5.39 (dd, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, 2H, H3/H5 $_{\text{Cym}}$), 5.68 (dd, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, 2H, H2/H6 $_{\text{Cym}}$), 7.33–7.36 (m, 2H, H4'/H7), 7.40 (dd, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, 1H, H5'), 7.55 (d, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, 1H, H8), 7.63–7.65 (m, 1H, H6), 8.21 (dd, $^4J(\text{H},\text{H}) = 1 \text{ Hz}$, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, 1H, H5), 8.41 (ddd, $^4J(\text{H},\text{H}) = 1 \text{ Hz}$, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, 1H, H6'), 8.72–8.73 (m, 1H, H2') ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): $\delta = 18.7$ (CH_3 , $\text{C}_{3\text{Cym}}$), 22.5 (2 CH_3 , $\text{C}_{3\text{Cym}}$), 31.3 (CH_{Cym}), 77.9 (C3/C5 $_{\text{Cym}}$), 81.2 (C2/C6 $_{\text{Cym}}$), 95.8 (C4 $_{\text{Cym}}$), 98.9 (C1 $_{\text{Cym}}$), 117.9 (C8), 119.9 (C8a), 124.2 (C7), 124.7 (C5), 124.8 (C6'), 127.1 (C2'), 128.9 (C4'), 129.4 (C5'), 133.0 (C6), 134.2 (C2/C3'), 147.3 (C1'), 154.0 (C4a), 154.9 (C3), 183.8 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{25}\text{H}_{22}\text{Cl}_2\text{O}_3\text{Ru}\cdot 0.15\text{H}_2\text{O}$: C 55.08, H 4.12%. Found: C 55.11, H 3.85%.

[Chlorido{3-(oxo- κO)-2-(2-chlorophenyl)-chromen-4-onato- κO }(η^6 -*p*-cymene)ruthenium(III)] (3h). The reaction was performed according to the general complexation procedure by using 2h (199 mg, 0.73 mmol), NaOMe (43 mg, 0.8 mmol), and $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol), affording 3h as brown powder (109 mg, 30%); mp 205–208 °C (decomp). Single crystals suitable for X-ray diffraction analysis were grown from MeOH/*n*-hexane. ^1H NMR (500.10 MHz, CDCl_3): $\delta = 1.35\text{--}1.39$ (m, 6H, CH_3 , $\text{C}_{3\text{Cym}}$), 2.37 (s, 3H, CH_3 , $\text{C}_{3\text{Cym}}$), 2.93–3.00 (m, 1H, CH_{Cym}), 5.35 (dd, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, 2H, H3/H5 $_{\text{Cym}}$), 5.63 (dd, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, 2H, H2/H6 $_{\text{Cym}}$), 7.34–7.38 (m, 3H, H3'/H5'/H7), 7.48–7.52 (m, 2H, H4'/H8), 7.60–7.62 (m, 1H, H6), 7.99–8.02 (m, 1H, H6'), 8.24 (dd, $^4J(\text{H},\text{H}) = 1 \text{ Hz}$, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, 1H, H5) ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): $\delta = 18.9$ (CH_3 , $\text{C}_{3\text{Cym}}$), 22.3 (2 CH_3 , $\text{C}_{3\text{Cym}}$), 31.2 (CH_{Cym}), 77.5 (C3/C5 $_{\text{Cym}}$), 80.5 (C2/C6 $_{\text{Cym}}$), 96.1 (C4 $_{\text{Cym}}$), 98.9 (C1 $_{\text{Cym}}$), 118.2 (C8), 120.2 (C8a), 124.1 (C7), 124.6 (C5), 126.3 (C5'), 130.3 (C4'), 130.5 (C3'), 130.7 (C2'), 132.7 (C6'), 132.8 (C6), 133.4 (C1'), 148.5 (C2), 154.3 (C3), 154.5 (C4a), 184.1 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{25}\text{H}_{22}\text{Cl}_2\text{O}_3\text{Ru}$: C 55.36, H 4.09%. Found: C 55.29, H 3.79%.

[Chlorido{3-(oxo- κO)-2-(4-bromophenyl)-chromen-4-onato- κO }(η^6 -*p*-cymene)ruthenium(III)] (3i). The reaction was performed according to the general complexation procedure, by using 2i (231 mg, 0.73 mmol), NaOMe (43 mg, 0.8 mmol), and $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol), affording 3i as red powder (179 mg, 46%); mp 228–233 °C (decomp). ^1H NMR (500.10 MHz, CDCl_3): $\delta = 1.40\text{--}$

1.45 (m, 6H, CH_3 , $\text{C}_{3\text{Cym}}$), 2.42 (s, 3H, CH_3 , $\text{C}_{3\text{Cym}}$), 2.98–3.04 (m, 1H, CH_{Cym}), 5.39 (dd, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, 2H, H3/H5 $_{\text{Cym}}$), 5.66 (dd, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, 2H, H2/H6 $_{\text{Cym}}$), 7.33–7.36 (m, 1H, H7), 7.54 (d, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, 1H, H8), 7.59–7.64 (m, 3H, H3'/H5'/H6), 8.21 (dd, $^4J(\text{H},\text{H}) = 1 \text{ Hz}$, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, 1H, H5), 8.48 (d, $^3J(\text{H},\text{H}) = 9 \text{ Hz}$, 2H, H2'/H6') ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): $\delta = 18.7$ (CH_3 , $\text{C}_{3\text{Cym}}$), 22.5 (CH_3 , $\text{C}_{3\text{Cym}}$), 31.3 (CH_{Cym}), 77.9 (C3/C5 $_{\text{Cym}}$), 80.0 (C2/C6 $_{\text{Cym}}$), 96.0 (C4 $_{\text{Cym}}$), 99.0 (C1 $_{\text{Cym}}$), 117.8 (C8), 120.0 (C8a), 123.4 (C4'), 124.1 (C7), 124.7 (C5), 128.6 (C2'/C6'), 131.4 (C3'/C5'), 131.7 (C2), 132.8 (C6), 148.0 (C1'), 153.9 (C4a), 154.7 (C3), 183.5 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{25}\text{H}_{22}\text{BrClO}_3\text{Ru}\cdot 0.25\text{H}_2\text{O}$: C 50.77, H 3.83%. Found: C 50.74, H 3.57%.

[Chlorido{3-(oxo- κO)-2-(3-bromophenyl)-chromen-4-onato- κO }(η^6 -*p*-cymene)ruthenium(III)] (3j). The reaction was performed according to the general complexation procedure by using 2j (231 mg, 0.73 mmol), NaOMe (43 mg, 0.8 mmol), and $[\text{Ru}(\eta^6\text{-}p\text{-cymene})\text{Cl}_2]_2$ (200 mg, 0.33 mmol), affording 3j as dark-brown crystals (153 mg, 40%); mp 213–219 °C (decomp). ^1H NMR (500.10 MHz, CDCl_3): $\delta = 1.44\text{--}1.49$ (m, 6H, CH_3 , $\text{C}_{3\text{Cym}}$), 2.44 (s, 3H, CH_3 , $\text{C}_{3\text{Cym}}$), 3.00–3.07 (m, 1H, CH_{Cym}), 5.39 (dd, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, 2H, H3/H5 $_{\text{Cym}}$), 5.68 (d, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, $^3J(\text{H},\text{H}) = 5 \text{ Hz}$, 2H, H2/H6 $_{\text{Cym}}$), 7.33–7.36 (m, 2H, H5'/H7), 7.40 (ddd, $^4J(\text{H},\text{H}) = 1 \text{ Hz}$, $^4J(\text{H},\text{H}) = 2 \text{ Hz}$, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, 1H, H4'), 7.56 (d, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, 1H, H8), 7.62–7.65 (m, 1H, H6), 8.21 (dd, $^4J(\text{H},\text{H}) = 1 \text{ Hz}$, $^3J(\text{H},\text{H}) = 8 \text{ Hz}$, 1H, H5), 8.45–8.47 (m, 1H, H6'), 8.87–8.89 (m, 1H, H2') ppm. $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): $\delta = 18.7$ (CH_3 , $\text{C}_{3\text{Cym}}$), 22.7 (2 CH_3 , $\text{C}_{3\text{Cym}}$), 31.3 (CH_{Cym}), 77.9 (C3/C5 $_{\text{Cym}}$), 81.2 (C2/C6 $_{\text{Cym}}$), 95.7 (C4 $_{\text{Cym}}$), 98.8 (C1 $_{\text{Cym}}$), 117.9 (C8), 119.8 (C8a), 122.4 (C3'), 124.2 (C7), 124.8 (C5), 125.3 (C6'), 129.7 (C2'), 129.9 (C5'), 131.8 (C4'), 133.0 (C6), 134.5 (C2), 147.1 (C1'), 154.0 (C4a), 154.9 (C3), 183.8 (C4) ppm. Elemental Anal. Calcd for $\text{C}_{25}\text{H}_{22}\text{BrClO}_3\text{Ru}\cdot 0.25\text{H}_2\text{O}$: C 49.64, H 4.00%. Found: C 49.40, H 3.85%.

Hydrolysis and Interaction with 5'-GMP. Hydrolysis and stability in water were investigated by ^1H NMR spectroscopy. Because of the lipophilic character of the organometallics, all experiments were performed in 10% DMSO- d_6 /D $_2$ O solutions. For the 5'-GMP binding studies, the complexes (ca. 0.1 mg/mL) were dissolved in 10% DMSO- d_6 /D $_2$ O, yielding the corresponding highly reactive aqua species. The aqua complexes were converted in situ by addition of 50 μL increments of 5'-GMP solution (10 mg/mL) into the respective 5'-GMP adduct, and the reaction was monitored by ^1H NMR spectroscopy until unconverted 5'-GMP was observed.

Cytotoxicity in Cancer Cell Lines. Cell Lines and Culture Conditions. CH1 cells originate from an ascites sample of a patient with a papillary cystadenocarcinoma of the ovary and were a gift from Lloyd R. Kelland, CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK. SW480 (human adenocarcinoma of the colon) and A549 (human non-small cell lung cancer) cells were provided by Brigitte Marian (Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Austria). The cell lines 5637 (bladder cancer), LCLC-103H (lung cancer), and DAN-G (pancreatic cancer) were obtained from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, FRG). Cells were grown in 75 cm^2 culture flasks (Iwaki) as adherent monolayer cultures in either Minimum Essential Medium (MEM) supplemented with 10% heat inactivated fetal calf serum (FCS), 1 mM sodium pyruvate, 4 mM L-glutamine, and 1% nonessential amino acids (from 100 $\times$ stock) (i.e., CH1, SW480, and A549) or in RPMI 1640 medium supplemented with 10% FCS (i.e., 5637, LCLC-103H, DAN-G). All cell culture reagents were obtained from Sigma-Aldrich. Cultures were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2 .

MTT Assay. Cytotoxicity was determined by the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Fluka] microculture assay. For this purpose, cells were harvested from culture flasks by trypsinization and seeded in 100 μL aliquots into 96-well microculture plates (Iwaki). Cell densities of 1.5×10^3 cells/well (CH1), 2.5×10^3 cells/well (SW480), and 4×10^3 cells/well (A549) were chosen in order to ensure exponential growth of

untreated controls throughout the experiment. Cells were allowed to settle and resume exponential growth in drug-free complete culture medium for 24 h. Stock solutions of the test compounds in DMSO were appropriately diluted in complete culture medium so that the maximum DMSO content did not exceed 1%. These dilutions were added in 100 μ L aliquots to the microcultures, and cells were exposed to the test compounds for 96 h. In case of the studies on time-dependent cytotoxicity, SW480 cells were treated for 1, 4, 24, and 96 h with **3j** followed by incubation in drug-free medium for the rest of a total incubation time of 96 h. At the end of incubation, all media were replaced by 100 μ L/well RPMI1640 culture medium (supplemented with 10% heat-inactivated FCS) plus 20 μ L/well MTT solution in phosphate-buffered saline (5 mg/mL). After incubation for 4 h, the supernatants were removed and the formazan crystals formed by viable cells were dissolved in 150 μ L of DMSO per well. Optical densities at $\lambda = 550$ nm were measured with a microplate reader (Tecan Spectra Classic) by using a reference wavelength of 690 nm to correct for unspecific absorption. The quantity of viable cells was expressed in terms of T/C values by comparison to untreated controls, and 50% inhibitory concentrations (IC_{50}) were calculated from concentration–effect curves by interpolation. Evaluation is based on means from at least three independent experiments, each comprising at least three replicates per concentration level.

Crystal Violet Assay Conditions. This assay has been described in detail elsewhere.⁴⁹ Culture conditions were the same as used in the MTT assay. Briefly, cells were seeded into 96-well microculture plates (Sarstedt, FRG) in cell densities of 1.0×10^3 cells/well, except for LCLC-103H, which was seeded at 250 cells/well. After a 24 h preincubation, cells were treated with the test substance for 96 h. Stock solutions of the test substance were prepared to 20 mM in DMF and diluted 1000-fold in RPMI 1640 culture medium containing 10% FCS. Substances that showed a $\geq 50\%$ growth inhibition at 20 μ M were tested at five serial dilutions in four wells/concentration to determine the IC_{50} values as described.⁴⁹ The staining of the cells was done for 30 min with a 0.02% crystal violet solution in water followed by washing out the excess dye. Cell bound dye was redissolved in 70% ethanol/water solution, and the optical densities at $\lambda = 570$ nm were measured with a microplate reader (Anthos 2010).

Cell Cycle Analysis. One million A549 cells were seeded into Petri dishes and allowed to recover for 24 h. Cells were then exposed for 48 h to the test compounds. Control and drug-treated cells were collected, washed with PBS, fixed in 70% ice-cold ethanol, and stored at -20°C . To determine cell cycle distribution, cells were transferred in physiological NaCl solution into PBS, incubated with 10 μ g/mL RNase A for 30 min at 37°C , followed by 30 min treatment with 5 μ g/mL propidium iodide. Fluorescence was measured by flow cytometry by using FACS Calibur (Becton Dickinson, Palo Alto, CA). The resulting DNA histograms were quantified by Cell Quest Pro software (Becton Dickinson and Company, New York, USA).

Determination of Topoisomerase II α Activity. Effects on the catalytic activity of topoisomerase II α were determined using a decatenation assay. Catenated kinetoplast DNA (kDNA) was used as a substrate. kDNA is an aggregate of interlocked DNA minicircles (mostly 2.5 kb), which can be released by topoisomerase II α . kDNA (200 ng, TopoGen, OH, USA) was incubated in a final volume of 30 μ L (containing 40 ng of topoisomerase II α ; 50 mM Tris, pH 7.9; 120 mM KCl; 10 mM MgCl₂; 1 mM ATP; 0.5 mM DTT; 0.5 mM EDTA; 0.03 mg/mL BSA) at 37°C for 60 min. The reaction was stopped by the addition of 1/10 volume of 1 mg/mL proteinase K in 10% (w/v) SDS and incubation at 37°C for further 30 min. Gel electrophoresis was performed in the absence of ethidium bromide at 60 V for 3 h in 1% (w/v) agarose gels with Tris acetate/EDTA buffer (40 mM Tris; 1 mM EDTA, pH 8.5; 20 mM acetic acid). Subsequently, the gel was stained in 10 μ g/mL ethidium bromide solution for 20 min. The fluorescence of ethidium bromide was detected with the LAS-4000 system (Fujifilm, Raytest, Germany).

CDK2/Cyclin A Protein Kinase Inhibition Assay. The CDK2 peptide substrate, HHASPRK, and the CDK2/Cyclin A protein complex were purchased from Enzo Life Sciences. Adenosine 5'-[γ -ferrocene] triphosphate (Fc-ATP) was synthesized according to the

procedure published elsewhere.³⁵ Gold disk electrodes (99.99% purity) with surface area of 0.02 cm² were obtained from CHInstruments. All experiments were conducted in aqueous conditions using ultrapure water (18.2 M Ω cm) from a Millipore Milli-Q system.

Fabrication of Kinase Biosensor. The gold electrodes were cleaned by polishing with slurry of 1 μ m Al₂O₃ until a mirror finish was obtained. After 5 min sonication in Milli-Q water, the gold electrodes were rinsed with water and ethanol. The electrodes were then cleaned electrochemically by cyclic voltammetry (CV) in the negative potential range from -0.6 to -2.3 V in 0.5 M KOH, followed by cycling in 0.5 M H₂SO₄ in the 0 – 1.2 V potential range. Next, the gold electrodes were incubated with 2 mM lipoic acid *N*-hydroxysuccinimide ester solution in ethanol for 3 days at 273 K. After extensive washing with freshly distilled ethanol, the gold electrodes were incubated with a 0.1 mM peptide solution in Milli-Q water for 18 h at 273 K. Consequently, the modified electrodes were rinsed with Milli-Q water and then incubated with 100 mM ethanolamine solution in absolute ethanol for 1 h. Finally, the electrodes were immersed in 10 mM dodecanethiol solution in ethanol for 20 min to block any unmodified gold surface.

Kinase-Catalyzed Fc-Phosphorylation Reaction. The peptide-modified gold electrodes were immersed in the kinase assay buffer based on 60 mM HEPES (pH 7.5), 3 mM MnCl₂, 3 mM MgCl₂, 0.5 μ g/ μ L of PEG 20000, 3 μ M sodium *ortho*-vanadate, 1 μ g/mL CDK2/cyclin A protein kinase and 200 μ M Fc-ATP. The phosphorylation reaction was performed for 6 h at 37°C in a heating block (VWR Scientific, USA). The modified gold electrodes were washed five times using the kinase assay buffer and 0.1 M phosphate buffer (pH 7.4) prior to the electrochemical measurement. For the inhibitor studies, the CDK2/cyclin A protein and compounds **3a–h** (1 mM, DMSO) were added to the kinase buffer while maintaining the working concentration of the protein and inhibitor at 1 μ g/mL and 10 μ M, respectively. The DMSO concentration was maintained at a low level ($<2\%$). After 30 min, the kinase reaction was initiated by the addition of Fc-ATP and was followed by the procedure outlined above.

Electrochemical Experiments. All electrochemical experiments were carried out using a CHInstrument 660B system potentiostat (Austin, TX) at a 100 mV/s scan rate unless otherwise specified. The electrochemical measurements were performed in 0.1 M phosphate buffer (pH 7.4). Typical electrochemical experimental set up included a three-electrode system: a modified gold electrode as the working electrode, Ag/AgCl in 3 M KCl as the reference electrode, which was connected with the electrolyte via a salt bridge, and platinum wire as the counter electrode. For each electrode, cyclic voltammetry was performed at a scan rate of 100 mV/s and square-wave voltammetry was recorded from 0.2 to 0.6 V, with an amplitude of 25 mV at 20 Hz frequency.

■ ASSOCIATED CONTENT

§ Supporting Information

Single crystal X-ray diffraction data for compounds **3d**, **3f**, and **3h**, NMR spectra demonstrating the stability of **3i** over two years and the binding ability of **3a** to the DNA model 5'-GMP, concentration–effect curves for different cell lines, the cell cycle distribution in dependence of the concentration of Ru complex, data on the topoisomerase II α inhibitory activity, and electrochemical data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +64 9 373 7599 ext 83220. Fax: +64 9 373 7599 87422. E-mail: c.hartinger@auckland.ac.nz. Web site: <http://hartinger.wordpress.fos.auckland.ac.nz/>. Address: The University of Auckland, School of Chemical Sciences, Private Bag 92019, Auckland 1142, New Zealand.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the University of Vienna, the Austrian Science Fund (FWF), the Johanna Mahlke geb. Obermann Foundation, and COST D39 for financial support. We gratefully acknowledge Alexander Roller for collecting the X-ray diffraction data. V. Dirsch and D. Schachner (Department of Pharmacognosy, University of Vienna) are gratefully acknowledged for providing the FACS instrument and for technical instructions, respectively.

■ ABBREVIATIONS USED

5'-GMP, guanosine 5'-monophosphate; CDK, cyclin dependent kinase; CV, cyclic voltammetry; cym, η^6 -*p*-cymene; Fc-ATP, adenosine 5'-[γ -ferrocene] triphosphate; IC₅₀, 50% inhibitory concentration; kDNA, kinetoplast DNA; pta, 1,3,5-triaza-7-phosphatricyclo-[3.3.1.1]decane; SWV, square-wave voltammetry

■ REFERENCES

- (1) Jakupec, M. A.; Galanski, M.; Arion, V. B.; Hartinger, C. G.; Keppler, B. K. Antitumor metal compounds: more than theme and variations. *Dalton Trans.* **2008**, 183–194.
- (2) Heffeter, P.; Jungwirth, U.; Jakupec, M.; Hartinger, C.; Galanski, M.; Elbling, L.; Micksche, M.; Keppler, B.; Berger, W. Resistance against novel anticancer metal compounds: differences and similarities. *Drug Resist. Updates* **2008**, *11*, 1–16.
- (3) Clarke, M. J. Ruthenium metallopharmaceuticals. *Coord. Chem. Rev.* **2003**, *236*, 209–233.
- (4) Hartinger, C. G.; Jakupec, M. A.; Zorbas-Seifried, S.; Groessl, M.; Egger, A.; Berger, W.; Zorbas, H.; Dyson, P. J.; Keppler, B. K. KP1019, a new redox-active anticancer agent—preclinical development and results of a clinical phase I study in tumor patients. *Chem. Biodiversity* **2008**, *5*, 2140–2155.
- (5) 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(1*H*-indazole)ruthenate(III)] (KP1019 or FFC14A). *J. Inorg. Biochem.* **2006**, *100*, 891–904.
- (6) Rademaker-Lakhai, J. M.; Van Den Bongard, D.; Pluim, D.; Beijnen, J. H.; Schellens, J. H. M. A phase I and pharmacological study with imidazolium-*trans*-DMSO-imidazole-tetrachlororuthenate, a novel ruthenium anticancer agent. *Clin. Cancer Res.* **2004**, *10*, 3717–3727.
- (7) Ang, W. H.; Casini, A.; Sava, G.; Dyson, P. J. Organometallic ruthenium-based antitumor compounds with novel modes of action. *J. Organomet. Chem.* **2011**, *696*, 989–998.
- (8) Hartinger, C. G.; Dyson, P. J. Bioorganometallic chemistry—from teaching paradigms to medicinal application. *Chem. Soc. Rev.* **2009**, *38*, 391–401.
- (9) Zimmermann, G. R.; Lehár, J.; Keith, C. T. Multi-target therapeutics: when the whole is greater than the sum of the parts. *Drug Discovery Today* **2007**, *12*, 34–42.
- (10) Kurzwernhart, A.; Kandioller, W.; Bartel, C.; Bächler, S.; Trondl, R.; Mühlgassner, G.; Jakupec, M. A.; Arion, V. B.; Marko, D.; Keppler, B. K.; Hartinger, C. G. Targeting the DNA-topoisomerase complex in a double-strike approach with a topoisomerase inhibiting moiety and covalent DNA binder. *Chem. Commun.* **2012**, *48*, 4839–4841.
- (11) Cushnie, T. P. T.; Lamb, A. J. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents* **2005**, *26*, 343–356.
- (12) Harborne, J. B.; Williams, C. A. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55*, 481–504.
- (13) Middleton, E., Jr.; 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.
- (14) Sathish, S.; Narayan, G.; Rao, N.; Janardhana, C. A self-organized ensemble of fluorescent 3-hydroxyflavone–Al(III) complex as sensor for fluoride and acetate ions. *J. Fluoresc.* **2007**, *17*, 1–5.
- (15) Mladěnká, P.; Macáková, K.; Filipický, T.; Zatloukalová, L.; Jahodář, L.; Bovicelli, P.; Silvestri, I. P.; Hrdina, R.; Saso, L. In vitro analysis of iron chelating activity of flavonoids. *J. Inorg. Biochem.* **2011**, *105*, 693–701.
- (16) Roshal, A. D.; Grigorovich, A. V.; Doroshenko, A. O.; Pivovarenko, V. G.; Demchenko, A. P. Flavonols as metal-ion chelators: complex formation with Mg²⁺ and Ba²⁺ cations in the excited state. *J. Photochem. Photobiol., A* **1999**, *127*, 89–100.
- (17) Grazul, M.; Budzisz, E. Biological activity of metal ions complexes of chromones, coumarins and flavones. *Coord. Chem. Rev.* **2009**, *253*, 2588–2598.
- (18) Bennett, M.; Burke, A. J.; O'Sullivan, W. I. Aspects of the Algar–Flynn–Oyamada (AFO) reaction. *Tetrahedron* **1996**, *52*, 7163–7178.
- (19) Geissman, T. A.; Fukushima, D. K. Flavanones and related compounds. V. The oxidation of 2'-hydroxychalcones with alkaline hydrogen peroxide. *J. Am. Chem. Soc.* **1948**, *70*, 1686–1689.
- (20) Qin, C. X.; Chen, X.; Hughes, R. A.; Williams, S. J.; Woodman, O. L. Understanding the cardioprotective effects of flavonols: discovery of relaxant flavonols without antioxidant activity. *J. Med. Chem.* **2008**, *51*, 1874–1884.
- (21) Kandioller, W.; Hartinger, C. G.; Nazarov, A. A.; Kasser, J.; John, R.; Jakupec, M. A.; Arion, V. B.; Dyson, P. J.; Keppler, B. K. Tuning the anticancer activity of maltol-derived ruthenium complexes by derivatization of the 3-hydroxy-4-pyrone moiety. *J. Organomet. Chem.* **2009**, *694*, 922–929.
- (22) 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.
- (23) Chen, H.; Parkinson, J. A.; Morris, R. E.; Sadler, P. J. Highly selective binding of organometallic ruthenium ethylenediamine complexes to nucleic acids: novel recognition mechanisms. *J. Am. Chem. Soc.* **2003**, *125*, 173–186.
- (24) Jakupec, M. A.; Galanski, M.; Keppler, B. K. Tumour-inhibiting platinum complexes—state of the art and future perspectives. *Rev. Physiol. Biochem. Pharmacol.* **2003**, *146*, 1–53.
- (25) 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. *Chem.—Eur. J.* **2009**, *15*, 12283–12291.
- (26) Hann, S.; Koellensperger, G.; Stefanka, Z.; Stingeder, G.; Fuerhacker, M.; Buchberger, W.; Mader, R. M. Application of HPLC-ICP-MS to speciation of cisplatin and its degradation products in water containing different chloride concentrations and in human urine. *J. Anal. At. Spectrom.* **2003**, *18*, 1391–1395.
- (27) Kurzwernhart, A.; Kandioller, W.; Enyedy, É. A.; Novak, M.; Jakupec, M. A.; Keppler, B. K.; Hartinger, C. G. 3-Hydroxyflavones vs. 3-hydroxyquinolinones: structure–activity relationships and stability studies on Ru^{II}(arene) anticancer complexes with biologically active ligands. *Dalton Trans.* **2012**, DOI: 10.1039/c2dt32206d.
- (28) Peacock, A. F. A.; Melchart, M.; Deeth, R. J.; Habtemariam, A.; Parsons, S.; Sadler, P. J. Osmium(II) and ruthenium(II) arene maltolato complexes: rapid hydrolysis and nucleobase binding. *Chem.—Eur. J.* **2007**, *13*, 2601–2613.
- (29) Luo, F. R.; Wyrick, S. D.; Chaney, S. G. Pharmacokinetics and biotransformations of oxaliplatin in comparison with ormaplatin following a single bolus intravenous injection in rats. *Cancer Chemother. Pharmacol.* **1999**, *44*, 19–28.
- (30) Groessl, M.; Hartinger, C. G.; Dyson, P. J.; Keppler, B. K. CZE-ICP-MS as a tool for studying the hydrolysis of ruthenium anticancer drug candidates and their reactivity towards the DNA model compound dGMP. *J. Inorg. Biochem.* **2008**, *102*, 1060–1065.

- (31) Spicer, J. A.; Gamage, S. A.; Atwell, G. J.; Finlay, G. J.; Baguley, B. C.; Denny, W. A. Structure–Activity Relationships for Acridine-Substituted Analogues of the Mixed Topoisomerase I/II Inhibitor N-[2-(Dimethylamino)ethyl]acridine-4-carboxamide. *J. Med. Chem.* **1997**, *40*, 1919–1929.
- (32) Mendoza-Ferri, M. G.; Hartinger, C. G.; Mendoza, M. A.; Groessl, M.; Egger, A. E.; Eichinger, R. E.; Mangrum, J. B.; Farrell, N. P.; Maruszak, M.; Bednarski, P. J.; Klein, F.; Jakupec, M. A.; Nazarov, A. A.; Severin, K.; Keppler, B. K. Transferring the concept of multinuclearity to ruthenium complexes for improvement of anticancer activity. *J. Med. Chem.* **2009**, *52*, 916–925.
- (33) Smith, G. S.; Therrien, B. Targeted and multifunctional arene ruthenium chemotherapeutics. *Dalton Trans.* **2011**, *40*, 10793–10800.
- (34) Senderowicz, A. M. Novel direct and indirect cyclin-dependent kinase modulators for the prevention and treatment of human neoplasms. *Cancer Chemother. Pharmacol.* **2003**, *52*, S61–S73.
- (35) Song, H. F.; Kerman, K.; Kraatz, H. B. Electrochemical detection of kinase-catalyzed phosphorylation using ferrocene-conjugated ATP. *Chem. Commun.* **2008**, 502–504.
- (36) Martić, S.; Labib, M.; Kraatz, H. B. Enzymatically modified peptide surfaces: towards general electrochemical sensor platform for protein kinase catalyzed phosphorylations. *Analyst* **2011**, *136*, 107–112.
- (37) Hanif, M.; Henke, H.; Meier, S. M.; Martić, S.; Labib, M.; Kandioller, W.; Jakupec, M. A.; Arion, V. B.; Kraatz, H.-B.; Keppler, B. K.; Hartinger, C. G. Is the reactivity of M(II)–arene complexes of 3-hydroxy-2(1H)-pyridones to biomolecules the anticancer activity determining parameter? *Inorg. Chem.* **2010**, *49*, 7953–7963.
- (38) Larsen, A. K.; Escargueil, A. E.; Skladanowski, A. Catalytic topoisomerase II inhibitors in cancer therapy. *Pharmacol. Ther.* **2003**, *99*, 167–181.
- (39) Liu, L. F. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* **1989**, *58*, 351–375.
- (40) Nijveldt, R. J.; van Nood, E.; van Hoorn, D. E.; Boelens, P. G.; van Norren, K.; van Leeuwen, P. A. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **2001**, *74*, 418–425.
- (41) Bandele, O. J.; Osheroff, N. Bioflavonoids as poisons of human topoisomerase II alpha and II beta. *Biochemistry* **2007**, *46*, 6097–6108.
- (42) Holden, J. A. DNA topoisomerases as anticancer drug targets: from the laboratory to the clinic. *Curr. Med. Chem. Anti-Cancer Agents* **2001**, *1*, 1–25.
- (43) Champoux, J. J. DNA topoisomerases: structure, function, and mechanism. *Annu. Rev. Biochem.* **2001**, *70*, 369–413.
- (44) Dorcier, A.; Hartinger, C. G.; Scopelliti, R.; Fish, R. H.; Keppler, B. K.; Dyson, P. J. Studies on the reactivity of organometallic Ru–, Rh–, and Os–pta complexes towards DNA model compounds. *J. Inorg. Biochem.* **2008**, *102*, 1066–1076.
- (45) Bennett, M. A.; Smith, A. K. Arene ruthenium(II) complexes formed by dehydrogenation by cyclohexadienes with ruthenium(III) trichloride. *J. Chem. Soc., Dalton Trans.* **1974**, 233–241.
- (46) Pressprich, M. R.; Chambers, J. SAINT+ Integration Engine, Program for Crystal Structure Integration; Bruker Analytical X-ray Systems: Madison, WI, 2004.
- (47) Sheldrick, G. M. A short history of SHELX. *Acta Crystallogr., Sect. A: Found. Crystallogr.* **2008**, *A64*, 112–122.
- (48) Farrugia, L. J. ORTEP-3 for windows—a version of ORTEP-III with a graphical user interface (GUI). *J. Appl. Crystallogr.* **1997**, *30*, 565.
- (49) Bracht, K.; Boubakari, Grünert, R.; Bednarski, P. J. Correlations between the activities of 19 anti-tumor agents and the intracellular glutathione concentrations in a panel of 14 human cancer cell lines: comparisons with the National Cancer Institute data. *Anti-Cancer Drugs* **2006**, *17*, 41–51.

SUPPORTING INFORMATION

Structure-activity relationships of targeted Ru^{II}(η^6 -*p*-cymene) anticancer complexes with flavonol-derived ligands

Andrea Kurzwernhart,^a Wolfgang Kandioller,^{a,b} Simone Bächler,^c Caroline Bartel,^a
Sanela Martić,^d Magdalena Buczkowska,^e Gerhard Mühlgassner,^a Michael A.
Jakupec,^{a,b} Heinz-Bernhard Kraatz,^d Patrick J. Bednarski,^e Vladimir B. Arion,^a Doris
Marko,^c Bernhard K. Keppler,^{a,b} and Christian G. Hartinger^{a,b,e,*}

^a Institute of Inorganic Chemistry, University of Vienna, Waehringer Str. 42, 1090 Vienna, Austria

^b Research Platform “Translational Cancer Therapy Research”, University of Vienna, Waehringer Str. 42, 1090 Vienna, Austria

^c Institute of Food Chemistry and Toxicology, University of Vienna, Waehringer Str. 38, 1090 Vienna, Austria

^d Department of Physical and Environmental Sciences, University of Toronto at Scarborough, Canada

^e Department of Pharmaceutical and Medicinal Chemistry, University of Greifswald, Germany

^f The University of Auckland, School of Chemical Sciences, Private Bag 92019, Auckland 1142, New Zealand

Table of Contents

1. Single crystal X-ray diffraction analysis data	2
2. Long-term stability of 3i	3
3. Studies on the interaction of 3a with 5'-GMP	3
4. <i>In vitro</i> anticancer activity	4
5. Cell cycle analysis.....	6
6. Topoisomerase II α inhibition.....	8
7. Electrochemical screening of the CDK/Cyclin A inhibition	9

1. Single crystal X-ray diffraction analysis data

Table S1. Crystallographic data of **3d**, **3f** and **3h**.

Compound	3d	3f	3h
Empirical formula	C ₂₅ H ₂₂ ClFO ₄ Ru	C ₂₅ H ₂₂ Cl ₂ O ₃ Ru	C ₂₅ H ₂₂ Cl ₂ O ₃ Ru
Formula weight / g/mol	525.95	542.40	542.40
Temperature / K	100(2)	100(2)	100(2)
Wavelength /	0.71073	0.71073	0.71073
Crystal size / mm	0.20 × 0.15 × 0.08	0.30 × 0.17 × 0.10	0.20 × 0.20 × 0.10
Crystal system	triclinic	monoclinic	monoclinic
space group	<i>P</i> -1	<i>P</i> 2 ₁ / <i>n</i>	<i>P</i> 2 ₁ / <i>n</i>
<i>a</i> / Å	8.0865(5)	9.7756(4)	8.1020(2)
<i>b</i> / Å	9.8272(7)	10.2548(5)	24.9929(6)
<i>c</i> / Å	14.1751(10)	22.6155(11)	10.7067(3)
α / °	74.256(4)		
β / °	77.550(3)	101.373(2)	94.829(1)
γ / °	85.699(3)		
Volume / Å ³	1058.54(12)	2222.61(18)	2160.33(10)
<i>Z</i>	2	4	4
Calculated density / mg/m ³	1.650	1.621	1.668
Absorption coefficient / mm ⁻¹	0.901	0.971	0.999
<i>F</i> (000)	532	1096	1096
θ range for data collection	2.15–29.91°	1.84–30.13°	2.08–25.98°
Index ranges	-11 ≤ <i>h</i> ≤ 11 -13 ≤ <i>k</i> ≤ 13 -19 ≤ <i>l</i> ≤ 19	-13 ≤ <i>h</i> ≤ 13 -13 ≤ <i>k</i> ≤ 14 -29 ≤ <i>l</i> ≤ 31	-9 ≤ <i>h</i> ≤ 9 -30 ≤ <i>k</i> ≤ 30 -12 ≤ <i>l</i> ≤ 12
Reflections collected / unique	41123 / 6072	68880 / 6520	52250 / 4031
Data / restraints / parameters	6077 / 0 / 281	6520 / 0 / 281	4031 / 0 / 281
<i>R</i> (int)	0.0972	0.0572	0.0612
Goodness-of-fit on <i>F</i> ² [1]	1.024	1.009	1.040
Final <i>R</i> indices [<i>I</i> > 2σ(<i>I</i>)] [2]			
<i>R</i> ₁	0.0305	0.0282	0.0261
<i>wR</i> ₂	0.0744	0.0614	0.0649

[1] $\text{GOF} = \{\sum[w(F_o^2 - F_c^2)^2]/(n - p)\}^{1/2}$, where *n* is the number of reflections and *p* is the total number of parameters refined.

[2] $R_1 = \sum||F_o| - |F_c||/\sum|F_o|$.

$wR_2 = \{\sum[w(F_o^2 - F_c^2)^2]/\sum[w(F_o^2)^2]\}^{1/2}$

2. Long-term stability of **3i**

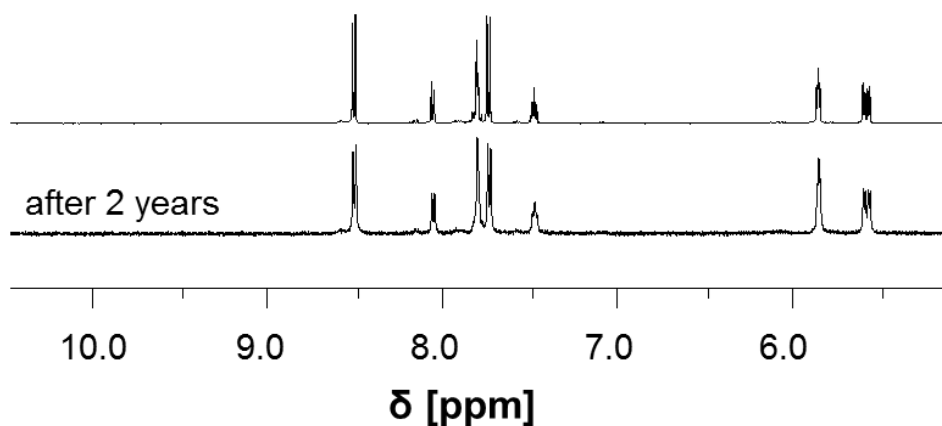


Figure S1. Aromatic regions of the ¹H NMR spectra of freshly prepared in *d*₆-DMSO solutions of **3i** measured immediately and after 2 years.

3. Studies on the interaction of **3a** with 5'-GMP

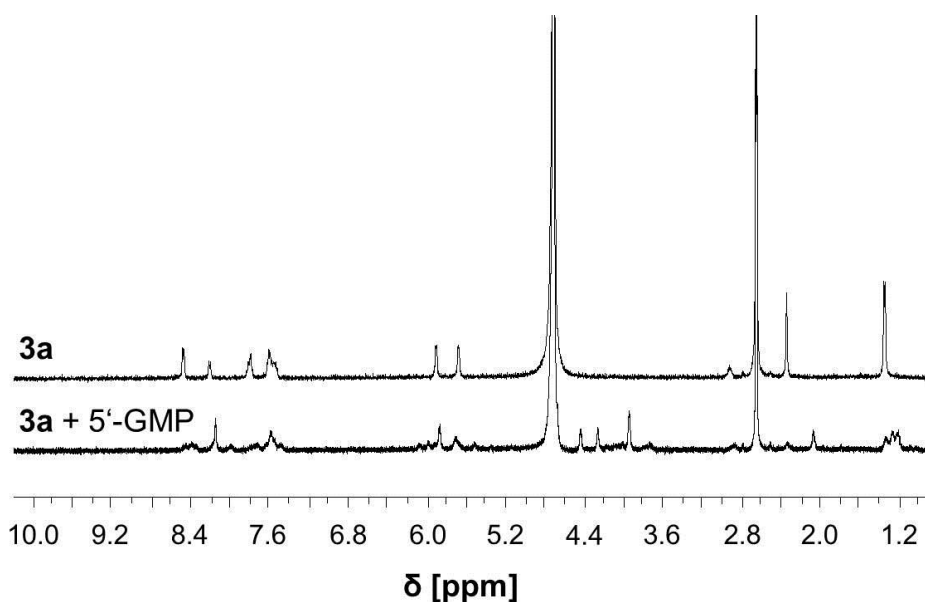


Figure S2. ¹H NMR spectra of **3a** and **3a** incubated with an excess of 5'-GMP in 10% *d*₆-DMSO/D₂O. The reaction of **3a** with 5'-GMP is indicated by the upfield shift of the *N*7 atom of 5'-GMP from approximately δ = 8.1 (free 5'-GMP) to 7.6 ppm (bound 5'-GMP) after addition of 5'-GMP in excess.

4. *In vitro* anticancer activity

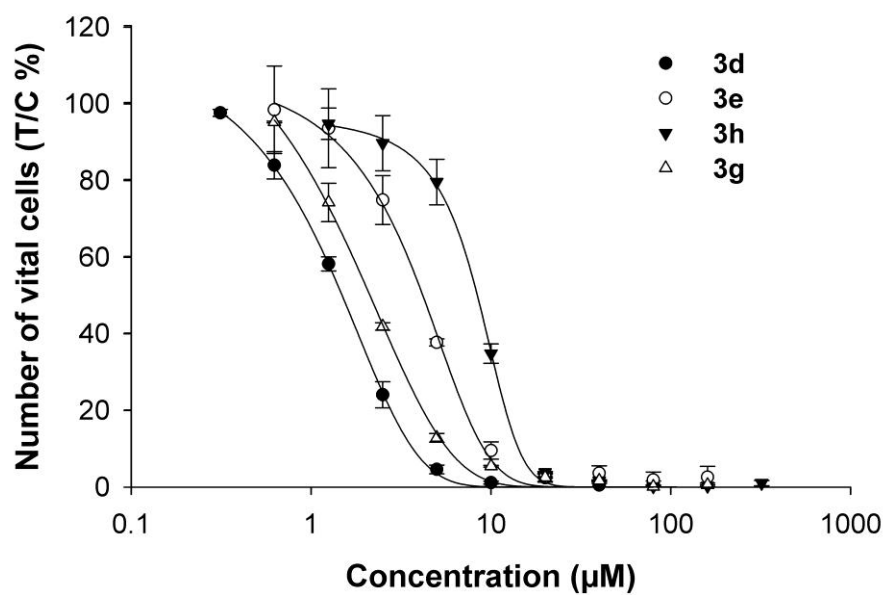


Figure S3. Concentration–effect curves of **3d**, **3e**, **3g** and **3h** in CH1 human ovarian carcinoma cells.

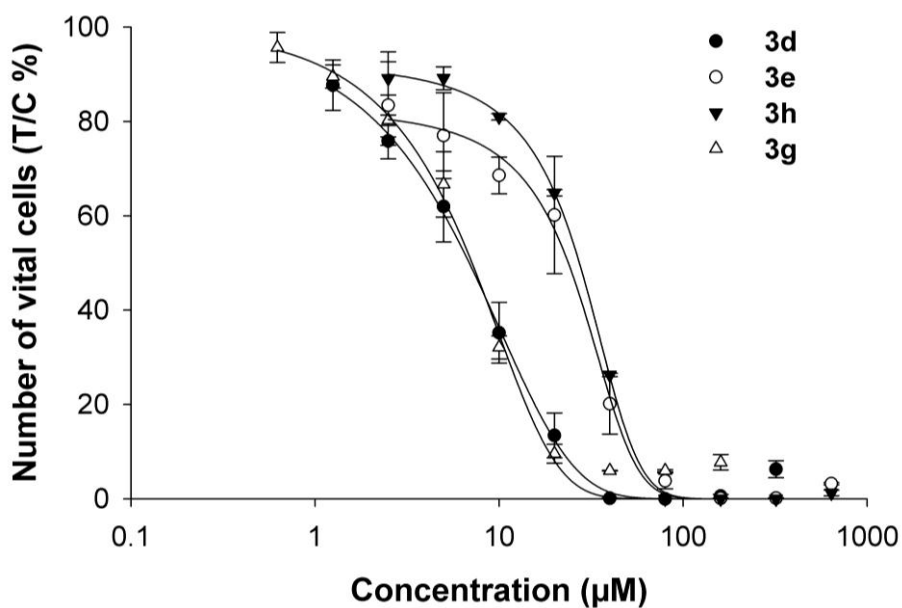


Figure S4. Concentration–effect curves of **3d**, **3e**, **3g** and **3h** in SW480 human colon carcinoma cells.

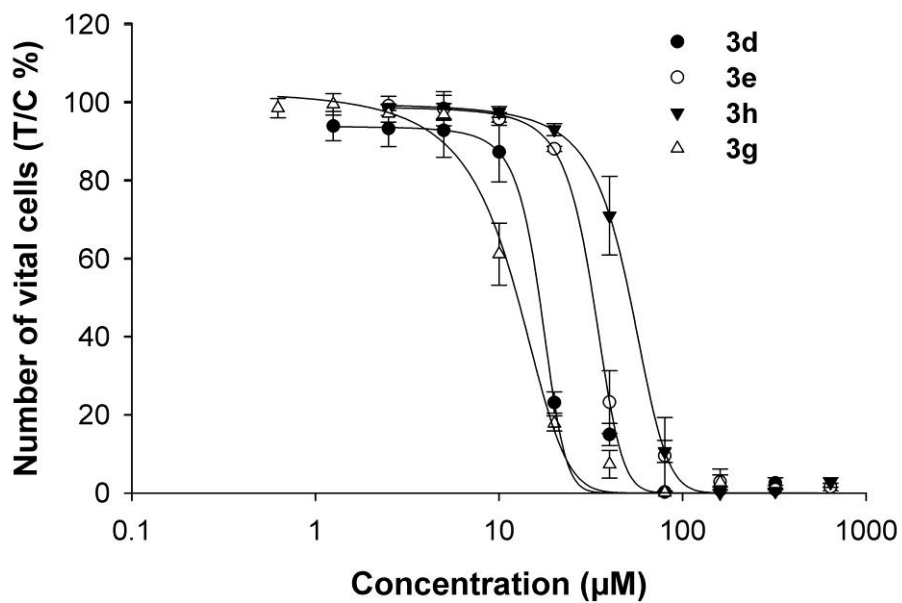


Figure S5. Concentration–effect curves of **3d**, **3e**, **3g** and **3h** in A549 human non-small cell lung carcinoma cells.

5. Cell cycle analysis

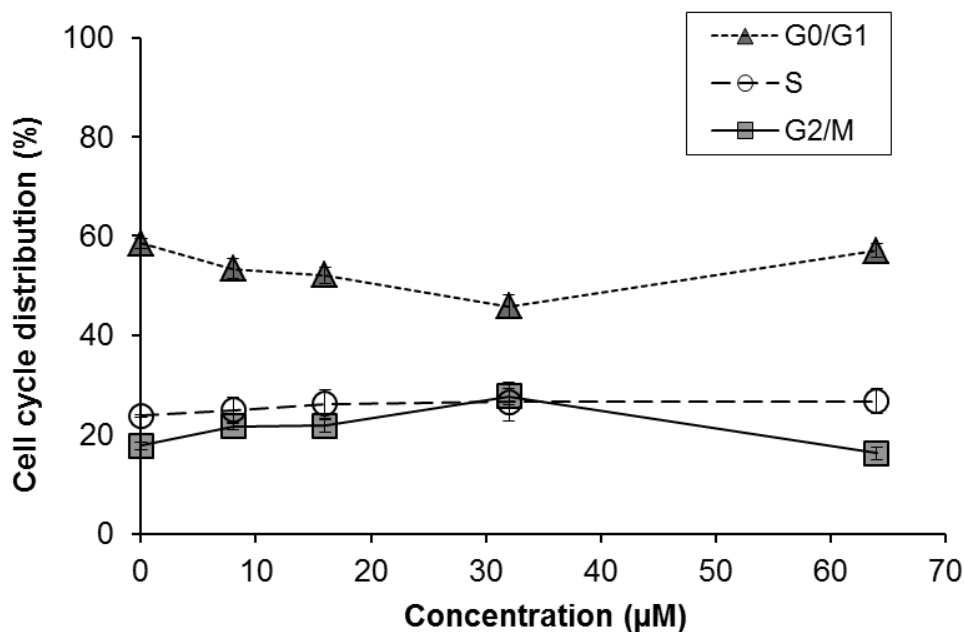


Figure S6. Concentration-dependent impact of **3a** on the cell cycle distribution of A549 cells after exposure for 48 h (values are means $\pm$ standard deviations of two independent experiments).

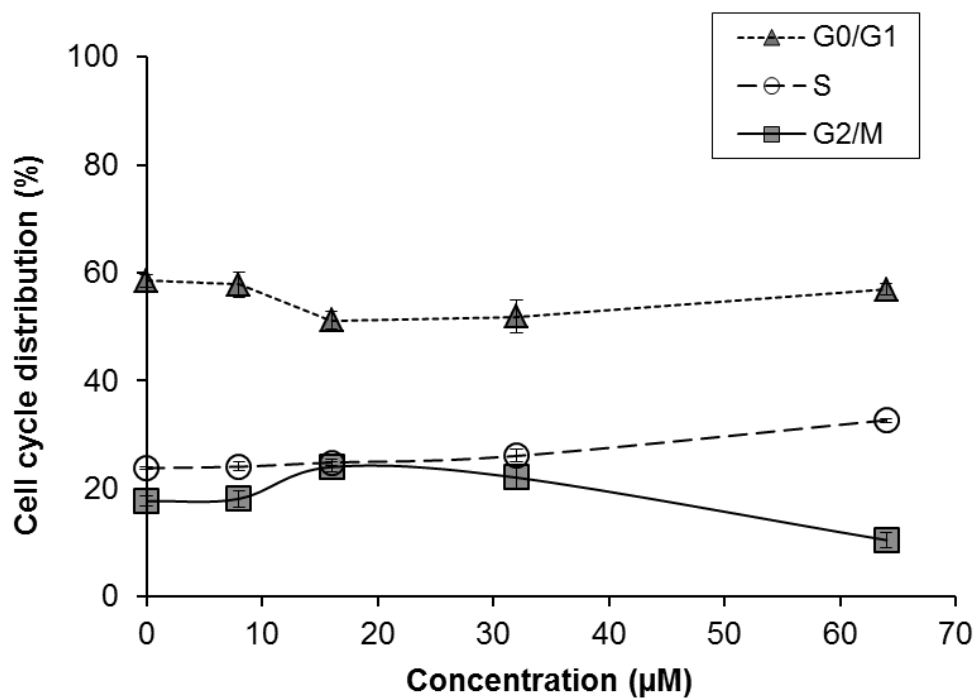


Figure S7. Concentration-dependent impact of **3b** on the cell cycle distribution of A549 cells after exposure for 48 h (values are means $\pm$ standard deviations of two independent experiments).

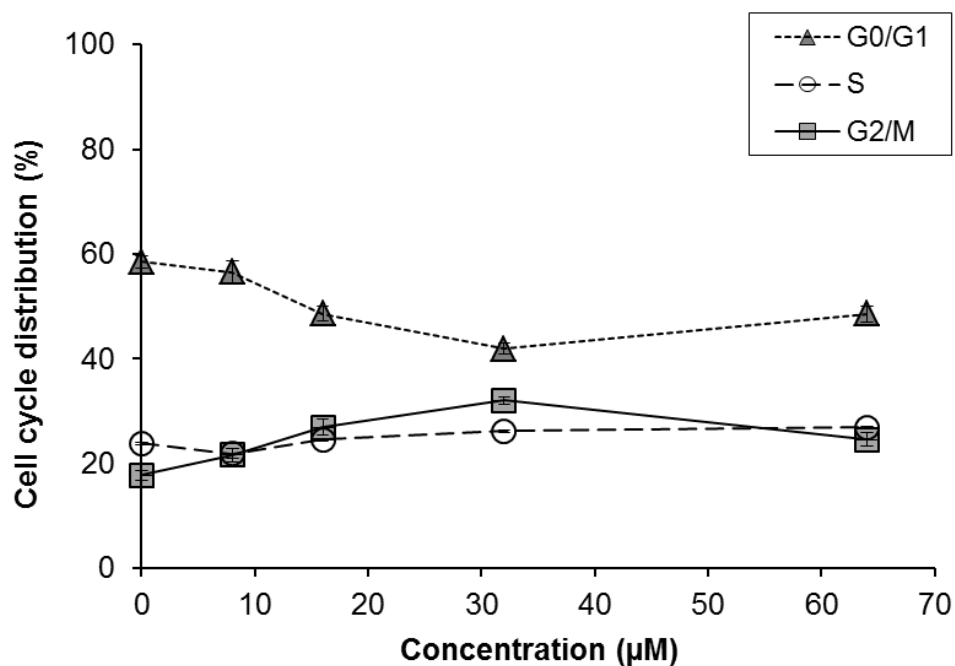


Figure S8. Concentration-dependent impact of **3c** on the cell cycle distribution of A549 cells after exposure for 48 h (values are means $\pm$ standard deviations of two independent experiments).

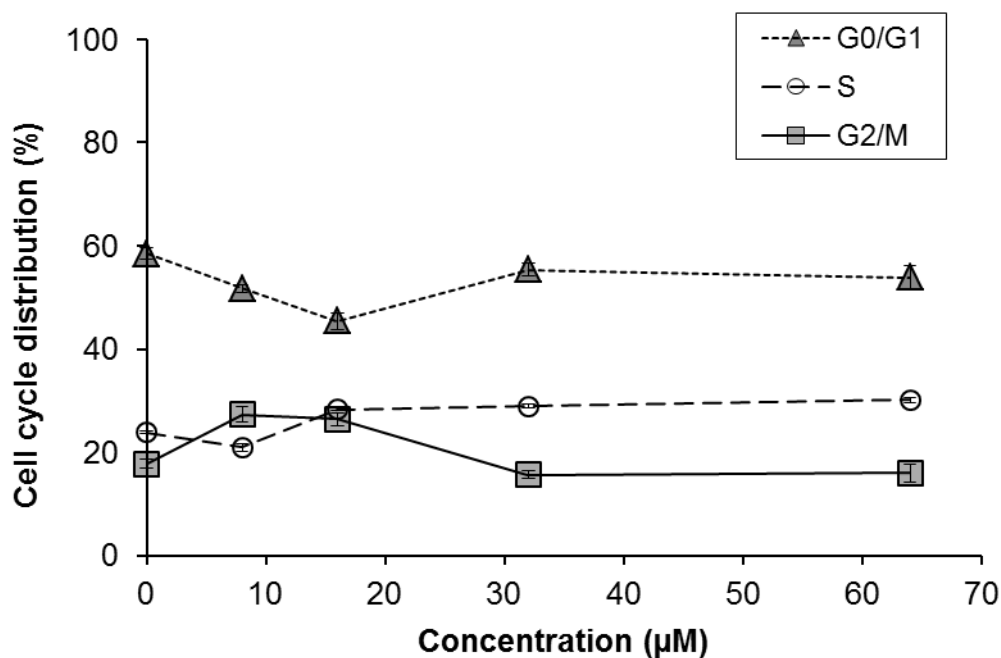


Figure S9. Concentration-dependent impact of **3f** on the cell cycle distribution of A549 cells after exposure for 48 h (values are means $\pm$ standard deviations of two independent experiments).

6. Topoisomerase II α inhibition

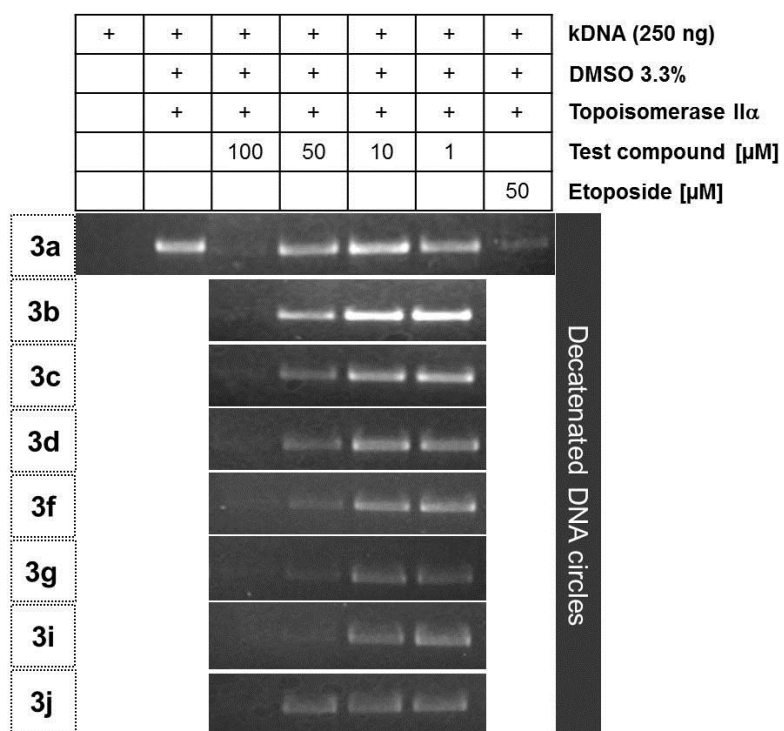


Figure S10. Concentration-dependent effect of the Ru complexes **3a–d**, **3f**, **3g**, **3i** and **3j** on the catalytic activity of topoisomerase II α , as determined by the decatenation assay.

7. Electrochemical screening of the CDK/Cyclin A inhibition

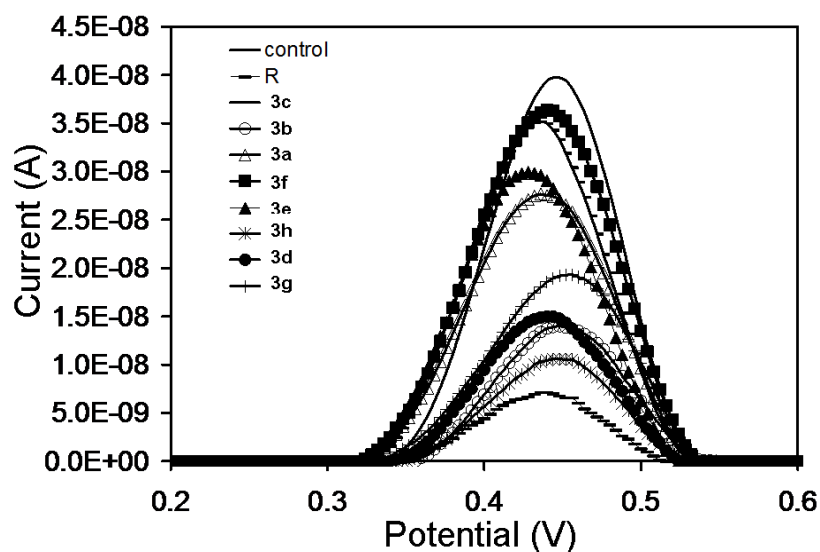
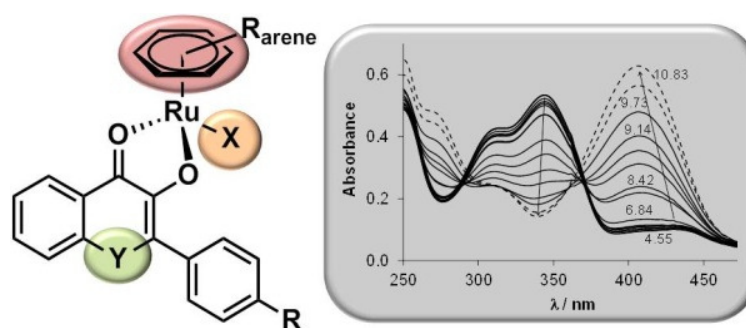


Figure S11. Square-wave voltammograms of peptide-modified gold electrodes following the Fc-phosphorylation with CDK2/cyclin A protein kinase and Fc-ATP in the absence of inhibitor (control), and in the presence of inhibitors: roscovitine (R) and organometallic compounds **3a–3h** (100 mV/s scan rate, Ag/AgCl as reference, Pt wire as auxiliary and peptide-modified gold electrode as working electrode, 0.1 M phosphate buffer, pH 7.4 as electrolyte).

3.4. 3-Hydroxyflavones vs. 3-Hydroxyquinolinones: Structure–Activity Relationships and Stability Studies on Ru^{II}(arene) Anticancer Complexes with Biologically Active Ligands

Dalton Transactions **2013**, DOI: 10.1039/C2DT32206D.

Graphical abstract



Ru^{II}(η⁶-arene) complexes, especially with bioactive ligands, are considered to be very promising compounds for anticancer drug design. We have shown recently that Ru^{II}(η⁶-*p*-cymene) complexes with 3-hydroxyflavone ligands exhibit very high *in vitro* cytotoxic activities correlating with a strong inhibition of topoisomerase IIα. In order to expand our knowledge about the structure–activity relationships and to determine the impact of lipophilicity of the arene ligand and of the hydrolysis rate on anticancer activity, a series of novel 3-hydroxyflavone derived Ru^{II}(η⁶-arene) complexes were synthesised. Furthermore, the impact of the heteroatom in the bioactive ligand backbone was studied by comparing the cytotoxic activity of Ru^{II}(η⁶-*p*-cymene) complexes of 3-hydroxyquinolinone ligands with that of their 3-hydroxyflavone analogues. To better understand the behaviour of these Ru^{II} complexes in aqueous solution, the stability constants and pK_a values for complexes and the corresponding ligands were determined. Furthermore, the interaction with the DNA model 5'-GMP and with a series of amino acids was studied in order to identify potential biological target structures.

3-Hydroxyflavones vs. 3-hydroxyquinolinones: structure–activity relationships and stability studies on Ru^{II}(arene) anticancer complexes with biologically active ligands†

Cite this: DOI: 10.1039/c2dt32206d

Andrea Kurzwernhart,^{a,b} Wolfgang Kandioller,^{a,b} Éva A. Enyedy,^c Maria Novak,^a Michael A. Jakupec,^{a,b} Bernhard K. Keppler^{a,b} and Christian G. Hartinger^{*a,b,d}

Ru^{II}(η^6 -arene) complexes, especially with bioactive ligands, are considered to be very promising compounds for anticancer drug design. We have shown recently that Ru^{II}(η^6 -*p*-cymene) complexes with 3-hydroxyflavone ligands exhibit very high *in vitro* cytotoxic activities correlating with a strong inhibition of topoisomerase II α . In order to expand our knowledge about the structure–activity relationships and to determine the impact of lipophilicity of the arene ligand and of the hydrolysis rate on anticancer activity, a series of novel 3-hydroxyflavone derived Ru^{II}(η^6 -arene) complexes were synthesised. Furthermore, the impact of the heteroatom in the bioactive ligand backbone was studied by comparing the cytotoxic activity of Ru^{II}(η^6 -*p*-cymene) complexes of 3-hydroxyquinolinone ligands with that of their 3-hydroxyflavone analogues. To better understand the behaviour of these Ru^{II} complexes in aqueous solution, the stability constants and pK_a values for complexes and the corresponding ligands were determined. Furthermore, the interaction with the DNA model 5'-GMP and with a series of amino acids was studied in order to identify potential biological target structures.

Received 21st September 2012,
Accepted 1st November 2012

DOI: 10.1039/c2dt32206d

www.rsc.org/dalton

Introduction

Ruthenium complexes represent a promising class of metal-based anticancer compounds. The octahedral geometry of ruthenium, its binding ability to plasma proteins and the number of possible oxidation states in biological environments make it well suitable for drug design.¹ Several ruthenium complexes have shown interesting properties *in vivo*, and a generally lower toxicity than for platinum drugs was

observed.² Two Ru^{III} compounds, namely [ImH][*trans*-Ru(DMSO)(Im)Cl₄] (NAMI-A, Im = imidazole) and [IndH][*trans*-Ru(Ind)₂Cl₄] (KP1019, Ind = indazole) (Chart 1), are currently undergoing clinical trials with very promising results.^{3–5}

In the course of ruthenium anticancer drug development programmes, organometallic and especially half-sandwich Ru^{II}(η^6 -arene) complexes have more and more demonstrated their potential.^{6–10} Their hydrophobic arene ligand is thought to facilitate the diffusion through the lipophilic cell membrane.¹¹ The three remaining Ru coordination sites can be filled with various mono-, bi- or tridentate ligands, which offers a number of possibilities to modulate biological and pharmacological properties by proper ligand selection.¹² Important examples for this substance type are Ru^{II}(η^6 -arene) complexes of bidentate ethylenediamine, such as RM175

^aInstitute of Inorganic Chemistry, University of Vienna, Waehringer Str. 42, 1090 Vienna, Austria

^bResearch Platform “Translational Cancer Therapy Research”, University of Vienna, Waehringer Str. 42, 1090 Vienna, Austria

^cDepartment of Inorganic and Analytical Chemistry, University of Szeged, Dóm tér 7, H-6720 Szeged, Hungary

^dThe University of Auckland, School of Chemical Sciences, Private Bag 92019, Auckland 1142, New Zealand. E-mail: c.hartinger@auckland.ac.nz; http://hartinger.wordpress.fos.auckland.ac.nz; Tel: +64 9 373 7955 ext 83220

†Electronic supplementary information (ESI) available: Fluorescence spectra of ligand **b** and complex **2** in aqueous solution, measured and calculated UV-vis absorbance spectra and concentration distribution curves of the Ru^{II}(cym)-ligand **b** system, NMR studies on the stability of **12** in aqueous solution, NMR spectra demonstrating the binding ability of **12'** to the DNA model 5'-GMP and NMR spectra showing the reactions of **1'**, **12'** and **13'** with amino acids. See DOI: 10.1039/c2dt32206d

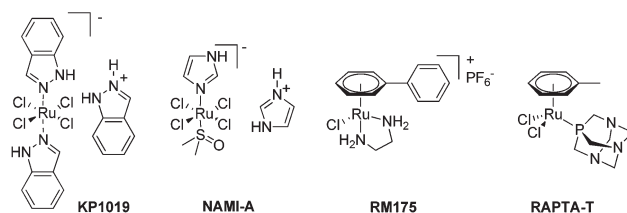


Chart 1 Structures of Ru anticancer agents.

(Chart 1), and the RAPTA-type compounds containing the monodentate 1,3,5-triaza-7-phosphatricyclo[3.3.1.1]decane (pta) ligand. RM175 binds to DNA either covalently *via* the N7 of guanine or non-covalently by intercalation of the arene, leading to cell death by modulation of the p53-p21-bax pathway.^{2,13} As opposed to this, the RAPTA compounds have very different chemical and biological properties. RAPTA-T (Chart 1) is selectively activated under the hypoxic conditions of solid tumours and is capable of inhibiting experimental metastasis models both *in vitro* and *in vivo*.^{4,14–16} Tethering ethacrynic acid to the arene ligand of RAPTA led to a compound capable of overcoming the glutathione transferase drug resistance mechanism of tumour cells and triggered several biological pathways involving either endonuclease G, caspases or c-Jun N-terminal kinase.¹⁷ This is an example of linking a biologically active molecule to a metal centre and modulating thereby its biological properties. Other related approaches involve Ru^{II}(arene) compounds with ligand systems that resemble the kinase inhibitor staurosporine¹⁸ or complexes of paullones, which are cyclin-dependent kinase (CDK) and glycogen synthase kinase-3 inhibitors.¹⁹ More recently, we have demonstrated that Ru^{II}(cym) (cym = η^6 -*p*-cymene) complexes of 3-hydroxyflavones are potent tumour cell growth inhibitors.²⁰

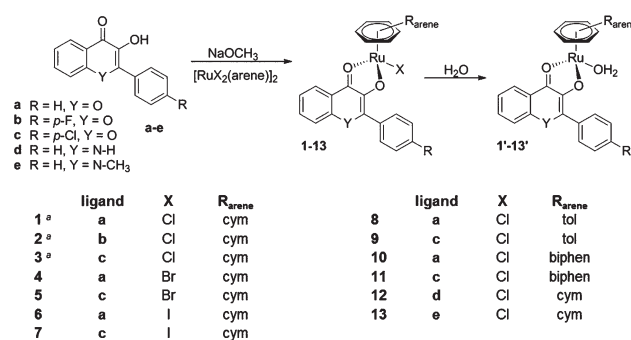
3-Hydroxyflavones belong to the naturally occurring class of flavonoids which are polyphenols of plants, fruits and vegetables. They are well known for their beneficial effects on health due to their antioxidant, antiinflammatory, antiviral and anticarcinogenic properties. These effects are caused primarily by the scavenging of free radicals by the flavonoid structure and by interaction with a number of enzymes.²¹ Flavonoids are capable of forming stable chelate complexes with a broad range of metal ions, which have already shown biological activity in the treatment of diseases like AIDS, diabetes mellitus, some genetic diseases and also cancer.²² The Ru^{II}(cym) complexes of 3-hydroxyflavones were found to exhibit not only high *in vitro* anticancer activity in human cancer cell lines but also to inhibit human topoisomerase II α activity, which correlates with their cytotoxic potency.²⁰

In order to study the impact of the nature of the arene and halogenido ligands on the stability and cytotoxic activity, a series of Ru^{II}(η^6 -arene)X complexes with 3-hydroxyflavones has been synthesised. These properties are compared with those of structurally related 3-hydroxyquinolinone complexes featuring a nitrogen atom in the heterocyclic ligand. These studies are complemented with UV-vis and fluorescence spectroscopy experiments to gain information on the stability and pK_a values of the hydrolysis products and ligand systems.

Results and discussion

Synthesis

Within the course of a project to prepare 3-hydroxy-4-pyrone complexes, we have reported the synthesis of ruthenium(II)-cymene complexes with various substituted 3-hydroxyflavones, and the influence of the substitution pattern and the nature of



Scheme 1 Synthesis of Ru^{II}(η^6 -arene) complexes **1–13** and formation of the hydrolysis products **1'–13'** in aqueous solution. ^aFrom ref. 20 and 23.

the substituent on the *in vitro* anticancer activity was studied.^{20,23} In order to extend our knowledge about the structure–activity relationships (SARs), a series of Ru^{II}(η^6 -arene) complexes with 3-hydroxyflavones **a–c** and 3-hydroxyquinolinones **d** and **e** was synthesised by deprotonation of the ligands with sodium methoxide and subsequent reaction with the respective bis[dihalido(η^6 -arene)ruthenium(II)] ([RuX₂(arene)]₂; η^6 -arene = cym, toluene, biphenyl; X = Cl, Br, I), yielding complexes **1–13** in good to very good yields (Scheme 1). The compounds were characterised by standard analytical methods (see the experimental part) and were stable for over one year though exposed to sunlight and air.

Behaviour and stability in aqueous solution

In order to study the properties of the 3-hydroxyflavone-derived Ru^{II}(cym) complexes in aqueous solution, the proton dissociation process of the *p*-fluoro-substituted ligand **b**, the hydrolysis of [Ru^{II}(cym)X₃]^{*n*} (*n* = −1 to 2; X = Cl[−], H₂O or DMSO, ESI⁺) and the complex formation process of the corresponding complex **2** were investigated and stability and dissociation constants were studied.

PROTON DISSOCIATION PROCESS OF LIGAND B. The proton dissociation constant (pK_a) of ligand **b** was determined by UV-vis spectrophotometry in 20% (w/w) dimethyl sulfoxide (DMSO)–H₂O because of the poor solubility of the ligand and its complex in pure water. Since flavonoids may suffer from photodegradation,²⁴ spectra were measured at various pH values employing the batch technique instead of continuous titrations. This guarantees minimal UV exposure and helps avoiding photolysis, especially at high pH values (Fig. 1). The pH-dependent spectra of the ligand show characteristic changes at increasing pH values. The deprotonation (HL $\rightleftharpoons$ L[−] + H⁺) attributed to the hydroxyl functional group is accompanied by a bathochromic shift of the $\lambda_{\max}$ and a small increase in intensity. The isosbestic point is constant at 366 nm up to pH 10.4 but shifts at higher pH most probably due to the photodegradation of the ligand. Therefore, the pK_a value of 8.70 ± 0.01 and the individual spectra of the ligand species (HL, L[−]; Fig. 1b) were calculated on the basis of deconvoluted spectra recorded at pH < 10.4. The $\lambda_{\max}$ values of both the protonated and the deprotonated forms of ligand **b** are

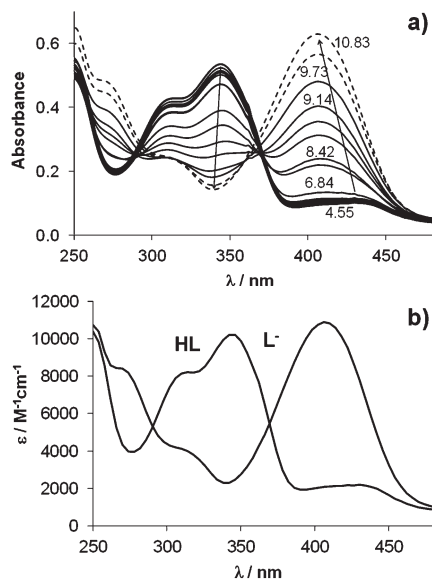


Fig. 1 UV-vis spectra of ligand **b** at various pH values (a) and calculated individual absorbance spectra of the HL and L⁻ species (b) ($c_{\text{ligand}} = 5 \times 10^{-5}$ M; $T = 25$ °C; $I = 0.20$ M (KCl); 20% (w/w) DMSO–H₂O); HL: $\lambda_{\text{max}} = 342$ nm ($\lambda_{342 \text{ nm}} = 10\,210 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$); L⁻: $\lambda_{\text{max}} = 402$ nm ($\lambda_{402 \text{ nm}} = 10\,755 \text{ mol}^{-1} \text{ dm}^3 \text{ cm}^{-1}$).

identical to those of the unsubstituted 3-hydroxyflavone **a**.²⁵ However, its pK_a value is significantly lower due to the electron withdrawing effect of the fluoro substituent. The pK_a of the structurally related pyrone ligand maltol (8.76 ± 0.01), which was also determined under the same conditions, was in the same range as that of **b**.²⁶

In addition, the proton dissociation process of **b** in aqueous phase was monitored by fluorimetry (Fig. S1a†) at a much lower concentration. The ligand excitation maximum was found at 342 nm, and the emission spectrum contains two maxima at 504 and 411 nm. The appearance of the two emission bands indicates two pathways for deactivation of the excited state. The pH dependence of the fluorescence emission spectra shows that the emission intensity is strongly sensitive to the pH, and deprotonation results in a significant decrease of the intensity. From the spectral changes in water a pK_a value of 8.30 ± 0.09 was obtained, which verifies the pK_a determined in 20% (w/w) DMSO–H₂O and which is again in the same range as the pK_a of maltol in aqueous solution (8.44).²⁵

SOLUTION EQUILIBRIA OF $[\text{Ru}^{\text{II}}(\text{cym})\text{X}_3]^n$ AND COMPLEX 2. In order to understand the behaviour of the flavonoid complex in aqueous solution, the equilibria of the hydrolysis of $[\text{Ru}^{\text{II}}(\text{cym})\text{X}_3]^n$ ($n = -1$ to 2; $\text{X} = \text{Cl}^-$, H₂O or DMSO) needed to be determined under the same conditions. This was studied in 20% (w/w) DMSO–H₂O by UV-vis spectrophotometric titrations (Fig. S2†). Based on the spectral changes, stability constants of the minor $[\text{Ru}_2(\text{cym})_2(\text{OH})_2\text{X}_m]^n$ ($m = 1, 2$) and major $[\text{Ru}_2(\text{cym})_2(\text{OH})_3]^+$ dinuclear hydrolysis products were determined as $\log \beta [(\text{Ru}(\text{cym}))_2\text{H}_{-2}]^{2+} = -9.85 \pm 0.06$ and $\log \beta [\text{Ru}_2(\text{cym})_2\text{H}_{-3}]^+ = -15.11 \pm 0.03$, respectively (ESI†). As the titrations were performed in the presence of 0.2 M KCl, these constants are regarded as conditional stability constants. Similar but not

identical speciation was found in pure aqueous solution.²⁷ The presence of DMSO can suppress the hydrolysis of $[\text{Ru}^{\text{II}}(\text{cym})\text{X}_3]^n$ which is then shifted to higher pH values (Fig. S2b†).

The complex formation processes of the ruthenium(II)–cym complex **2** were studied under the same conditions as for $[\text{Ru}^{\text{II}}(\text{cym})\text{X}_3]^n$ (Fig. 2a) and are compared to the maltol–ruthenium(II)–cym system (Fig. 2b).¹¹ The pH-dependent spectral changes of the ruthenium(II)–cym-containing systems (Fig. 2c) compared to the free ligands reveal that the complex formation starts at $\text{pH} > \sim 4$ in both cases. The complex formation results in a significant shift of the λ_{max} values, and this new band is different from the bands belonging to the protonated and deprotonated forms of the metal-free ligands. This band is especially well-separated in the case of **2** (Fig. 2a) (*i.e.* λ_{max} of complex: 436 nm, HL: 342 nm, L⁻: 402 nm). Analysis of changes in the overlapping ligand and charge transfer (CT) bands shows the exclusive formation of mononuclear species $[\text{Ru}^{\text{II}}(\text{cym})(\text{L})\text{X}]^n$ with a 1 : 1 metal-to-ligand ratio. By deconvolution of the UV-vis spectra (Fig. S3†), a stability constant $\log \beta [(\text{Ru}^{\text{II}}(\text{cym})(\text{L})\text{X})^n] = 7.13 \pm 0.08$ for **2** was determined, which is in about the same range as that of the maltolato complex ($\log \beta = 7.04 \pm 0.05$).

At neutral and alkaline pH various parallel processes take place, namely the complex $[\text{Ru}^{\text{II}}(\text{cym})(\text{L})\text{X}]^n$ starts to hydrolyse forming the mixed hydroxido species $[\text{Ru}^{\text{II}}(\text{cym})(\text{L})(\text{OH})]$ and to dissociate giving the tris-hydroxido-bridged dinuclear species $[\text{Ru}_2(\text{cym})_2(\text{OH})_3]^+$ and the metal-free ligand (Fig. 2d). The dissociation of (O,O)-pyrone ligands such as maltol of *mono*-ligand complexes is relatively slow.²⁸ However, in the case of flavonoid complexes, the photodegradation of the ligand is a possible side reaction at $\text{pH} > \sim 10$. For these reasons the deconvolution of the spectra becomes more difficult and stability data of the $[\text{Ru}^{\text{II}}(\text{cym})(\text{L})(\text{OH})]$ species could only be obtained with lower accuracy as $\log \beta = 0.3 \pm 0.1$ for **2** and 0.1 ± 0.1 for maltol.

Based on the increased proton dissociation constants of ligand **b** and maltol (see above), higher stability constants of $[\text{Ru}^{\text{II}}(\text{cym})(\text{L})\text{X}]^n$ are expected in 20% (w/w) DMSO–H₂O than in pure aqueous solution. However, a $\log \beta = 9.05$ was reported for the maltolato complex in water,²⁹ which is actually two orders of magnitude higher than the constant obtained in a 20% (w/w) DMSO–H₂O mixture. DMSO complexes of Ru^{II} are known, and DMSO coordination can suppress the formation of $[\text{Ru}^{\text{II}}(\text{cym})(\text{L})\text{X}]^n$ complexes. The speciation and the stability of **2** and the maltolato complex show very strong similarities due to similar metal binding sites of the ligands. The fluorescence spectra of ligand **b** (Fig. S1a†) and complex **2** in aqueous solution (Fig. S1b†) show similar features up to $\text{pH} \sim 4$. Upon further increasing the pH, a band with high intensity at 448 nm develops reaching a maximum at $\text{pH} \sim 5$ and decreasing upon increasing pH. The appearance of this strong new band is most probably related to the formation of $[\text{Ru}^{\text{II}}(\text{cym})(\text{L})\text{X}]^n$, while the formation of the mixed hydroxido species $[\text{Ru}^{\text{II}}(\text{cym})(\text{L})(\text{OH})]$ is accompanied by a considerable loss of intensity. Therefore, this latter species seems to be

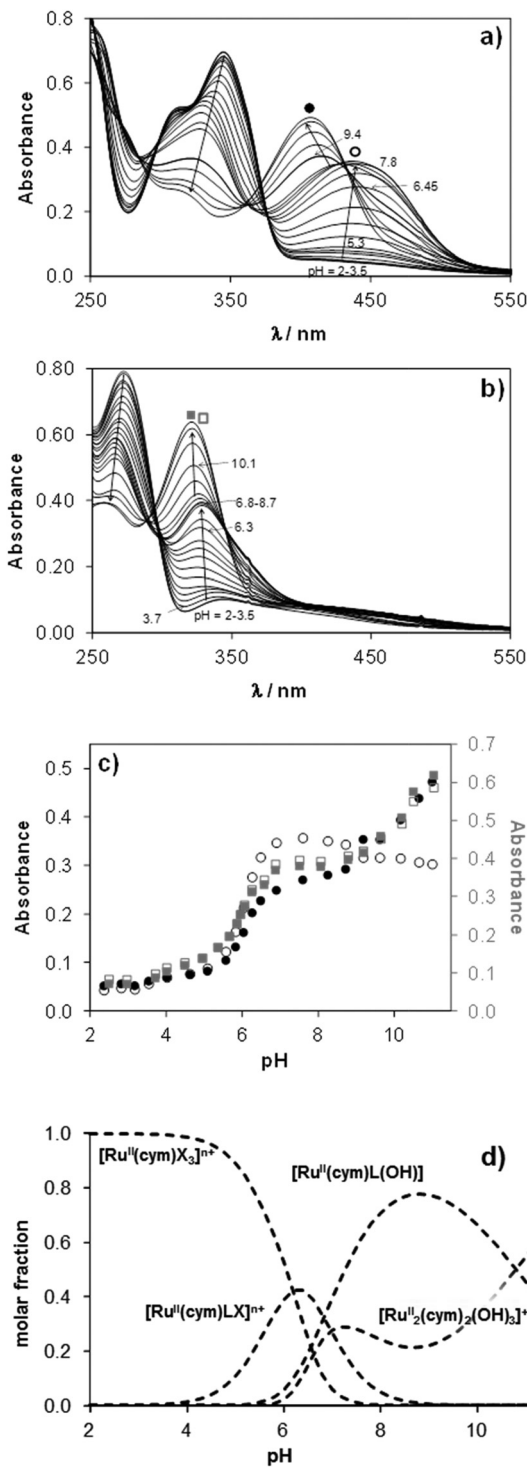


Fig. 2 (a) UV-vis spectra of **2** and (b) for comparison of a maltolato $\text{Ru}^{\text{II}}(\text{cym})$ complex at various pH values. (c) Absorbance values at 402 nm (●) and at 436 nm (○) for complex **2** and at 322 nm (■) and at 328 nm (□) for the maltolato $\text{Ru}^{\text{II}}(\text{cym})$ complex plotted against the pH value. (d) Concentration distribution curves of the complex **2** ($c_{\text{complex}} = 5 \times 10^{-5} \text{ M}$ ($8 \times 10^{-5} \text{ M}$ in the case of maltol); $T = 25^\circ \text{C}$; $I = 0.20 \text{ M}$ (KCl); 20% (w/w) DMSO– H_2O ; pH = 2.5–11.5).

much less fluorescent than $[\text{Ru}^{\text{II}}(\text{cym})(\text{L})\text{X}]^n$, but somewhat more fluorescent than the metal-free ligand. As also found for

the maltolato complex, partial hydrolysis and dissociation of **2** are probable at physiological pH.

Reactivity towards biomolecules

In aqueous solution, compounds **1–3**,²⁰ **5**, **7**, **9** and **11** are aquated immediately to the charged aqua species **1'–3'**, **5'**, **7'**, **9'** and **11'**, which can further react with biomolecules. The solubility of **4**, **6**, **8** and **10** in aqueous solution limited investigations, however, due to the structural similarity comparable behaviour is expectable. Several $\text{Ru}^{\text{II}}(\text{arene})$ complexes are known to bind to the DNA model compound 5'-GMP and therefore are also able to form adducts with DNA, which is a possible target for metal-based anticancer agents.^{1,2,11,30–33} Similarly, **1–3**,²⁰ **5**, **7**, **9** and **11** show interactions with 5'-GMP, as observed in ^1H NMR spectroscopy studies. However, due to their low solubility and even lower solubility of their 5'-GMP adducts, the binding mode and stability of the adducts are elusive.

The 3-hydroxyquinolinone-derived $\text{Ru}^{\text{II}}(\text{arene})$ complexes **12** (Fig. S4†) and **13** show the same aquation behaviour, but already 5 min after addition of D_2O the first signs of the hydrolysis side product $[\text{Ru}_2(\eta^6\text{-arene})_2(\text{OH})_3]^+$ were observed in the ^1H NMR spectrum, which increased within 24 h. This side product is thermodynamically stable and unreactive towards nucleophiles.⁷ Compounds **12** and **13** bind immediately to the N7 atom of 5'-GMP as indicated by an upfield shift of the H8 signal of 5'-GMP from approximately $\delta = 8.1$ to 7.6 ppm (Fig. S5†).

To gain more insight into possible interactions with proteins and pharmacokinetic pathways, the reactions of the representative hydrolysis products **1'**, **12'** and **13'** with the amino acids L-methionine, L-histidine, L-cysteine and glycine were investigated (Fig. S6–S12†). The reactivity was found to be similar to pyrone-derived $\text{Ru}^{\text{II}}(\text{cym})$ complexes. All compounds reacted immediately with Met and His by replacement of the aqua ligand with the respective amino acid, which is coordinated to the Ru^{II} centre *via* the sulphur atom or *via* the N1 or N3 atoms of the imidazole moiety, respectively.¹¹ In the case of **1'**, the ligand was cleaved off and precipitated completely within 24 h. The same behaviour was observed for the 3-hydroxyquinolinone-derived complexes. However, after 24 h especially for **12'** still signals of coordinated quinolinone ligands were visible. This may be due to a slightly higher stability of the 3-hydroxyquinolinone complexes towards the reaction with amino acids. Addition of Cys led to immediate decomposition of **1'** and to a lower extent of **13'**. For **12'** a reaction with Cys was observed (Fig. 3), but the compound also decomposed partly within 24 h. In the case of glycine, also differing behaviour between 3-hydroxyflavone and quinolinone complexes was observed. Glycine reacted immediately with **1'**, whereas the reaction with **12'** and especially **13'** was significantly slower. Two minutes after addition only traces of coordinated glycine (two doublets at approximately $\delta = 3.1 \text{ ppm}$)¹¹ were observed in **12'** and only after 18 h in **13'**, indicating again higher stability of the 3-hydroxyquinolinone complexes concerning reactions with amino acids. However, the cytotoxicity

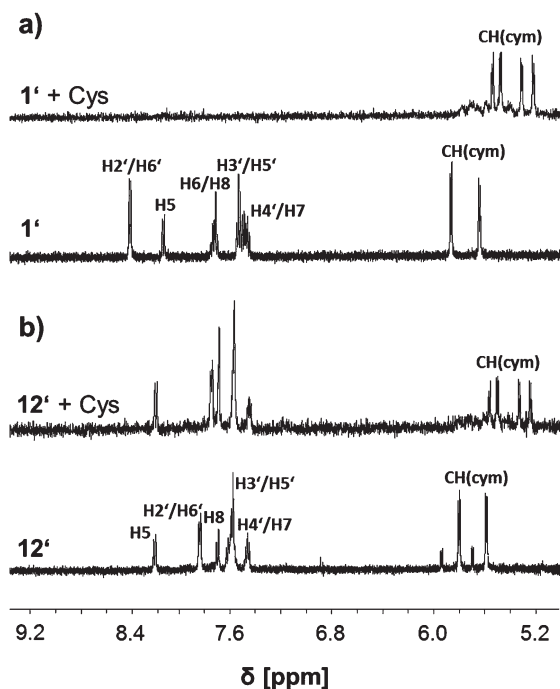


Fig. 3 Reaction mixtures of **1'** (a) and **12'** (b) with equimolar amounts of L-cysteine analysed by ^1H NMR spectroscopy after 5 min show immediate decomposition of **1'** after addition of Cys, whereas minor effects on the quinolinone signals of **12'** were observed.

of 3-hydroxyflavone and quinolinone $\text{Ru}^{\text{II}}(\text{cym})$ complexes was similar (see below), although the MTT assay to determine the IC_{50} values is carried out in an amino acid-containing medium. This indicates that the reaction with amino acids does not seem to significantly alter their *in vitro* anticancer potency, most probably due to their higher lipophilicity which may result in enhanced cellular uptake.

In vitro anticancer activity

The cytotoxic activity of the $\text{Ru}^{\text{II}}(\text{arene})$ complexes was determined in the human cancer cell lines CH1 (ovarian

carcinoma), SW480 (colon carcinoma) and A549 (non-small cell lung carcinoma) by means of the colorimetric MTT assay (Table 1). Recently, we have shown that the type and especially the position of the substituent on the phenyl ring of the ligand have a crucial impact on their biological activity.²⁰ *Meta*- and *para*-substitution led to more cytotoxic compounds, whereas *ortho*-substituted or unsubstituted ligand structures showed lower *in vitro* potency (Table 1, compare compounds **2** and **3** with **1**). These data correlate well with the inhibition of topoisomerase $\text{II}\alpha$ activity.²⁰ All synthesised complexes exhibit promising tumour-inhibiting properties with IC_{50} values in the low μM range, which is very remarkable for $\text{Ru}^{\text{II}}(\eta^6\text{-arene})$ complexes. In order to determine the effect of the lipophilicity on the anticancer activity, complexes bearing different arene ligands were synthesised. The toluene derivatives **8** and **9** exhibit a similar activity to their $\text{Ru}^{\text{II}}(\text{cym})$ analogues **1** and **3**, whereas the biphenyl complexes **10** and **11** are slightly less cytotoxic. Therefore, the influence of the arene ligands seems to be of minor importance for this type of compound. The same activity pattern was observed for pyrone and especially thiopyrone-derived $\text{Ru}^{\text{II}}(\text{arene})$ complexes,¹⁹ which is in contrast to for example ethylenediamine complexes. The latter compound class showed a strong dependence of cytotoxicity on the coordinated arene. The change from benzene to *p*-cymene to biphenyl resulted in a large increase of their growth inhibitory activity related to an increasing size and hydrophobicity.³⁴ It may be that the change in lipophilicity by the modification of the arene ligand is too marginal in case of lipophilic complexes to outperform the contribution of the flavonoid ligand to the lipophilicity. Furthermore, as already shown for analogous pyrone- and thiopyrone $\text{Ru}^{\text{II}}(\eta^6\text{-arene})$ derivatives, different halides as leaving groups show only little or no impact on the antiproliferative activity (compare **1**, **3**, **4**–**7**). This can be explained by the quick aquation of the Ru centre, leading to the same aqua products.

When changing from 3-hydroxyflavones to 3-hydroxyquinolinones as ligands, no improvement of the *in vitro* anticancer activity was observed. The quinolinone complexes **12** and **13**

Table 1 *In vitro* anticancer activity of **1**–**13** in ovarian (CH1), colon (SW480) and non-small cell lung carcinoma (A549) cell lines^a

	R	Y	X	Arene	IC_{50} [μM]		
					CH1	SW480	A549
1 ^b	H	O	Cl	cym	2.1 ± 0.2	9.6 ± 1.5	20 ± 2
2 ^b	<i>p</i> -F	O	Cl	cym	1.7 ± 0.4	7.9 ± 2.1	18 ± 1
3 ^b	<i>p</i> -Cl	O	Cl	cym	0.86 ± 0.06	3.8 ± 0.5	9.5 ± 0.5
4	H	O	Br	cym	2.8 ± 0.4	12 ± 1	27 ± 4
5	<i>p</i> -Cl	O	Br	cym	0.86 ± 0.04	3.4 ± 0.4	7.9 ± 0.6
6	H	O	I	cym	1.6 ± 0.2	9.6 ± 1.5	16 ± 1
7	<i>p</i> -Cl	O	I	cym	1.2 ± 0.3	4.7 ± 0.9	8.9 ± 0.8
8	H	O	Cl	tol	3.2 ± 0.1	12 ± 3	19 ± 1
9	<i>p</i> -Cl	O	Cl	tol	0.88 ± 0.17	4.7 ± 0.6	7.8 ± 2.5
10	H	O	Cl	biphen	5.5 ± 1.2	9.2 ± 1.9	28 ± 5
11	<i>p</i> -Cl	O	Cl	biphen	6.3 ± 1.1	21 ± 4	59 ± 1
12	H	N–H	Cl	cym	4.0 ± 0.2	14 ± 1	17 ± 2
13	H	N–CH ₃	Cl	cym	5.3 ± 0.2	12 ± 2	19 ± 1

^a IC_{50} = 50% inhibitory concentration, 96 h exposure. ^b Taken from ref. 20 and 23. tol = toluene, biphen = biphenyl.

exhibit cytotoxic activities in the same range as **1**. Also variation of the unsubstituted 3-hydroxyquinolinone **12** to the 1-methylated form in **13** showed no impact on the cytotoxic activity, indicating that the backbone of the ligand rather than the functional group seems to be crucial for the biological activity of this type of Ru^{II}(arene) complex.

Conclusions

Ru^{II}(η^6 -arene) complexes bearing biologically active ligand systems exhibit very interesting features and promising properties for anticancer drug design.¹² 3-Hydroxyflavone-derived Ru^{II}(η^6 -arene) complexes are potent cytotoxic agents with good correlation to their topoisomerase II α inhibitory activity.²⁶ We have extended the series of compounds by varying the arene and halido ligands to learn about their influence on the biological activity, as well as compared the 3-hydroxyflavone complexes to quinolinone analogues in terms of cytotoxicity and reactivity towards biomolecules. All compounds exhibit *in vitro* anticancer activity mostly in the low μ M range and showed interaction with the DNA model compound 5'-GMP. Substitution of the arene and halido ligands had only a minor effect on the cytotoxic activity. The 3-hydroxyquinolinone analogues behave similarly to the flavones in aqueous solutions and in anticancer activity assays, but are more stable in the presence of amino acids. Extensive solution phase studies by NMR, UV-vis and fluorescence spectroscopy revealed that the *para*-fluoro substituted 3-hydroxyflavone **b** [2-(4-fluorophenyl)-3-hydroxy-4*H*-chromen-4-one] exhibits a proton dissociation constant (pK_a) of 8.70 ± 0.01 in 20% (w/w) DMSO-H₂O and of 8.30 ± 0.09 in aqueous solution. The complex formation processes of the corresponding ruthenium(II)-cym complex **2** start at pH > ~4, forming mononuclear species [Ru^{II}(cym)(L)X]^{*n*} with a stability constant of $\log \beta = 7.13 \pm 0.08$. At pH ≥ 7 , hydrolysis of [Ru^{II}(cym)(L)X]^{*n*} leads to the mixed hydroxido species [Ru^{II}(cym)(L)(OH)] ($\log \beta = 0.3 \pm 0.1$) and partial dissociation giving the tris-hydroxido-bridged dinuclear species [Ru₂(cym)₂(OH)₃]⁺ and the metal-free ligand. The stability constants of the hydroxyflavone-derived ruthenium(II)-cym compounds are therefore in the range of structurally-related maltolato complexes.

Considering stability data and *in vitro* anticancer activity, 3-hydroxyflavones seem to be a well-suited ligand system for anticancer Ru^{II}(cym)(chlorido) complexes and those represent a promising compound class for further drug design.

Experimental part

Materials and methods

All solvents were dried and distilled prior to use. All chemicals were purchased from commercial suppliers and used without further purification. Bis[(η^6 -*p*-cymene)dichloridoruthenium(II)], bis[dichlorido(η^6 -toluene)ruthenium(II)],³⁵ bis[(η^6 -biphenyl)dichloridoruthenium(II)], bis[dibromido(η^6 -*p*-cymene)-

ruthenium(II)], bis[(η^6 -*p*-cymene)diiodidoruthenium(II)],³⁶ 3-hydroxy-2-phenyl-4*H*-chromen-4-one (**a**), 2-(4-fluorophenyl)-3-hydroxy-4*H*-chromen-4-one (**b**), 2-(4-chlorophenyl)-3-hydroxy-4*H*-chromen-4-one (**c**), [chlorido{3-(oxo- κ O)-2-phenyl-chromen-4(1*H*)-onato- κ O}](η^6 -*p*-cymene)ruthenium(II)] (**1**), [chlorido{3-(oxo- κ O)-2-(4-fluorophenyl)-chromen-4(1*H*)-onato- κ O}](η^6 -*p*-cymene)ruthenium(II)] (**2**), [chlorido{3-(oxo- κ O)-2-(4-chlorophenyl)-chromen-4(1*H*)-onato- κ O}](η^6 -*p*-cymene)ruthenium(II)] (**3**),²³ 3-hydroxy-2-phenyl-1*H*-quinolin-4-one (**d**) and 3-hydroxy-1-methyl-2-phenyl-1*H*-quinolin-4-one (**e**)^{37,38} were synthesised according to literature procedures.

Melting points were determined with a Büchi Melting Point B-540 apparatus. Elemental analyses were carried out with a Perkin Elmer 2400 CHN Elemental Analyser at the Microanalytical Laboratory of the University of Vienna. NMR spectra were recorded at 25 °C using a Bruker FT-NMR spectrometer Avance IIITM 500 MHz. ¹H NMR spectra were measured in CDCl₃ at 500.10 MHz and ¹³C{¹H} NMR spectra at 125.75 MHz. The 2D NMR spectra were recorded in a gradient-enhanced mode.

Synthetic procedures

GENERAL COMPLEXATION PROCEDURE. A solution of [(η^6 -arene)RuX(μ -X)]₂ (η^6 -arene = *p*-cymene, toluene, biphenyl; X = Cl, Br, I) in methanol (20 mL) was added to a solution of the ligand and sodium methoxide in methanol (20 mL). The reaction mixture was stirred at room temperature and under an argon atmosphere for 20 h (except for **8** and **10** which were stirred for 6 h and **11** and **12** which were stirred for 5 h). The solvent was evaporated in a vacuum; the residue was dissolved in dichloromethane, filtered and concentrated. Pure complexes were obtained by recrystallisation from methanol or precipitation from methanol with diethyl ether.

[BROMIDO{3-(OXO- κ O)-2-PHENYL-CHROMEN-4(1*H*)-ONATO- κ O}](η^6 -*P-CYME*NE)-RUTHENIUM(II)] (**4**). The reaction was performed according to the general complexation procedure using **a** (159 mg, 0.67 mmol), NaOMe (40 mg, 0.73 mmol) and [Ru(η^6 -*p*-cymene)Br₂]₂ (200 mg, 0.25 mmol) affording **4** as an orange powder (130 mg, 47%). Mp: 169–171 °C (decomp.); ¹H NMR (500.10 MHz, CDCl₃): δ = 1.44–1.45 (m, 6H, CH₃,C_{ym}), 2.44 (s, 3H, CH₃,C_{ym}), 3.02–3.08 (m, 1H, CH_{Cym}), 5.40–5.41 (m, 2H, H3/H5_{Cym}), 5.68 (dd, ³J(H,H) = 5 Hz, ³J(H,H) = 5 Hz, 2H, H2/H6_{Cym}), 7.33–7.36 (m, 1H, H7), 7.38–7.41 (m, 1H, H4'), 7.46–7.50 (m, 2H, H3'/H5'), 7.56 (d, ³J(H,H) = 8 Hz, 1H, H8), 7.59–7.63 (m, 1H, H6), 8.22 (dd, ⁴J(H,H) = 1 Hz, ³J(H,H) = 8 Hz, 1H, H5), 8.60 (dd, ⁴J(H,H) = 1 Hz, ³J(H,H) = 8 Hz, 2H, H2'/H6') ppm; ¹³C{¹H} NMR (125.75 MHz, CDCl₃): δ = 18.9 (CH₃,C_{ym}), 22.7 (CH₃,C_{ym}), 31.3 (CH_{Cym}), 78.4 (C3/C5_{Cym}), 81.0 (C2/C6_{Cym}), 95.5 (C4_{Cym}), 99.3 (C1_{Cym}), 117.9 (C8), 120.1 (C8a), 124.1 (C7), 124.6 (C5), 127.3 (C2'/C6'), 128.2 (C3'/C5'), 129.3 (C4'), 132.5 (C2), 132.6 (C6), 149.1 (C1'), 153.8 (C4a), 154.8 (C3), 183.5 (C4) ppm; elemental analysis calcd for C₂₅H₂₃BrO₃Ru: C 54.35, H 4.20%; found: C 54.36, H 4.25%.

[[BROMIDO{3-(OXO- κ O)-2-(4-CHLOROPHENYL)-CHROMEN-4(1*H*)-ONATO- κ O}](η^6 -*P-CYME*NE)RUTHENIUM(II)] (**5**). The reaction was performed according to the general complexation procedure using **c** (191 mg, 0.70 mmol), NaOMe (44 mg, 0.81 mmol) and [Ru-

(η^6 -*p*-cymene) Br_2] $_2$ (220 mg, 0.28 mmol) affording **5** as a red powder (210 mg, 64%). Mp: 164–167 °C (decomp.); ^1H NMR (500.10 MHz, CDCl_3): δ = 1.43–1.45 (m, 6H, CH_3 , $_{\text{Cym}}$), 2.43 (s, 3H, CH_3 , $_{\text{Cym}}$), 3.00–3.07 (m, 1H, CH_{Cym}), 5.41 (dd, $^3J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 2H, H3/H5 $_{\text{Cym}}$), 5.68 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H2/H6 $_{\text{Cym}}$), 7.33–7.36 (m, 1H, H7), 7.44 (d, $^3J(\text{H,H})$ = 9 Hz, 2H, H3'/H5'), 7.54 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.60–7.64 (m, 1H, H6), 8.21 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.55 (d, $^3J(\text{H,H})$ = 9 Hz, 2H, H2'/H6') ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 19.1 (CH_3 , $_{\text{Cym}}$), 22.5 (CH_3 , $_{\text{Cym}}$), 31.3 (CH_{Cym}), 78.4 (C3/C5 $_{\text{Cym}}$), 81.0 (C2/C6 $_{\text{Cym}}$), 95.9 (C4 $_{\text{Cym}}$), 99.3 (C1 $_{\text{Cym}}$), 117.8 (C8), 120.0 (C8a), 124.2 (C7), 124.7 (C5), 128.4 (C2'/C6'), 128.5 (C3'/C5'), 131.0 (C4'), 132.8 (C6), 134.9 (C2), 147.9 (C1'), 153.8 (C4a), 154.8 (C3), 183.7 (C4) ppm; elemental analysis calcd for $\text{C}_{25}\text{H}_{22}\text{ClBrO}_3\text{Ru}\cdot 0.25\text{H}_2\text{O}$: C 50.77, H 3.83%; found: C 50.79, H 3.77%.

[IODOID{3-(OXO- κ O)-2-PHENYL-CHROMEN-4(1H)-ONATO- κ O}](η^6 -*P*-CYMENE)-RUTHENIUM(II)] (**6**). The reaction was performed according to the general complexation procedure using **a** (128 mg, 0.54 mmol), NaOMe (33 mg, 0.61 mmol) and $[\text{Ru}(\eta^6$ -*p*-cymene) I_2] $_2$ (208 mg, 0.21 mmol) affording **6** as red crystals (177 mg, 70%). Mp: 131–134 °C (decomp.); ^1H NMR (500.10 MHz, CDCl_3): δ = 1.47–1.48 (m, 6H, CH_3 , $_{\text{Cym}}$), 2.45 (s, 3H, CH_3 , $_{\text{Cym}}$), 3.05–3.12 (m, 1H, CH_{Cym}), 5.45 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H3/H5 $_{\text{Cym}}$), 5.73 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H2/H6 $_{\text{Cym}}$), 7.34–7.37 (m, 1H, H7), 7.39–7.42 (m, 1H, H4'), 7.47–7.50 (m, 2H, H3'/H5'), 7.58 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.61–7.64 (m, 1H, H6), 8.20 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.61 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 2H, H2'/H6') ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 18.6 (CH_3 , $_{\text{Cym}}$), 22.7 (CH_3 , $_{\text{Cym}}$), 31.9 (CH_{Cym}), 77.7 (C3/C5 $_{\text{Cym}}$), 80.8 (C2/C6 $_{\text{Cym}}$), 95.0 (C4 $_{\text{Cym}}$), 99.5 (C1 $_{\text{Cym}}$), 117.9 (C8), 120.1 (C8a), 124.1 (C7), 124.6 (C5), 127.2 (C2'/C6'), 128.2 (C3'/C5'), 129.3 (C4'), 132.5 (C2), 132.6 (C6), 149.1 (C1'), 153.9 (C4a), 155.1 (C3), 183.7 (C4) ppm; elemental analysis calcd for $\text{C}_{25}\text{H}_{23}\text{IO}_3\text{Ru}\cdot 0.25\text{H}_2\text{O}$: C 49.72, H 3.92%; found: C 49.61, H 3.68%.

[IODOID{3-(OXO- κ O)-2-(4-CHLOROPHENYL)-CHROMEN-4(1H)-ONATO- κ O}](η^6 -*P*-CYMENE)-RUTHENIUM(II)] (**7**). The reaction was performed according to the general complexation procedure using **c** (151 mg, 0.55 mmol), NaOMe (36 mg, 0.67 mmol) and $[\text{Ru}(\eta^6$ -*p*-cymene) I_2] $_2$ (217 mg, 0.22 mmol) affording **7** as a deep red powder (190 mg, 68%). Mp: 93–95 °C (decomp.); ^1H NMR (500.10 MHz, CDCl_3): δ = 1.45–1.46 (m, 6H, CH_3 , $_{\text{Cym}}$), 2.42 (s, 3H, CH_3 , $_{\text{Cym}}$), 3.03–3.09 (m, 1H, CH_{Cym}), 5.44 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H3/H5 $_{\text{Cym}}$), 5.72 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H2/H6 $_{\text{Cym}}$), 7.33–7.36 (m, 1H, H7), 7.43 (d, $^3J(\text{H,H})$ = 9 Hz, 2H, H3'/H5'), 7.54 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.60–7.63 (m, 1H, H6), 8.18 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.54 (d, $^3J(\text{H,H})$ = 9 Hz, 2H, H2'/H6') ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 19.1 (CH_3 , $_{\text{Cym}}$), 22.6 (CH_3 , $_{\text{Cym}}$), 31.5 (CH_{Cym}), 78.0 (C3/C5 $_{\text{Cym}}$), 80.9 (C2/C6 $_{\text{Cym}}$), 95.6 (C4 $_{\text{Cym}}$), 99.3 (C1 $_{\text{Cym}}$), 117.9 (C8), 120.0 (C8a), 124.3 (C7), 124.6 (C5), 128.3 (C2'/C6'), 128.5 (C3'/C5'), 131.0 (C4'), 132.9 (C6), 134.9 (C2), 148.0 (C1'), 153.9 (C4a), 155.0 (C3), 183.8 (C4) ppm; elemental analysis calcd for $\text{C}_{25}\text{H}_{22}\text{ClIO}_3\text{Ru}\cdot 0.25\text{H}_2\text{O}$: C 47.03, H 3.55%; found: C 46.95, H 3.50%.

[CHLORIDO{3-(OXO- κ O)-2-PHENYL-CHROMEN-4(1H)-ONATO- κ O}](η^6 -TOLUENE)-RUTHENIUM(II)] (**8**). The reaction was performed according to the general complexation procedure using **a** (180 mg, 0.76 mmol), NaOMe (45 mg, 0.84 mmol) and $[\text{Ru}(\eta^6$ -toluene) Cl_2] $_2$ (200 mg, 0.38 mmol) affording **8** as an orange powder (148 mg, 42%). Mp: 218–220 °C (decomp.); ^1H NMR (500.10 MHz, CDCl_3): δ = 2.41 (s, 3H, CH_3 , $_{\text{Tol}}$), 5.39 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H2/H6 $_{\text{Tol}}$), 5.61 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 1H, H1 $_{\text{Tol}}$), 5.88–5.90 (m, 2H, H3/H5 $_{\text{Tol}}$), 7.34–7.36 (m, 1H, H7), 7.39–7.42 (m, 1H, H4'), 7.48–7.51 (m, 2H, H3'/H5'), 7.57 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.61–7.64 (m, 1H, H6), 8.24 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.61 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 2H, H2'/H6') ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 19.1 (CH_3 , $_{\text{Tol}}$), 29.9 (CH_{Tol}), 75.1 (C1 $_{\text{Tol}}$), 76.7 (C2/C6 $_{\text{Tol}}$), 85.2 (C3/C5 $_{\text{Tol}}$), 98.9 (C4 $_{\text{Tol}}$), 117.8 (C8), 119.9 (C8a), 124.2 (C7), 124.6 (C5), 127.4 (C2'/C6'), 128.3 (C3'/C5'), 129.4 (C4'), 132.3 (C2), 132.7 (C6), 149.4 (C1'), 153.9 (C4a), 154.6 (C3), 183.4 (C4) ppm; elemental analysis calcd for $\text{C}_{22}\text{H}_{17}\text{ClO}_3\text{Ru}\cdot 0.5\text{H}_2\text{O}$: C 55.64, H 3.82%; found: C 55.87, H 3.72%.

[CHLORIDO{3-(OXO- κ O)-2-(4-CHLOROPHENYL)-CHROMEN-4(1H)-ONATO- κ O}](η^6 -TOLUENE)-RUTHENIUM(II)] (**9**). The reaction was performed according to the general complexation procedure using **c** (206 mg, 0.76 mmol), NaOMe (45 mg, 0.84 mmol) and $[\text{Ru}(\eta^6$ -*p*-cymene) Cl_2] $_2$ (200 mg, 0.38 mmol) affording **9** as red crystals (281 mg, 74%). Mp: 217–219 °C (decomp.); ^1H NMR (500.10 MHz, CDCl_3): δ = 2.40 (s, 3H, CH_3 , $_{\text{Tol}}$), 5.39 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 2H, H2/H6 $_{\text{Tol}}$), 5.61 (dd, $^3J(\text{H,H})$ = 5 Hz, $^3J(\text{H,H})$ = 5 Hz, 1H, H1 $_{\text{Tol}}$), 5.88–5.91 (m, 2H, H3/H5 $_{\text{Tol}}$), 7.34–7.38 (m, 1H, H7), 7.50 (d, $^3J(\text{H,H})$ = 8 Hz, 2H, H3'/H5'), 7.52–7.54 (m, 1H, H4'), 7.56 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.62–7.65 (m, 1H, H6), 8.24 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.56 (d, $^3J(\text{H,H})$ = 8 Hz, 2H, H2'/H6') ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 19.1 (CH_3 , $_{\text{Tol}}$), 29.9 (CH_{Tol}), 75.1 (C1 $_{\text{Tol}}$), 76.7 (C2/C6 $_{\text{Tol}}$), 85.2 (C3/C5 $_{\text{Tol}}$), 98.6 (C4 $_{\text{Tol}}$), 117.9 (C8), 120.0 (C8a), 124.3 (C7), 124.7 (C5), 128.6 (C2'/C6'/C3'/C5'), 130.8 (C2), 133.0 (C6), 135.1 (C4'), 148.2 (C1'), 153.9 (C4a), 154.6 (C3), 183.6 (C4); elemental analysis calcd for $\text{C}_{22}\text{H}_{16}\text{Cl}_2\text{O}_3\text{Ru}$: C 52.81, H 3.22%; found: C 52.62, H 3.14%.

[CHLORIDO{3-(OXO- κ O)-2-PHENYL-CHROMEN-4(1H)-ONATO- κ O}](η^6 -BIPHENYL)-RUTHENIUM(II)] (**10**). The reaction was performed according to the general complexation procedure using **a** (170 mg, 0.71 mmol), NaOMe (43 mg, 0.80 mmol) and $[\text{Ru}(\eta^6$ -biphenyl)- Cl_2] $_2$ (200 mg, 0.31 mmol) affording **10** as a deep red powder (279 mg, 86%). Mp: 203–206 °C (decomp.); ^1H NMR (500.10 MHz, CDCl_3): δ = 5.91–5.93 (m, 1H, H1 $_{\text{Biphen}}$), 5.96–5.97 (m, 2H, H2/H6 $_{\text{Biphen}}$), 6.01–6.04 (m, 2H, H3/H5 $_{\text{Biphen}}$), 7.32–7.35 (m, 1H, H7), 7.39–7.44 (m, 3H, H3'/H5', H10 $_{\text{Biphen}}$), 7.47–7.51 (m, 3H, H4', H9/H11 $_{\text{Biphen}}$), 7.55 (d, $^3J(\text{H,H})$ = 8 Hz, 1H, H8), 7.60–7.63 (m, 1H, H6), 7.90 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H8/H12 $_{\text{Biphen}}$), 8.16 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 1H, H5), 8.47 (dd, $^4J(\text{H,H})$ = 1 Hz, $^3J(\text{H,H})$ = 8 Hz, 2H, H2'/H6') ppm; $^{13}\text{C}\{^1\text{H}\}$ NMR (125.75 MHz, CDCl_3): δ = 78.4 (C2/C6 $_{\text{Biphen}}$), 78.8 (C1 $_{\text{Biphen}}$), 83.0 (C3/C5 $_{\text{Biphen}}$), 96.9 (C4 $_{\text{Biphen}}$), 117.8 (C8), 120.0 (C8a), 124.2 (C7), 124.5 (C5), 127.4 (C2'/C6'), 128.2 (C3'/C5'), 128.8 (C9/C11 $_{\text{Biphen}}$), 129.1 (C8/

C12_{Biphen}), 129.4 (C4'), 129.6 (C10_{Biphen}), 132.1 (C2), 132.7 (C6), 135.2 (C7_{Biphen}), 149.5 (C1'), 153.9 (C4a), 154.4 (C3), 183.3 (C4) ppm; elemental analysis calcd for C₂₇H₁₉ClO₃Ru: C 61.42, H 3.63%; found: C 61.16, H 3.62%.

[CHLORIDO{3-(OXO-κO)-2-(4-CHLOROPHENYL)-CHROMEN-4(1H)-ONATO-κO}-(η⁶-BIPHENYL)RUTHENIUM(II)] (11). The reaction was performed according to the general complexation procedure using **c** (193 mg, 0.71 mmol), NaOMe (43 mg, 0.80 mmol) and [Ru(η⁶-*p*-cymene)Cl₂]₂ (200 mg, 0.32 mmol) affording **11** as deep red crystals (245 mg, 68%). Mp: 194–197 °C (decomp.); ¹H NMR (500.10 MHz, CDCl₃): δ = 5.91–5.93 (m, 1H, H1_{Biphen}), 5.95–5.97 (m, 2H, H2/H6_{Biphen}), 6.02–6.05 (m, 2H, H3/H5_{Biphen}), 7.33–7.38 (m, 3H, H3'/H5'/H7), 7.49–7.55 (m, 4H, H6/H8/H9/H11_{Biphen}), 7.60–7.64 (m, 1H, H10_{Biphen}), 7.88–7.90 (m, 1H, H8/H12_{Biphen}), 8.16 (dd, ⁴J(H,H) = 1 Hz, ³J(H,H) = 8 Hz, 1H, H5), 8.41 (d, ³J(H,H) = 9 Hz, 2H, H2'/H6') ppm; ¹³C{¹H} NMR (125.75 MHz, CDCl₃): δ = 78.3 (C2/C6_{Biphen}), 78.5 (C1_{Biphen}), 83.0 (C3/C5_{Biphen}), 97.1 (C4_{Biphen}), 117.8 (C8), 120.0 (C8a), 124.3 (C7), 124.6 (C5), 128.4 (C2'/C6'), 128.6 (C3'/C5'), 128.9 (C9/C11_{Biphen}), 129.1 (C8/C12_{Biphen}), 129.7 (C10_{Biphen}), 130.6 (C2), 132.9 (C6), 135.1 (C4', C7_{Biphen}), 148.4 (C1'), 153.9 (C4a), 154.4 (C3), 183.5 (C4); elemental analysis calcd for C₂₇H₁₈Cl₂O₃Ru·H₂O: C 55.87, H 3.47%; found: C 55.86, H 3.17%.

[CHLORIDO{3-(OXO-κO)-2-PHENYL-QUINOLON-4(1H)-ONATO-κO}-(η⁶-*p*-CYMENE)RUTHENIUM(II)] (12). The reaction was performed according to the general complexation procedure using **d** (172 mg, 0.73 mmol), NaOMe (43 mg, 0.8 mmol) and [Ru(η⁶-*p*-cymene)Cl₂]₂ (200 mg, 0.33 mmol) to afford **12** as an orange powder (195 mg, 59%). Mp: 177–180 °C (decomp.); ¹H NMR (500.10 MHz, CD₃OD): δ = 1.41 (m, 6H, CH₃,_{Cym}), 2.37 (s, 3H, CH₃,_{Cym}), 2.88–2.96 (m, 1H, CH_{Cym}), 5.57 (d, ³J(H,H) = 5 Hz, 2H, H3/H5_{Cym}), 5.81 (d, ³J(H,H) = 6 Hz, 2H, H2/H6_{Cym}), 7.40–7.44 (m, 1H, H7), 7.55–7.63 (m, 4H, H3'/H4'/H5'/H6), 7.76 (d, ³J(H,H) = 8 Hz, 1H, H8), 8.07–8.09 (m, 2H, H2'/H6'), 8.30 (dd, ⁴J(H,H) = 1 Hz, ³J(H,H) = 8 Hz, 1H, H5) ppm; ¹³C{¹H} NMR (125.75 MHz, CD₃OD): δ = 17.2 (CH₃,_{Cym}), 21.3 (CH₃,_{Cym}), 31.2 (CH_{Cym}), 77.3 (C3/C5_{Cym}), 79.6 (C2/C6_{Cym}), 95.9 (C4_{Cym}), 98.3 (C1_{Cym}), 117.9 (C8), 120.0 (C8a), 122.4 (C5), 123.4 (C7), 128.2 (C3'/C5'), 129.0 (C2'/C6'), 129.4 (C4'), 129.6 (C6), 132.3 (C2), 135.3 (C4a), 136.3 (C1'), 152.6 (C3), 174.9 (C4) ppm; elemental analysis calcd for C₂₅H₂₄ClNO₂Ru·0.8CH₂Cl₂: C 53.90, H 4.49%, N 2.44%; found: C 54.01, H 4.78%, N 2.27%.

[CHLORIDO{3-(OXO-κO)-1-METHYL-2-PHENYL-QUINOLON-4(1H)-ONATO-κO}-(η⁶-*p*-CYMENE)RUTHENIUM(II)] (13). The reaction was performed according to the general complexation procedure using **e** (180 mg, 0.73 mmol), NaOMe (43 mg, 0.8 mmol) and [Ru(η⁶-*p*-cymene)Cl₂]₂ (200 mg, 0.33 mmol) to afford **13** as an orange powder (157 mg, 46%). Mp: 188–190 °C (decomp.); ¹H NMR (500.10 MHz, CD₃OD): δ = 1.31–1.33 (m, 6H, CH₃,_{Cym}), 2.27 (s, 3H, CH₃,_{Cym}), 2.77–2.85 (m, 1H, CH_{Cym}), 3.74 (N-CH₃), 5.45 (d, ³J(H,H) = 5 Hz, 2H, H3/H5_{Cym}), 5.67 (d, ³J(H,H) = 6 Hz, 2H, H2/H6_{Cym}), 7.50–7.53 (m, 3H, H3'/H5'/H7), 7.61–7.66 (m, 3H, H2'/H4'/H6'), 7.72–7.75 (m, 1H, H6), 7.87 (d, ³J(H,H) = 8 Hz, 1H, H8), 8.44 (dd, ⁴J(H,H) = 1 Hz, ³J(H,H) = 8 Hz, 1H, H5) ppm; ¹³C{¹H} NMR (125.75 MHz, CD₃OD): δ = 17.1 (CH₃,_{Cym}), 21.3

(CH₃,_{Cym}), 31.2 (CH_{Cym}), 37.6 (N-CH₃), 77.6 (C3/C5_{Cym}), 79.5 (C2/C6_{Cym}), 96.4 (C4_{Cym}), 97.9 (C1_{Cym}), 116.8 (C8), 120.9 (C8a), 123.3 (C5), 123.5 (C7), 128.5 (C3'/C5'), 129.2 (C2'/C6'), 130.1 (C4'), 130.3 (C6), 132.3 (C2), 136.3 (C1'), 141.8 (C4a), 152.9 (C3), 174.0 (C4) ppm; elemental analysis calcd for C₂₅H₂₄ClNO₂Ru·CH₂Cl₂: C 53.52, H 4.66%, N 2.31%; found: C 53.48, H 4.52%, N 2.20%.

UV-vis spectrophotometric and spectrofluorimetric measurements

Maltol, KCl, KOH, HCl and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Stock solutions of maltol, **b** and **2** were prepared in a 20% (w/w) DMSO–H₂O mixture or in H₂O. The stock solution of [Ru^{II}(cym)X₃]ⁿ was obtained by dissolving a known amount of [Ru^{II}(cym)Cl₂]₂ in water and the exact concentration (~5 × 10^{−3} M) was determined with pH-potentiometric titrations in aqueous solution at 25.0 ± 0.1 °C at an ionic strength of 0.20 M (KCl) employing literature data for [Ru₂(cym)₂(OH)₂X_m]ⁿ (m = 1, 2) complexes.²⁹

A Hewlett Packard 8452A diode array spectrophotometer was used to record the UV-vis spectra in the interval 200–800 nm. The path length was 1 cm. The measurements for determination of the protonation constants of the ligands and the overall stability constants of the metal complexes were carried out at 25.0 ± 0.1 °C in a 20% (w/w) DMSO–H₂O mixture and at an ionic strength of 0.20 M. The titrations were performed with carbonate-free KOH solutions of known concentration (0.20 M). The concentrations of the KOH and HCl solutions were determined by pH-potentiometric titrations. An Orion 710A pH-meter equipped with a Metrohm combined electrode (type 6.0234.100) and a Metrohm 665 Dosimat burette was used for the pH-potentiometric measurements. The electrode system was calibrated to the pH = −log[H⁺] scale in DMSO–water solvent mixtures by means of blank titrations (strong acid vs. strong base; HCl vs. KOH), similarly to the method suggested by Irving *et al.* in pure aqueous solutions.²⁵ The average water ionisation constant, pK_w, was determined as 14.30 ± 0.02 at 25.0 °C and I = 0.20 M (KCl), which corresponds well to literature data.³⁹ Protonation and stability constants and the individual spectra of the species were calculated with the computer program PSEQUAD.⁴⁰ β (M_pL_qH_r) is defined for the general equilibrium pM + qL + rH ⇌ M_pL_qH_r as β(M_pL_qH_r) = [M_pL_qH_r]/[M]^p[L]^q[H]^r where M denotes [Ru^{II}(cym)X₃]ⁿ and L the completely deprotonated ligand.

The spectrophotometric titrations were performed on samples containing either ligand **b**, maltol or [Ru^{II}(cym)X₃]ⁿ, [Ru^{II}(cym)X₃]ⁿ and maltol, or complex **2** in 20% (w/w) DMSO–H₂O. The concentration of ligands was 5–8 × 10^{−5} M and the metal-to-ligand ratios were 1 : 1 and 1 : 2 in the case of maltol over the pH range 2.0–11.5. Complex **2** was titrated at a concentration of 5 × 10^{−5} M and [Ru^{II}(cym)X₃]ⁿ at 1.8 × 10^{−4} M.

The pH-dependent fluorescence measurements of **b** and **2** were carried out on a Hitachi-4500 spectrofluorimeter with the excitation at 342 nm in aqueous solution at 25.0 ± 0.1 °C and an ionic strength of 0.20 M (KCl). The emission spectra were recorded in a 1 cm quartz cell in the pH range 2.0–11.5 using

10 nm/10 nm slit widths. The samples contained the compounds at 1.5×10^{-5} M concentration.

Due to the photosensitivity of **b** and **2**, the batch technique was used for recording the UV-vis and fluorimetric spectra instead of continuous titrations and the solutions were kept in the dark.

Hydrolysis, interaction with 5'-GMP and amino acids

Hydrolysis and stability in water were investigated by ^1H NMR spectroscopy. Due to the lipophilic character of the organometallics, all experiments were performed in 10% (v/v) $\text{d}_6\text{-DMSO}/\text{D}_2\text{O}$ solutions. For the interaction with 5'-GMP, the complexes (*ca.* 0.1 mg mL^{-1}) were dissolved in 10% (v/v) $\text{d}_6\text{-DMSO}/\text{D}_2\text{O}$, yielding the corresponding highly reactive aqua species. The aqua complexes were converted *in situ* by addition of 50 μL aliquots of 5'-GMP solution (10 mg mL^{-1}) to the respective 5'-GMP adduct and the reaction was monitored by ^1H NMR spectroscopy. To investigate the reactivity towards amino acids, the aqua complexes (*ca.* 0.1 mg mL^{-1}) were treated with equimolar amounts of amino acids and ^1H NMR spectra were recorded after 5 min and 24 h.

Cytotoxicity in cancer cell lines

CELL LINES AND CULTURE CONDITIONS. CH1 cells originate from an ascites sample of a patient with papillary cystadenocarcinoma of the ovary and were a gift from Lloyd R. Kelland, CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK. SW480 (human adenocarcinoma of the colon) and A549 (human non-small cell lung cancer) cells were provided by Brigitte Marian (Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Austria). All cell culture reagents were obtained from Sigma-Aldrich Austria. Cells were grown in 75 cm^2 culture flasks (Iwaki) as adherent monolayer cultures in a Minimum Essential Medium (MEM) supplemented with 10% heat inactivated fetal calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine and 1% non-essential amino acids (from 100 $\times$ stock). Cultures were maintained at 37 $^\circ\text{C}$ in a humidified atmosphere containing 95% air and 5% CO_2 .

MTT ASSAY. Cytotoxicity was determined by the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma] microculture assay. For this purpose, cells were harvested from culture flasks by trypsinisation and seeded in 100 μL per well aliquots into 96-well microculture plates (Iwaki). Cell densities of 1.5×10^3 cells per well (CH1), 2.5×10^3 cells per well (SW480) and 4×10^3 cells per well (A549) were chosen in order to ensure exponential growth of untreated controls throughout the experiment. Cells were allowed to settle and resume exponential growth in a drug-free complete culture medium for 24 h. Stock solutions of the test compounds in DMSO were diluted in a complete culture medium so that the maximum DMSO content did not exceed 1%. These dilutions were added in 100 μL per well aliquots to the microcultures and cells were exposed to the test compounds for 96 h. At the end of exposure, all media were replaced by 100 μL per well RPMI1640 culture medium

(supplemented with 10% heat-inactivated fetal bovine serum) plus 20 μL per well MTT solution in phosphate-buffered saline (5 mg mL^{-1}). After incubation for 4 h, the supernatants were removed, and the formazan crystals formed by viable cells were dissolved in 150 μL DMSO per well. Optical densities at 550 nm were measured with a microplate reader (Tecan Spectra Classic), using a reference wavelength of 690 nm to correct for unspecific absorption. The quantity of viable cells was expressed in terms of *T/C* values by comparison to untreated controls, and 50% inhibitory concentrations (IC_{50}) were calculated from concentration–effect curves by interpolation. Evaluation is based on means from at least three independent experiments, each comprising at least three replicates per concentration level.

Acknowledgements

This work was supported by the Hungarian Research Foundation OTKA 103905 and É.A. Enyedy gratefully acknowledges the financial support of J. Bolyai research fellowship. We thank the University of Vienna, the Austrian Science Fund (FWF), the Johanna Mahlke geb. Obermann Foundation, and COST D39 for financial support. We gratefully acknowledge Filip Groznica for doing parts of the synthetic work.

Notes and references

- 1 M. J. Clarke, F. C. Zhu and D. R. Frasca, *Chem. Rev.*, 1999, **99**, 2511–2533.
- 2 A. Bergamo, C. Gaiddon, J. H. M. Schellens, J. H. Beijnen and G. Sava, *J. Inorg. Biochem.*, 2012, **106**, 90–99.
- 3 J. M. Rademaker-Lakhai, D. Van Den Bongard, D. Pluim, J. H. Beijnen and J. H. M. Schellens, *Clin. Cancer Res.*, 2004, **10**, 3717–3727.
- 4 C. G. Hartinger, S. Zorbas-Seifried, M. A. Jakupiec, B. Kynast, H. Zorbas and B. K. Keppler, *J. Inorg. Biochem.*, 2006, **100**, 891–904.
- 5 C. G. Hartinger, M. A. Jakupiec, S. Zorbas-Seifried, M. Groessl, A. Egger, W. Berger, H. Zorbas, P. J. Dyson and B. K. Keppler, *Chem. Biodiversity*, 2008, **5**, 2140–2155.
- 6 P. J. Dyson, *Chimia*, 2007, **61**, 698–703.
- 7 A. F. A. Peacock and P. J. Sadler, *Chem.-Asian J.*, 2008, **3**, 1890–1899.
- 8 C. G. Hartinger and P. J. Dyson, *Chem. Soc. Rev.*, 2009, **38**, 391–401.
- 9 G. Gasser, I. Ott and N. Metzler-Nolte, *J. Med. Chem.*, 2011, **54**, 3–25.
- 10 C. G. Hartinger, N. Metzler-Nolte and P. J. Dyson, *Organometallics*, 2012, **31**, 5677–5685.
- 11 W. Kandioller, C. G. Hartinger, A. A. Nazarov, C. Bartel, M. Skocic, M. A. Jakupiec, V. B. Arion and B. K. Keppler, *Chem.-Eur. J.*, 2009, **15**, 12283–12291.
- 12 G. S. Smith and B. Therrien, *Dalton Trans.*, 2011, **40**, 10793–10800.

- 13 R. L. Hayward, Q. C. Schornagel, R. Tente, J. C. Macpherson, R. E. Aird, S. Guichard, A. Habtemariam, P. Sadler and D. I. Jodrell, *Cancer Chemother. Pharmacol.*, 2005, **55**, 577–583.
- 14 C. S. Allardyce, P. J. Dyson, D. J. Ellis and S. L. Heath, *Chem. Commun.*, 2001, 1396–1397.
- 15 A. Bergamo, A. Masi, P. J. Dyson and G. Sava, *Int. J. Oncol.*, 2008, **33**, 1281–1289.
- 16 S. Chatterjee, S. Kundu, A. Bhattacharyya, C. G. Hartinger and P. J. Dyson, *J. Biol. Inorg. Chem.*, 2008, **13**, 1149–1155.
- 17 S. Chatterjee, I. Biondi, P. J. Dyson and A. Bhattacharyya, *J. Biol. Inorg. Chem.*, 2011, **16**, 715–724.
- 18 E. Meggers, G. E. Atilla-Gokcumen, K. Grundler, C. Frias and A. Prokop, *Dalton Trans.*, 2009, 10882–10888.
- 19 W. F. Schmid, R. O. John, V. B. Arion, M. A. Jakupec and B. K. Keppler, *Organometallics*, 2007, **26**, 6643–6652.
- 20 A. Kurzwernhart, W. Kandioller, C. Bartel, S. Bachler, R. Trondl, G. Muhlgassner, M. A. Jakupec, V. B. Arion, D. Marko, B. K. Keppler and C. G. Hartinger, *Chem. Commun.*, 2012, **48**, 4839–4841.
- 21 R. J. Nijveldt, E. van Nood, D. E. van Hoorn, P. G. Boelens, K. van Norren and P. A. van Leeuwen, *Am. J. Clin. Nutr.*, 2001, **74**, 418–425.
- 22 M. Grazul and E. Budzisz, *Coord. Chem. Rev.*, 2009, **253**, 2588–2598.
- 23 A. Kurzwernhart, W. Kandioller, S. Bächler, C. Bartel, S. Martic, M. Buczkowska, G. Mühlgassner, M. A. Jakupec, H.-B. Kraatz, P. J. Bednarski, V. B. Arion, D. Marko, B. K. Keppler and C. G. Hartinger, *J. Med. Chem.*, DOI: 10.1021/jm301376a.
- 24 S. Tommasini, M. L. Calabro, P. Donato, D. Raneri, G. Guglielmo, P. Ficarra and R. Ficarra, *J. Pharm. Biomed. Anal.*, 2004, **35**, 389–397.
- 25 O. S. Wolfbeis, A. Knierzinger and R. Schipfer, *J. Photochem.*, 1983, **21**, 67–79.
- 26 E. A. Enyedy, L. Horvath, K. Gajda-Schranz, G. Galbacs and T. Kiss, *J. Inorg. Biochem.*, 2006, **100**, 1936–1945.
- 27 P. Buglyo and E. Farkas, *Dalton Trans.*, 2009, 8063–8070.
- 28 E. A. Enyedy, E. Sija, T. Jakusch, C. G. Hartinger, B. K. Keppler and T. Kiss, unpublished data.
- 29 L. Biro, E. Farkas and P. Buglyo, *Dalton Trans.*, 2010, **39**, 10272–10278.
- 30 J. Reedijk, *Chem. Rev.*, 1999, **99**, 2499–2510.
- 31 H. Chen, J. A. Parkinson, S. Parsons, R. A. Coxall, R. O. Gould and P. J. Sadler, *J. Am. Chem. Soc.*, 2002, **124**, 3064–3082.
- 32 M. Hanif, H. Henke, S. M. Meier, S. Martic, M. Labib, W. Kandioller, M. A. Jakupec, V. B. Arion, H. B. Kraatz, B. K. Keppler and C. G. Hartinger, *Inorg. Chem.*, 2010, **49**, 7953–7963.
- 33 M. Hanif, P. Schaaf, W. Kandioller, M. Hejl, M. A. Jakupec, A. Roller, B. K. Keppler and C. G. Hartinger, *Aust. J. Chem.*, 2010, **63**, 1521–1528.
- 34 R. E. Aird, J. Cummings, A. A. Ritchie, M. Muir, R. E. Morris, H. Chen, P. J. Sadler and D. I. Jodrell, *Br. J. Cancer*, 2002, **86**, 1652–1657.
- 35 M. A. Bennett and A. K. Smith, *J. Chem. Soc., Dalton Trans.*, 1974, 233–241.
- 36 M. G. Mendoza-Ferri, C. G. Hartinger, A. A. Nazarov, R. E. Eichinger, M. A. Jakupec, K. Severin and B. K. Keppler, *Organometallics*, 2009, **28**, 6260–6265.
- 37 H. M. Irving, M. G. Miles and L. D. Pettit, *Anal. Chim. Acta*, 1967, **38**, 475–488.
- 38 P. Hradil, J. Hlavac and K. Lemr, *J. Heterocycl. Chem.*, 1999, **36**, 141–144.
- 39 SCQuery, *The IUPAC Stability Constants Database, Academic Software (Version 5.5)*, Royal Society of Chemistry, Saurby Old Farm, Timble, Otley, Yorks, 1993–2005.
- 40 L. Zékány and I. Nagypál, in *Computational Methods for the Determination of Stability Constants*, ed. D. L. Leggett, Plenum Press, New York, 1985, p. 291.

Supporting Information

3-Hydroxyflavones vs. 3-Hydroxyquinolinones: Structure-Activity Relationships and Stability Studies on Ru^{II}(arene) Anticancer Complexes with Biologically Active Ligands

Andrea Kurzwernhart,^{a,b} Wolfgang Kandioller,^{a,b} Éva A. Enyedy,^c Maria Novak,^a Michael A. Jakupec,^{a,b} Bernhard K. Keppler^{a,b} and Christian G. Hartinger^{a,b,d,*}

^a Institute of Inorganic Chemistry, University of Vienna, Waehringer Str. 42, 1090 Vienna, Austria.

^b Research Platform “Translational Cancer Therapy Research”, University of Vienna, Waehringer Str. 42, 1090 Vienna, Austria

^c Department of Inorganic and Analytical Chemistry, University of Szeged, Dóm tér 7. H-6720 Szeged, Hungary.

^d The University of Auckland, School of Chemical Sciences, Private Bag 92019, Auckland 1142, New Zealand;
Tel: +64 9 373 7955 ext 83220; E-mail: c.hartinger@auckland.ac.nz

Table of contents

1.1.	Solution equilibria: General formulae and definitions of formation equilibria	3
1.2.	Fluorescence spectra of ligand b and complex 2 in aqueous solution	4
1.3.	Measured and calculated UV-vis absorbance spectra and concentration distribution curves of the Ru ^{II} (cym)/ligand b system	5
1.4.	NMR studies on the stability of 12 in aqueous solution	6
1.5.	¹ H NMR spectroscopy studies on the interaction of 12' with 5'-GMP	7
1.6.	Reactions of 1' , 12' and 13' with amino acids	7
1.7.	The reactions of 12' with amino acids	9
1.7.1	Reaction of 12' with an equimolar amount of L-methionine.....	9
1.7.2	Reaction of 12' with an equimolar amount of L-histidine.....	9
1.7.3	Reaction of 12' with an equimolar amount of glycine	10
1.7.4	Reaction of 12' with an equimolar amount of L-cysteine.....	10

1.1. Solution equilibria: General formulae and definitions of formation equilibria

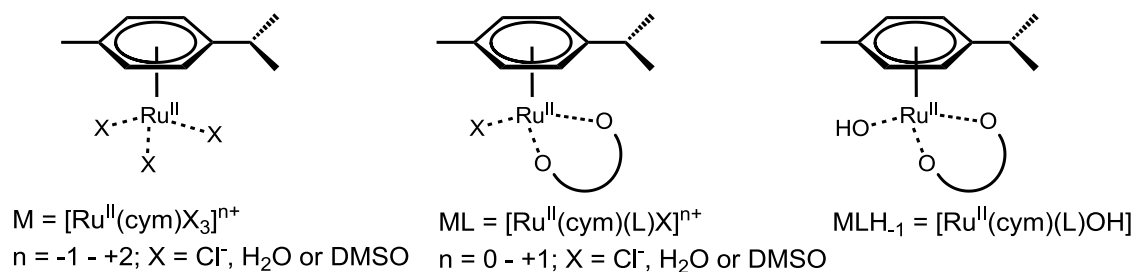


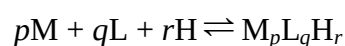
Figure S1. General formulae of the ruthenium(II)- η^6 -*p*-cymene complexes

Definition of the stability constants of the metal complexes and equilibria

General formula:

$M = [Ru^{II}(cym)X_3]^{n+}$; L = deprotonated form of the ligand (L^-); $H = H^+$ (charges were omitted for simplicity).

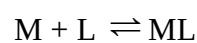
$$\beta(M_pL_qH_r) = [M_pL_qH_r]/[M]^p[L]^q[H]^r$$



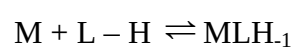
Constant:

Equilibrium:

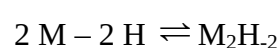
$$\beta(ML \text{ or } [Ru^{II}(cym)(L)X]) = [ML]/[M][L]$$



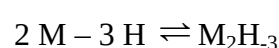
$$\beta(MLH_{-1} \text{ or } [Ru^{II}(cym)(L)OH]) = [MLH_{-1}]/[M][L][H]^{-1}$$



$$\beta(M_2H_2 \text{ or } [(Ru^{II}(cym))_2(OH)_2]) = [M_2H_2]/[M]^2[H]^{-2}$$



$$\beta(M_2H_3 \text{ or } [(Ru^{II}(cym))_2(OH)_3]) = [M_2H_3]/[M]^2[H]^{-3}$$



(H_x = deprotonation of a coordinated water molecule)

1.2. Fluorescence spectra of ligand **b** and complex **2** in aqueous solution

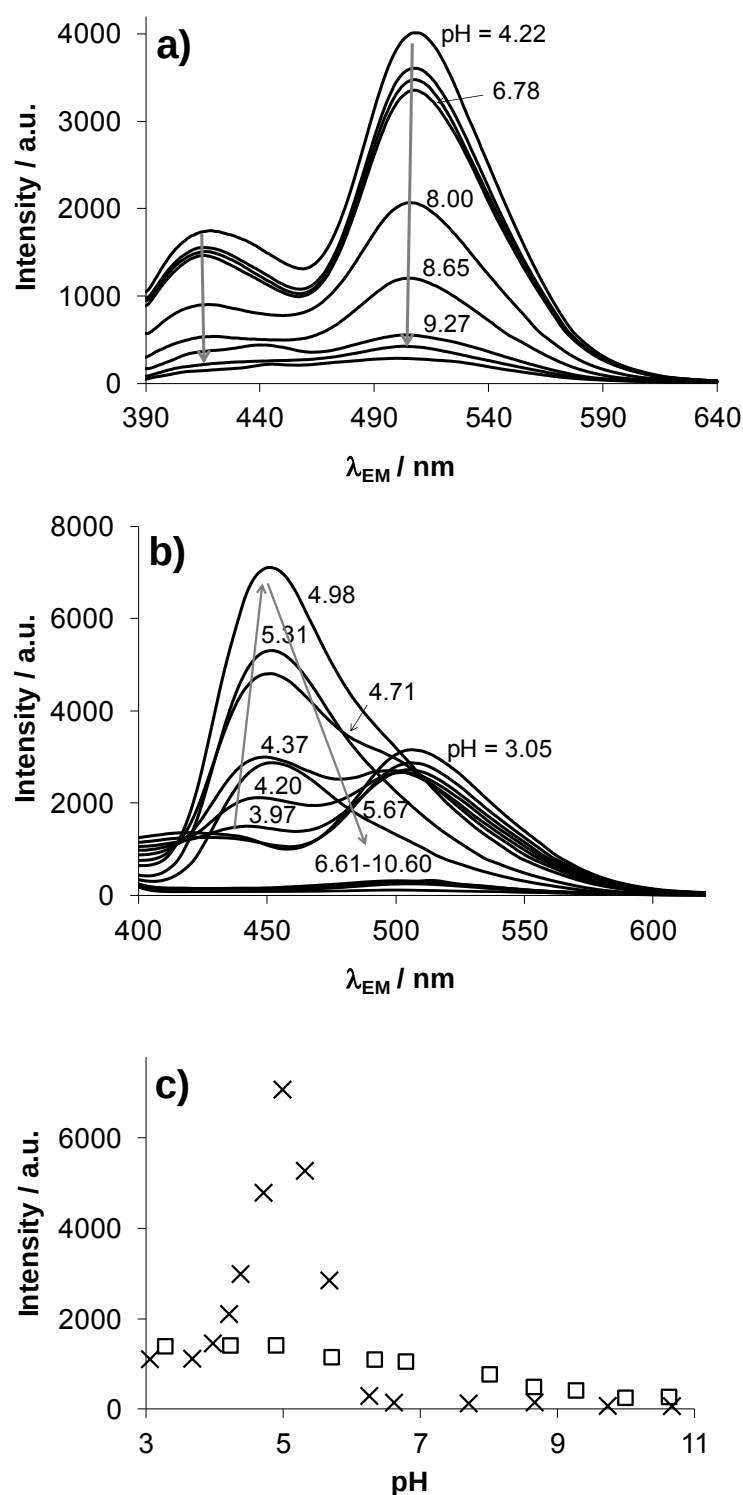


Figure S2. (a) pH-Dependent fluorescence emission spectra of ligand **b** ($c = 1.5 \times 10^{-5}$ M) and (b) of complex **2** ($c = 1.5 \times 10^{-5}$ M). (c) pH-dependent intensity changes at 448 nm of the ligand (□) and the complex (×) { $T = 25$ °C; $I = 0.20$ M (KCl); $\lambda_{EX} = 342$ nm; PTM = 700 V; Slits: 10/10 nm, in H_2O }.

1.3. Measured and calculated UV-vis absorbance spectra and concentration distribution curves of the $\text{Ru}^{\text{II}}(\text{cym})/\text{ligand b}$ system

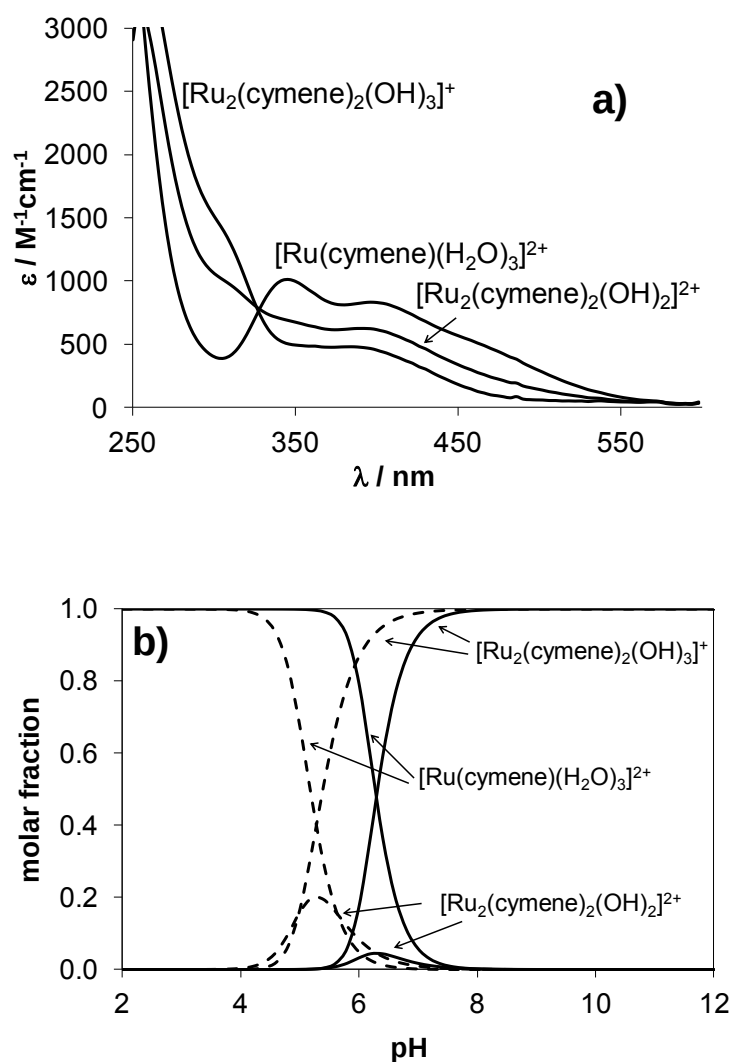


Figure S3. (a) Calculated individual UV-vis absorbance spectra of the species of $[\text{Ru}^{\text{II}}(\text{cym})\text{X}_3]^{n+}$ divided by the number of Ru^{II} in each species in the DMSO/ H_2O mixture; (b) Concentration distribution curves of the $[\text{Ru}^{\text{II}}(\text{cym})\text{X}_3]^{n+}$ system at a wide range of pH values in the DMSO/ H_2O mixture at $c_{\text{Ru}} = 1.8 \times 10^{-4} \text{ M}$ and for comparison speciation in pure aqueous solution (dashed lines) $\{T = 25 \text{ }^\circ\text{C}; I = 0.20 \text{ M (KCl); 20\% (w/w) DMSO/H}_2\text{O}\}$.

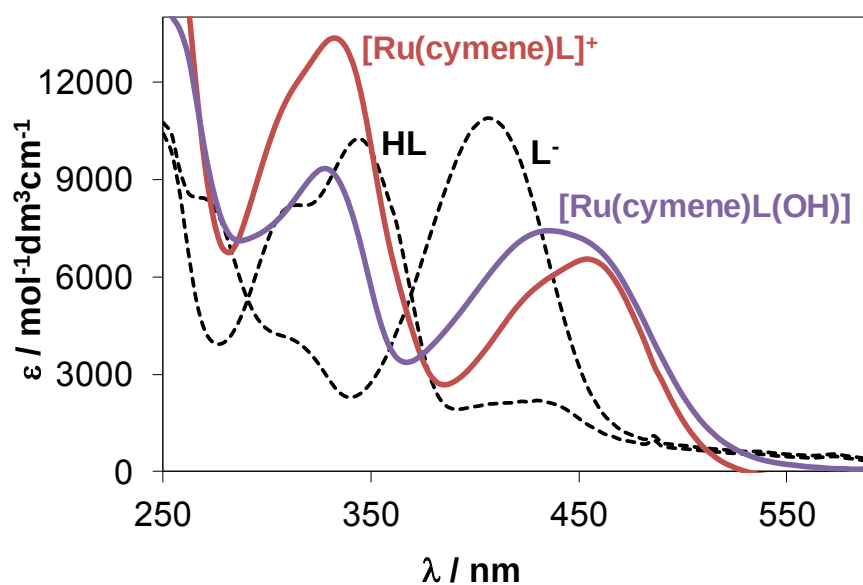


Figure S4. Calculated individual UV-vis absorbance spectra of the species formed in the $\text{Ru}^{\text{II}}(\text{cym})$ -ligand **b** system $\{T = 25\text{ }^{\circ}\text{C}; I = 0.20\text{ M (KCl)}; 20\% \text{ (w/w) DMSO/H}_2\text{O}\}$.

1.4. NMR studies on the stability of **12** in aqueous solution

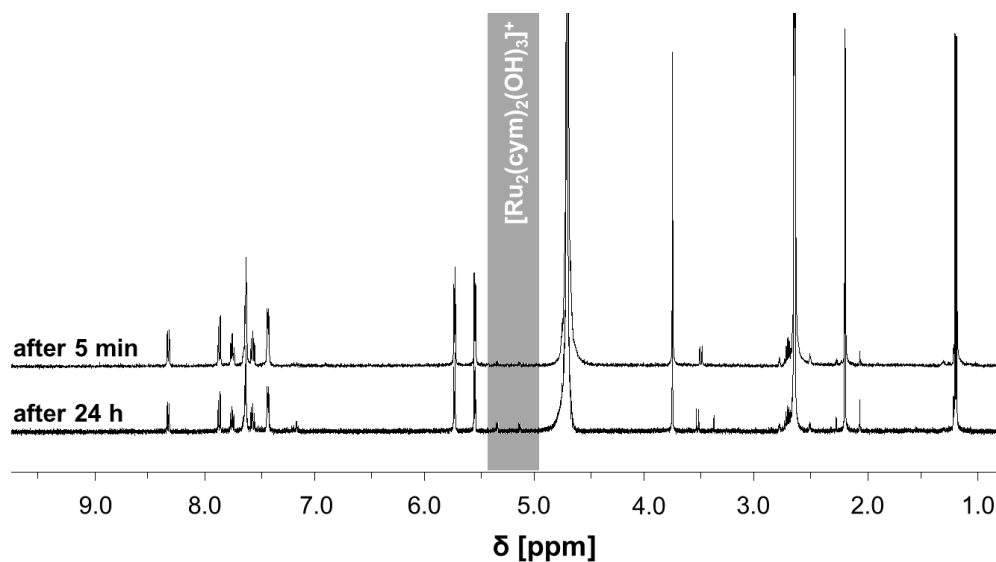


Figure S5. ^1H NMR spectra of **12** in 10% d_6 -DMSO/ D_2O after 5 min and 24 h.

1.5. ^1H NMR spectroscopy studies on the interaction of **12'** with 5'-GMP

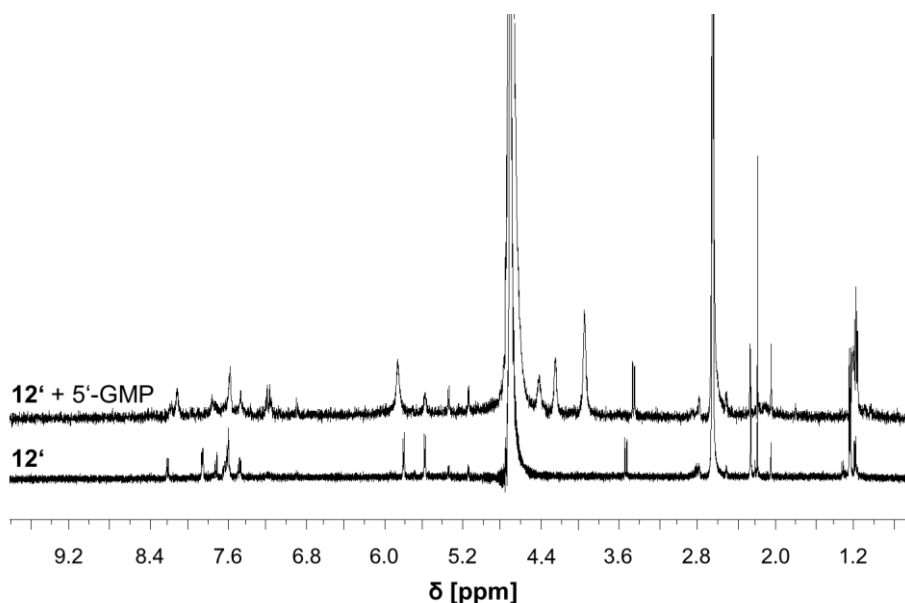


Figure S6. ^1H NMR spectra of **12'** and **12'** incubated with an excess of 5'-GMP in 10% d_6 -DMSO/ D_2O . The reaction of **12'** with 5'-GMP is indicated by the upfield shift of the N7 atom of 5'-GMP from approximately $\delta = 8.1$ (free 5'-GMP) to 7.6 ppm (bound 5'-GMP) after addition of 5'-GMP in excess.

1.6. Reactions of **1'**, **12'** and **13'** with amino acids

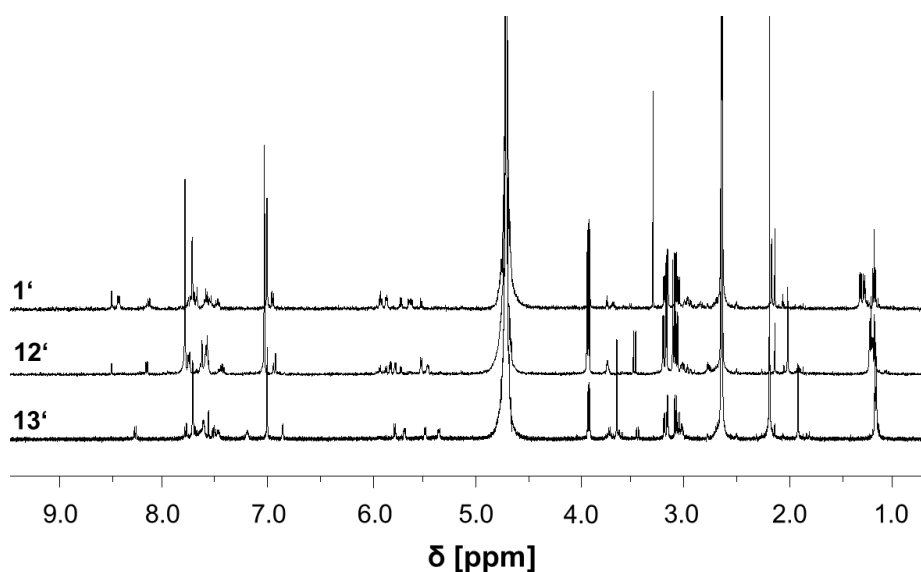


Figure S7. ^1H NMR spectra of the reactions of **1'**, **12'** and **13'** with an equimolar amount of L-histidine after 5 min.

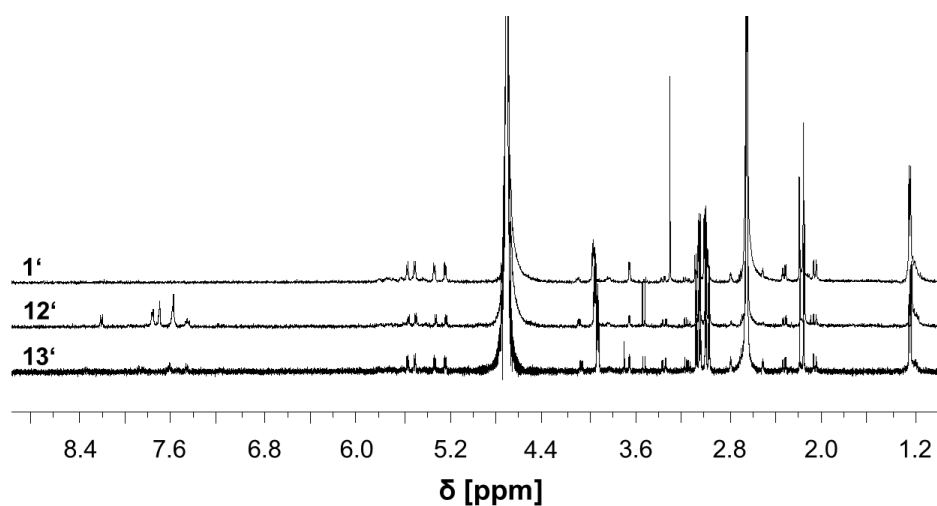


Figure S8. ^1H NMR spectra of the reactions of 1', 12' and 13' with an equimolar amount of L-cysteine after 5 min.

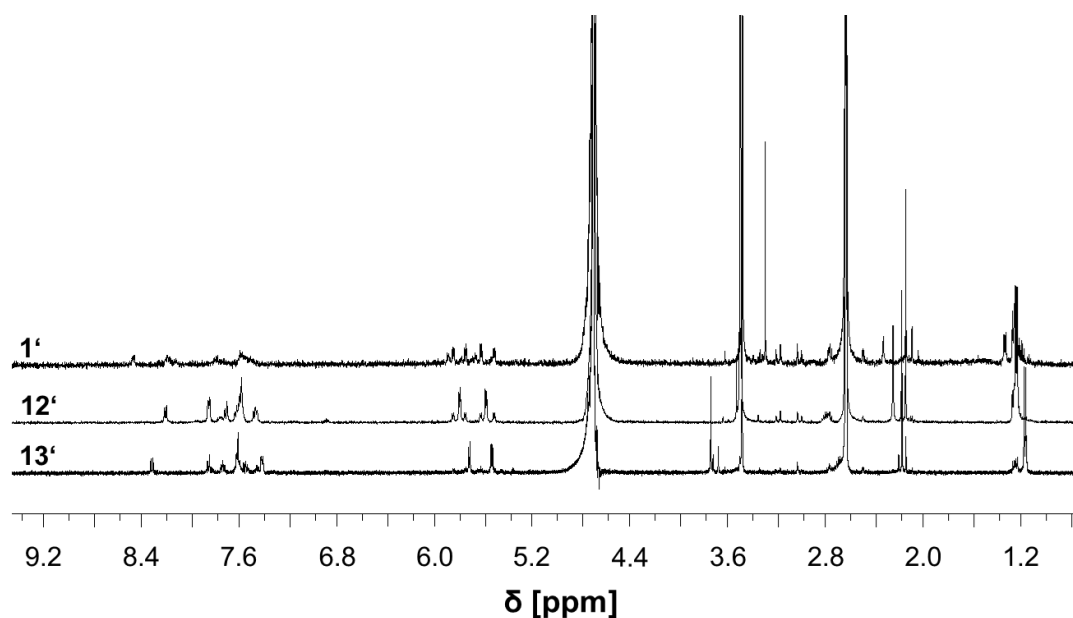


Figure S9. ^1H NMR spectra of the reactions of 1', 12' and 13' with an equimolar amount of glycine after 5 min.

1.7. The reactions of 12' with amino acids

1.7.1 Reaction of 12' with an equimolar amount of L-methionine

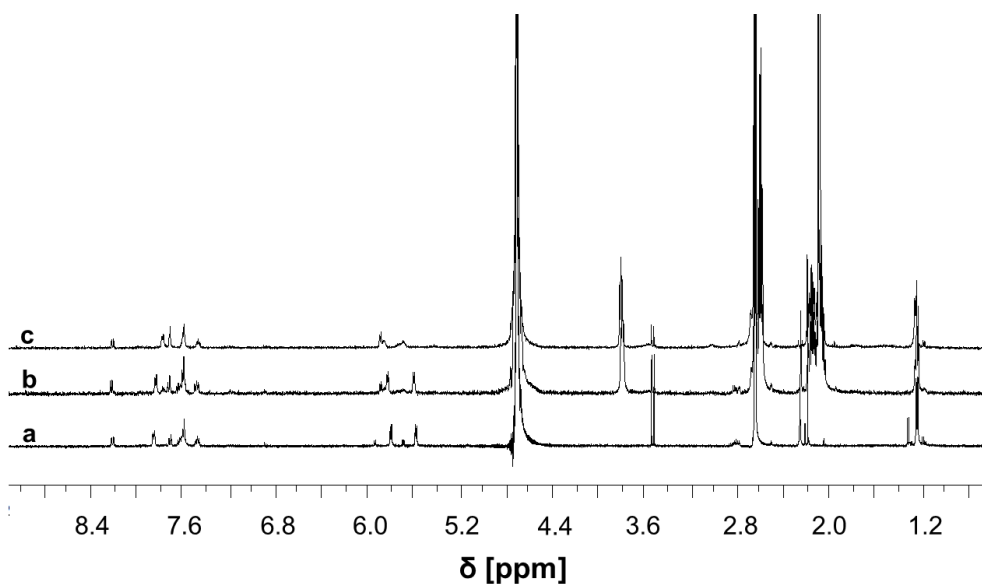


Figure S10. ^1H NMR spectra of a) 12', b) 12' + 1 eq L-methionine after 5 min and c) after 24 h.

1.7.2 Reaction of 12' with an equimolar amount of L-histidine

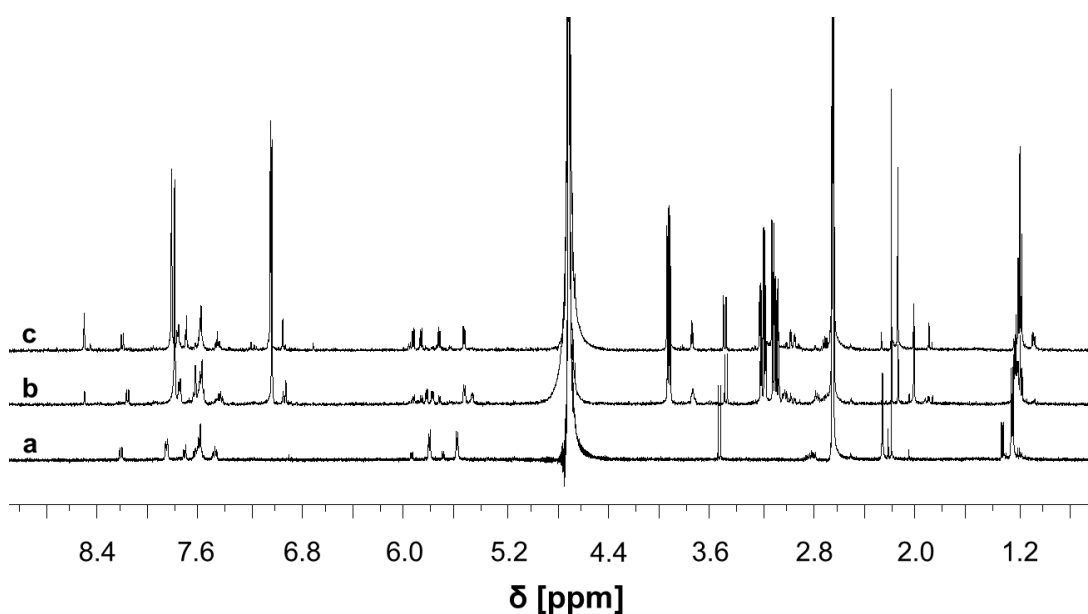


Figure S11. ^1H NMR spectra of a) 12', b) 12' + 1 eq L-histidine after 5 min and c) after 24 h.

1.7.3 Reaction of 12' with an equimolar amount of glycine

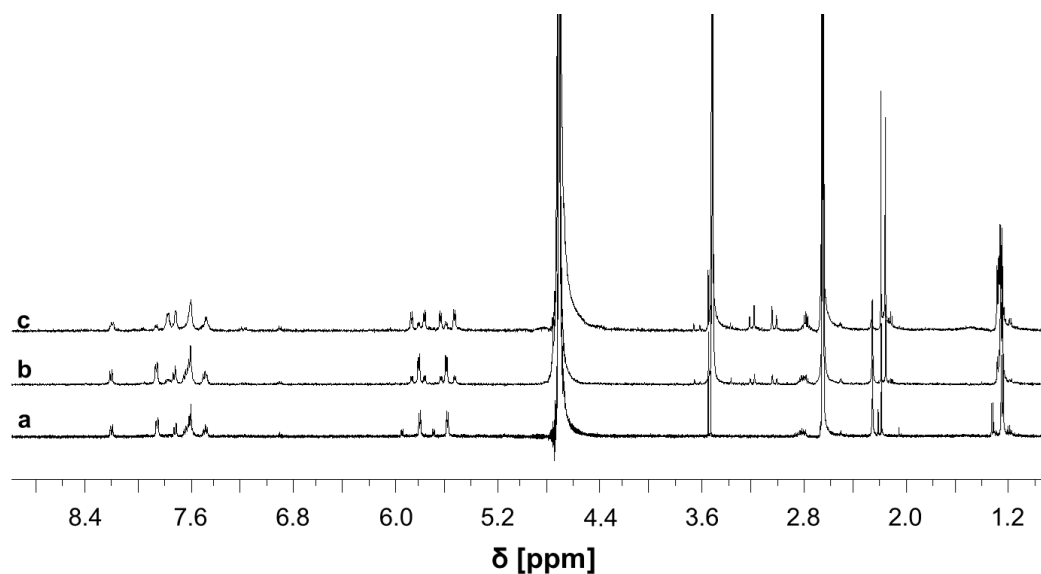


Figure S12. ¹H NMR spectra of a) 12', b) 12' + 1 eq glycine after 5 min and c) after 24 h.

1.7.4 Reaction of 12' with an equimolar amount of L-cysteine

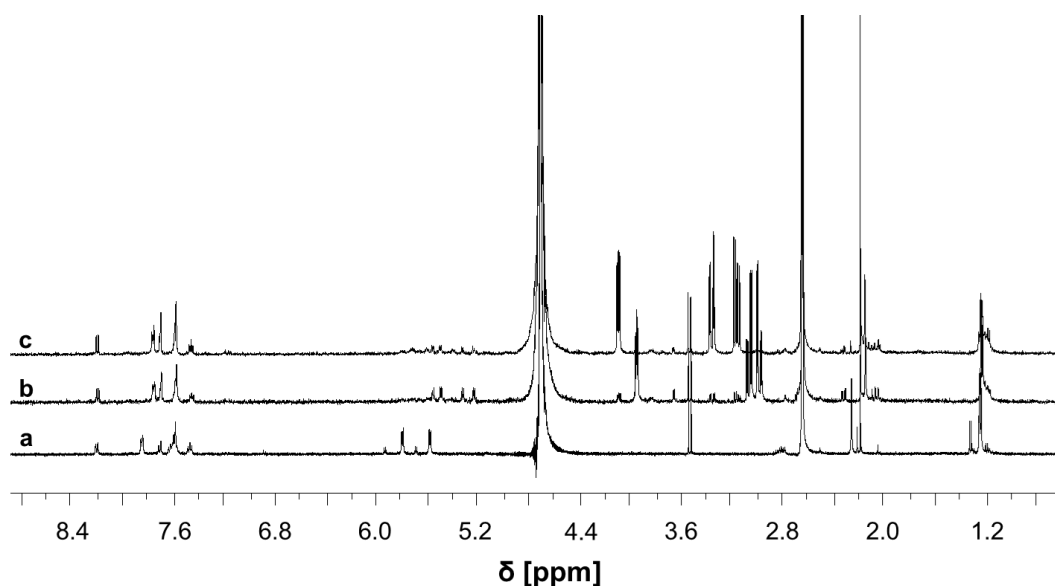


Figure S12. ¹H NMR spectra of a) 12', b) 12' + 1 eq L-cysteine after 5 min and c) after 24 h.

4. Conclusions and Outlook

Flavonoids are natural compounds which exhibit very interesting biological effects including antioxidant and anticancer activity and members of this compound class have already been investigated in clinical trials as anticancer agents. By combination of these versatile small molecules with ruthenium moieties we aimed for new anticancer agents that are able to interact with multiple targets, which could be an approach for single-molecular combination therapy. Within this Ph.D. thesis, a series of Ru^{II}(arene) complexes, bearing bioactive flavonoid ligands, was synthesized, and their behavior in aqueous solution and the activity towards small biomolecules was studied. The anticancer activity was determined in different human cancer cell lines. Furthermore the inhibitory activity on CDK2 and topoisomerase II α as potential target molecules was investigated, and flow cytometry analyses of the cell cycle were conducted.

The Ru^{II}(arene) complexes were synthesized in moderate to good yields and are stable in solid state for more than one year even under exposition to light and air. The compounds were characterized by standard analytical methods and single crystals were analyzed by X-ray diffraction methods confirming the typical pseudo-octahedral “piano-stool” configuration of the substances. In aqueous solution the complexes hydrolyze quickly by exchange of the halido ligand with a water molecule leading to the more reactive charged aqua species, which has been shown to be stable in solution for about 24 h. After 6 days, the formation of the dimeric hydrolysis side product [Ru₂(cym)₂(OH)₃]⁺ was observed, which is known to be thermodynamically stable and is biological inactive. More extensive solution phase studies by UV-VIS and fluorescence spectroscopy revealed that the stability constants of the 3-hydroxyflavone derived Ru^{II}(η^6 -*p*-cymene) complexes are in the range of the structurally related maltolato complexes with a stability constant of $\beta = 7.13 \pm 0.08$.

The *in vitro* anticancer activity was determined in six human cancer cell lines, namely CH1 (ovarian carcinoma), SW480 (colon carcinoma) and A549 (non-small cell lung carcinoma) by means of the colorimetric MTT assay and 5637 (urinary bladder), LCLC-103H (large cell lung) and DAN-G (pancreatic carcinoma) by means of the crystal violet assay. The obtained IC₅₀ values were in the low μ M range, which is notable for Ru^{II}(arene) complexes, but this indicates the central role of the flavonoid ligand system as the main anticancer activity de-

terminating factor. The ligand structure plays an important role for the cytotoxic activity of these compounds. Compared to the unsubstituted ligand structure, *ortho* substitution of the phenyl ring appears unfavorable, whereas *meta* and *para* substitution increases the antiproliferative activity. In contrast to this, variation of the arene ligand and the halido leaving group had only minor to no effect on anticancer activity. Compared to the free ligands, the complexes were slightly more active *in vitro*, but it also has to be mentioned in this context that the complexes exhibit approximately 10-fold better solubility in water than the respective ligands. When changing from 3-hydroxyflavone- to 3-hydroxyquinolinone-derived compounds, no improvement of the *in vitro* anticancer activity was observed.

To gain more insight about pharmacokinetic pathways and possible reactions with small biomolecules, the reactions of the compounds, or rather the respective aqua species, with the DNA model 5'-GMP and several amino acids were investigated. All compounds reacted immediately with 5'-GMP, forming monofunctional *N7*-adducts, indicating DNA as a possible binding partner for this substance class. The 3-hydroxyflavone complexes reacted immediately with Met, His and Gly by replacement of the aqua ligand with the amino acid, and the flavonol ligand was released within 24 h leading to precipitation in the test tubes. Addition of Cys led to immediate decomposition of the 3-hydroxyflavone complexes. In contrast to this the 3-hydroxyquinolinone-derived compounds showed a higher stability concerning the reaction with amino acids as for Met, His and Gly still signals of coordinated ligands were visible in the NMR spectra after 24 h and also a reaction with Cys was observed.

Due to the intrinsic fluorescence of the flavonoids and their Ru^{II}(arene) complexes it is possible to perform fluorescence studies on the compounds' distribution and enrichment in the cells. In preliminary co-staining experiments with fluorescence confocal laser scanning microscopy the tested flavonol-derived Ru^{II}(*p*-cymene) complex were found to be located in the endoplasmatic reticulum, which is thought to be the primarily targeted organelle and might act as a kind of reservoir for the lipophilic species.

In order to gain information on the mode of action of the complexes and to reveal possible biological target structures, the inhibitory activity on CDK2 and topoisomerase II α was assayed and flow cytometry analyses of the cell cycle were conducted. The compounds showed a slight influence on the cell cycle; especially around the IC₅₀ values an increase in the G₀/G₁ fraction was observed. Furthermore all tested compounds inhibited CDK2 and

were nearly as active as the well-known CDK2 inhibitor roscovitine. However, there is no direct correlation to the *in vitro* anticancer data set and also due to the minor influence on the cell cycle, CDK2 and CDKs in general are not considered as a main target for this substance class.

As flavonoids themselves are known topoisomerase inhibitors, the catalytic activity of topoisomerase II α in the presence of varying complex concentration was determined by means of the decatenation assay. All complexes inhibit topoisomerase II α at concentrations $\geq 10\mu\text{M}$ with the extent of inhibition being well correlated to the *in vitro* anticancer potency. Compared to the free flavonol ligands, the complexes showed to be more potent inhibitors, demonstrating that the inhibition of topoisomerase II α is enhanced by the linkage of the biological active flavonol scaffold to the Ru^{II}(η^6 -*p*-cymene) moiety. This observation can be explained by the additional ability of the ruthenium center to interact with the DNA. Therefore the complexes are thought to act in a bifunctional, multi-targeted manner, which could be an approach for single-molecular combination therapy and a promising strategy to overcome drug resistance and to overcome drawbacks of current chemotherapeutics in general.

The Ru^{II}(arene)-flavonoid system offers multiple possibilities for the design of novel anticancer drugs. Studies about the role of the metal center and the preparation of thioflavonol-derived complexes are already in progress. Further investigations will be on the synthesis of even better water soluble flavonol-derived Ru^{II}(arene) compounds. In order to learn more about the mode of action of this substance class, detailed studies about the cellular uptake and the interactions with other biological targets will be conducted. Furthermore, *in vivo* tests with the by now most active compound are already in progress.

In conclusion, Ru^{II}(arene) complexes with flavonoid-type ligands are promising development candidates for multi-targeted anticancer drugs.

Curriculum vitae

Mag. Andrea Kurzwernhart

Email: andrea.kurzwernhart@univie.ac.at



Date and Place of Birth: 14.03.1986, Vienna

Nationality: Austria

Education

since 09.2011	Apprentice trainer at the Institute of Inorganic Chemistry, University of Vienna
since 01. 2010	Teaching assistant („prae doc“) at the Institute of Inorganic Chemistry, University of Vienna
since 10. 2009	Ph.D. Research at the Institute of Inorganic Chemistry, University of Vienna
27.10. 2009	Diploma graduation with Mag. rer. nat.
03. 2009 – 10. 2009	Diploma Thesis at the Institute of Inorganic Chemistry, University of Vienna, under supervision of Prof. Dr. Dr. Bernhard Keppler: <i>“Organometallic anticancer compounds targeted by linking the flavopiridol backbone to ruthenium-cymene moieties”</i>
03. 2009 – 07. 2009	Tutor at the Institute of Inorganic Chemistry, University of Vienna
09. 2004 – 10. 2009	Diploma study Chemistry, University of Vienna
07.06. 2004	High-school graduation
09. 1996 – 06. 2004	High-school Mater Salvatoris, 1070 Vienna
09. 1992 – 06. 1996	Elementary school Mater Salvatoris, 1070 Vienna

National and International Presentations

- 29.03.2010 6th Workshop for Inorganic Chemistry in Austria (WACÖ), Johannes Kepler University Linz; Oral presentation:
"Organometallic anticancer compounds targeted by linking the flavopiridol backbone to ruthenium-cymene moieties"
- 28.09.2011 14th Austrian Chemistry Days, Johannes Kepler University Linz; Oral presentation:
"Ruthenium^{II}(arene)-flavonoid complexes as multi-targeted anticancer compounds"
- 02.-05.12.2011 11th International Symposium on Applied Bioinorganic Chemistry (ISABC), Barcelona; Poster presentation:
"DNA-binding and Topoisomerase II α -inhibiting Ru(cymene) Complexes with Flavone-derived Ligands"
- 02.-03.04.2012 7th Workshop for Inorganic Chemistry in Austria (WACÖ), Leopold-Franzens-Universität Innsbruck; Oral presentation:
"DNA-binding and Topoisomerase II α -inhibiting Ru^{II}(arene) Complexes with Flavone-derived Ligands"
- 18.-22.06.2012 International Symposium on Metal Complexes 2012 (ISMEC), Lissabon; Poster presentation:
"Structure-activity relationship studies for flavonol-derived Ru^{II}(arene) anticancer complexes"
- 07.-08.12.2012 International conference "Metallodrugs I: Design and mechanism of action", Olomouc, Czech Republic; Oral presentation:
"Structure-activity relationship studies and biological activity of flavonol-derived Ru^{II}(arene) anticancer complexes"

Publications

“Pyrone derivatives and metals: From natural products to metal-based drugs”

Wolfgang Kandioller, Andrea Kurzwernhart, Muhammad Hanif, Samuel M. Meier, Helena Henke, Bernhard K. Keppler, Christian G. Hartinger, *Journal of Organometallic Chemistry* **2011**, 696, 999-1010.

“Targeting the DNA-topoisomerase complex in a double-strike approach with a topoisomerase inhibiting moiety and covalent DNA binder”

Andrea Kurzwernhart, Wolfgang Kandioller, Caroline Bartel, Simone Bächler, Robert Trondl, Gerhard Mühlgassner, Michael A. Jakupiec, Vladimir B. Arion, Doris Marko, Bernhard K. Keppler, Christian G. Hartinger, *Chemical Communications* **2012**, 48, 4839–4841.

“Structure–Activity Relationships of Targeted Ru^{II}(η^6 -*p*-Cymene) Anticancer Complexes with Flavonol-Derived Ligands”

Andrea Kurzwernhart, Wolfgang Kandioller, Simone Bächler, Caroline Bartel, Sanela Martić, Magdalena Buczkowska, Gerhard Mühlgassner, Michael A. Jakupiec, Heinz-Bernhard Kraatz, Patrick J. Bednarski, Vladimir B. Arion, Doris Marko, Bernhard K. Keppler, and Christian G. Hartinger, *Journal of Medicinal Chemistry* **2012**, 55 (23), 10512–10522.

“3-Hydroxyflavones vs. 3-Hydroxyquinolinones: Structure–Activity Relationships and Stability Studies on Ru^{II}(arene) Anticancer Complexes with Biologically Active Ligands”

Andrea Kurzwernhart, Wolfgang Kandioller, Éva A. Enyedy, Maria Novak, Michael A. Jakupiec, Bernhard K. Keppler and Christian G. Hartinger, *Dalton Transactions* **2013**, DOI: 10.1039/C2DT32206D.