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„New Hybrid Ligands and Their Metal(II)
Complexes as Potential Anticancer Drugs“

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Abstract

Cancer is one of the major health problems in today's society. Metal (especially platinum(II)) containing drugs became an important weapon in the fight against cancer in the past few decades. Despite impressive curing rates in some cancer types, these drugs bear several drawbacks, such as severe side effects and resistances in many cancer types. The design of novel non-platinum based metallodrugs is a promising strategy for the creation of safer anticancer therapies with activity against platinum(II) resistant cancers. The main focus is on ruthenium(II) and ruthenium(III) based metallodrugs at the moment, but also complexes with other metals, for example gallium(III), copper(II), nickel(II), palladium(II), zinc(II) show promising results.

Thiosemicarbazones (TSCs) are long known to be active against cancer. In addition, they are excellent ligands for first row transition metals. The best developed TSC to date is Triapine (3-AP) which is currently in clinical phase II. TSCs are possible inhibitors of the enzymes ribonucleotide reductase (RNR) and Topoisomerase II α (Topo II α). Both enzymes are attractive targets for cancer treatment since they are crucial for cell division. A general problem of TSCs is their poor aqueous solubility and low therapeutic index.

We prepared TSC-proline hybrids and their copper(II) complexes previously. These compounds were highly water soluble, but their anticancer activity was much lower than that of the parent TSCs. One of the reasons for this was most probably their very low lipophilicity. The topic of this work was to find the optimal balance between lipophilicity and water solubility, and to increase the therapeutic index. Several modifications were performed in order to enhance the lipophilicity and improve the therapeutic index, (i) methylation at the terminal thiosemicarbazone nitrogen, (ii) complexation with other metals than copper(II), (iii) using other water soluble substituents than proline, i. e. morpholine and methylpiperazine.

Zusammenfassung

Krebs ist eines der größten Probleme im heutigen Gesundheitssystem. Metall- (insbesondere Platin(II)-) haltige Chemotherapeutika entwickelten sich im Laufe der letzten Jahrzehnte zu einer wichtigen Waffe im Kampf gegen Krebs. Trotz beeindruckender Heilungsraten bei manchen Krebs-Typen gibt es schwerwiegende Nachteile, wie etwa schwere Nebenwirkungen und Resistenzen bei mehreren Krebsarten. Die Entwicklung neuer metallhaltiger Wirkstoffe, welche nicht auf Platin(II) basiert sind ist ein vielversprechender Weg, um neue, sicherere Chemotherapeutika mit einem breiteren Wirkungsspektrum zu entwickeln. Das größte Augenmerk liegt im Moment auf Ruthenium(II) und (III) basierten Verbindungen, aber auch Komplexe mit anderen Metallen, z. B. Gallium(III), Kupfer(II), Nickel(II), Palladium(II), Zink(II) zeigen vielversprechende Resultate.

Die krebsinhibierende Wirkung von Thiosemicarbazonen (TSCs) ist seit langem bekannt, außerdem sind TSCs exzellente Liganden für die Metalle der ersten Übergangsmetallreihe. Das heutzutage am weitesten entwickelte TSC ist Triapin (oder 3-AP), welches sich zurzeit in klinischer Phase(II) befindet. TSCs sind mögliche Inhibitoren der Enzyme Ribonukleotidreduktase (RNR) und Topoisomerase II α (Topo II α). Beide Enzyme sind attraktive Angriffsziele in der Krebstherapie, da sie essentiell für die Zellteilung sind. Ein generelles Problem von TSCs ist ihre geringe Wasserlöslichkeit, was Studien in wässriger Lösung, sowie ihr niedriger therapeutischer Index.

Vor kurzem haben wir TSC-Prolin Hybride und ihre Kupfer(II)komplexe dargestellt. Diese neuen Verbindungen waren höchst wasserlöslich, aber ihre Anti-Krebs-Wirkung war wesentlich niedriger als jene ihrer Vorläufer. Der Grund hierfür war höchstwahrscheinlich ihre geringe Lipophilie. Die Zielsetzung dieser Arbeit war ein optimales Gleichgewicht zwischen Lipophilie und Wasserlöslichkeit zu finden, sowie den therapeutischen Index zu erhöhen. Verschiedene Modifikationen wurden durchgeführt um die Lipophilie zu erhöhen und den therapeutischen Index zu verbessern, (i) Methylierung des terminalen Thiosemicarbazon Stickstoffes, (ii) Komplexierung mit anderen Metallen als Kupfer(II), (iii) Benützung anderer wasserlöslicher Substituenten als Prolin, z. B. Morpholin und Methylpiperazin.

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1 Introduction

1.1 Cancer

1.1.1 Cancer mortality worldwide and in Austria

8.2 million people died because of cancer in 2012 worldwide, moreover 14.1 new cancer cases were diagnosed and 32.6 million people were living with cancer in the same year.¹ Cancer is one of the most spread death causes worldwide, outnumbered only by cardiovascular and infectious/parasitic diseases and clearly a strong burden for everyone who is affected. Cancer incidence is sex related, the most frequent type of cancer for men is lung cancer, followed by stomach and liver cancer, while for women it is breast cancer followed by lung cancer and stomach cancer.² In Austria, which can be seen as typical example for high income countries, cancer is the second most frequent cause of death, behind cardiovascular diseases (see Figure 1). 28% of men and 22% of women who died in Austria in 2013, died because of cancer.³

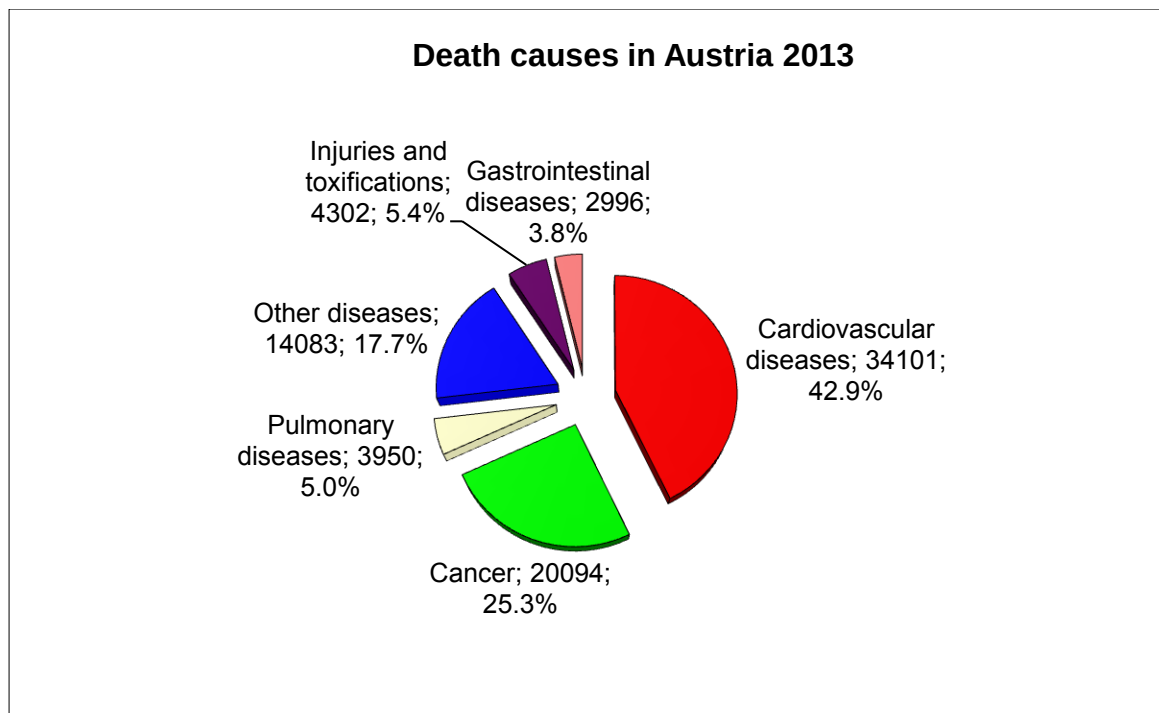


Figure 1: Death causes in Austria in 2013.

The risk to die because of cancer strongly increases with the age (see Figure 2).⁴

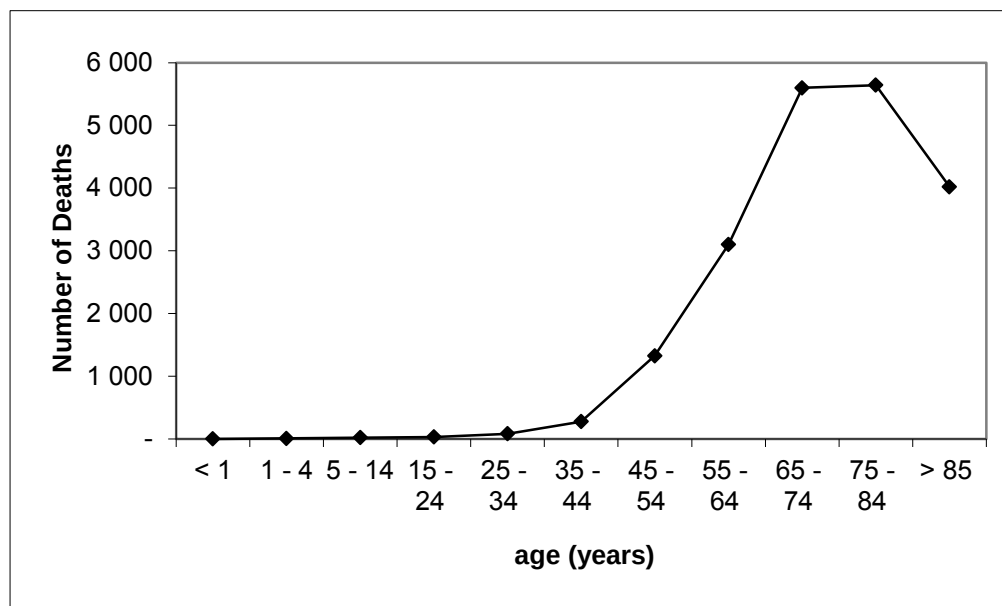


Figure 2: Age related cancer death rate in Austria in 2013.

In fact we live in an ageing society; the lifespan for men in Austria is expected to increase from 77.6 in 2008 to 85.9 years in 2050 and for women from 83.0 to 89.5 years respectively.⁵ This means that cancer will become a growing problem and the development of new, safer anticancer strategies is of prime importance.

1.1.2 Cancer development

The term cancer denotes a very heterogeneous group of over 100 diseases consisting of malign neoformations. Their common features are: (i) uncontrolled cell growth, (ii) “immortal” cells (normal cells have a certain lifespan, after which they undergo a controlled cell death called apoptosis, in cancer cells this mechanism is jammed), (iii) the ability of cells to invade into other tissues (building of metastases).

Cancer development is a slow process, three steps can be distinguished: initiation, promotion and progression. Normally clinical signs are evident only several years after the first mutations took place.

Initiation:

Cancer development starts with mutations in the cellular genome. If there are too many mutations or a gene which is essential for cell viability is impacted the cell will undergo apoptosis and cancer development will not take place. Especially dangerous are mutations in genes that control apoptosis and cell division, initiation may occur if genes of this type are inflicted. Genomic damages can be caused by exogenetic or endogenetic

factors. Endogenetic factors are usually inherited and responsible only for a small fraction of cancers (e. g. some breast cancers).⁶ Exogenetic factors are responsible for the vast majority of cancers; they can be divided into three groups: physical, biological and chemical factors.

Physical factors are for example ionizing radiation, like γ - and X-ray and UV-radiation or nanoparticles for example asbestos or traffic originated fine dust.

An example for biological factors is the human papilloma virus that can cause cervical cancer.

Chemical factors are mutagenic substances, such as benzene, certain metals, organochlorides and others.⁷ Tobacco consumption and unhealthy nutrition lead to a larger exposure to these harmful substances, which are responsible for the majority of cancer cases.

Promotion:

The initiated cell starts to divide in this step. Certain chemicals, the so called promoters, such 12-O-tetradecanoylphorbol 13-acetate (TPA) and alcohol accelerate this process.⁸⁻
¹⁰ A healthy lifestyle may slow down or even stop tumor promotion, since this is a reversible process that can take several years and involves several mutations which lead to the irreversible step of progression.

Progression:

After the mutations that took place during promotion, the cells lose their normal function, they start to divide in an uncontrolled manner and invade other tissues (metastases). The gene p53 which is involved in apoptosis and cell cycle regulation seems to play an important role within this process; it is mutated in over 50% of all cancers.¹¹ Cancers are usually not detected before progression took place, which largely exacerbates healing chances.

1.1.3 Cancer therapy

Three main cancer therapy strategies are used in mainstream medicine today: surgery, radiation therapy and chemotherapy.

Surgery:

Introduction

This technique is the oldest form of anticancer therapy. It was already used by the old Greeks to cure breast cancer and is still today the first line therapy for solid tumors. The drawbacks of this anticancer strategy is that it is only applicable to solid tumors before they built up metastases, non-solid tumors, e. g. leukemia, are not treatable.

Radiation therapy:

In this therapy hard irradiation is applied to the tumor tissue to kill the cancer cells. The principle of this technique is that cancer cells are generally more vulnerable for ionizing irradiation than normal cells, since their DNA repair mechanisms are hampered. Radiation sources are X- or γ -rays, or radioactive nucleotides for *in situ* therapy, for example ^{131}I for treatment of thyroid cancer. Problems related to radiotherapy are that the tumor has to be solid and localized and that the applied irradiation may lead to genomic lesions, which can cause secondary tumors.¹²

Chemotherapy:

In this therapy form chemical substances are applied to the cancer cells, which inhibit essential cell functions and lead to apoptosis. In general they interfere with the cell division mechanism. Chemotherapy is the only strategy to cure non-solid cancers and metastases. Chemotherapeutics do not act exclusively on cancer cells but also to healthy, especially fast dividing tissues e.g. hair follicles, red bone marrow, intestinal mucosa, explaining the common side effects of chemotherapeutics, i. e. hair loss, anemia, nausea and vomiting.

The major goal for today's scientists is to find cytotoxic substances that accumulate selectively within the tumor tissue and/or are selectively activated within the cancer cells, according to the magic bullet concept defined by Paul Ehrlich more than 100 years ago. Ehrlich was awarded the Nobel Prize for physiology and medicine in 1908, he is considered to be the founder of modern chemotherapy.¹³ The ATCⁱ classifies anticancer drugs into the following groups: alkylating agents, antimetabolites, plant alkaloids and other natural products, cytotoxic antibiotics and related substances and other antineoplastic agents (metallo drugs belong to this last class).¹⁴

ⁱ ATC stands for Anatomical Therapeutic Chemical classification system. It is hosted by the WHO.

1.2 Platinum based anticancer drugs

Today around 50% of all chemotherapeutic regimes contain at least one platinum based anticancer drug.¹⁵

The first worldwide approved platinum based anticancer drug is cisplatin (*cis*-diamminedichloridoplatinum(II), **1**), which was first synthesized by Michelle Peyrone in 1844.¹⁶ However, its anticancer potential was coincidentally discovered only more than 100 years later by Barnett Rosenberg in the 1960s.¹⁷ Rosenberg studied the effect of electric fields on dividing cells. He dipped platinum electrodes in an ammonium chloride containing medium containing *Escherichia coli* bacteria. Surprisingly the bacteria did not divide, but they grew to filaments up to 300 times as long as their normal size.¹⁷ Accurate scientific work led to the conclusion that the cell division was not inhibited by the electric field, but by a small amount of cisplatin, originated from tiny corrosions of the platinum electrodes, which was formed within the culture medium of the bacteria under the influence of sunlight.¹⁸ Cisplatin showed impressing anticancer activity, first in mouse models and then in clinical trials, which culminated in a fast admission for clinical use by the FDA.^{ii 19–21}

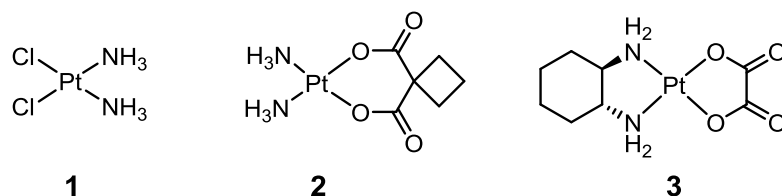


Chart 1: Worldwide approved platinum drugs: **1**, cisplatin, *cis*-diamminedichloridoplatinum(II); **2**, carboplatin, *cis*-diammine-(1,1-cyclobutanedicarboxylato)platinum(II); **3**, oxaliplatin, (1R,2R)diaminocyclohexane oxalatoplatinum(II).

The mode of action of Cisplatin is most probably the formation of crosslinks between DNA bases, especially guanine-guanine intrastrand crosslinks. Platinated DNA cannot be replicated during cell division, which leads to apoptosis.²² Cisplatin is especially effective against cancers of the genitourinary system, testicle cancer is one example with an impressing healing chance of more than 90%. However there are also problems related to cisplatin, namely severe side effects, especially nephrotoxicity, which is doselimiting and resistances in some cancer types, e. g. colon cancer. In order to

ⁱⁱ FDA stands for US Food and Drug Administration.

alleviate these disadvantages second and third generation platinum based anticancer drugs were developed.²³

Carboplatin bears a more stable leaving group, namely a 1,1-cyclobutanedicarboxylate ligand, which hydrolyzes much slower compared to the two chlorido ligands in cisplatin. This means that carboplatin can be administered in much higher doses, while myelosuppression is the doselimiting side effect. On the other hand carboplatin is less active than cisplatin and shows crossresistances to the latter since the active hydrolyzed species is identical for both the complexes.^{24,25}

Oxaliplatin is a third generation platinum(II) compound, in which not only the leaving group but also the stable N,N' moiety is modified. The diaminocyclohexane (DACH) ligand creates a hydrophobic environment around the Pt(II)-DNA bonds, what may hinder the function of DNA repair mechanisms. Oxaliplatin is therefore also active in cisplatin resistant cancers, and in particular, colorectal cancer.^{26–28}

Other platinum(II) based drugs have been approved with regional restrictions: lobaplatin (**4**, in China), nedaplatin (**5**, in Japan) and hepaplatin (**6**, in South Korea).

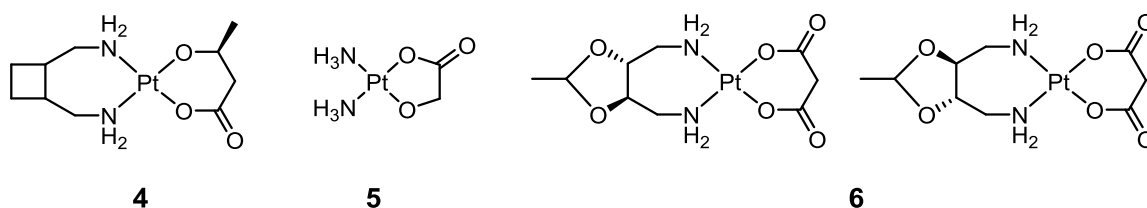


Chart 2: Regionally approved platinum drugs: **4**, lobaplatin; **5**, nedaplatin; **6**, hepaplatin.

Thousands of other platinum-based anticancer drugs,ⁱⁱⁱ matching the structure-activity relationships (SARs) of cisplatin which is mononuclear and uncharged, with square-planar cis coordination geometry and oxidation state +II, have been developed and failed either in preclinical development or in clinical trials. It soon became clear that also metal-based substances that do not match these criteria have the potential for clinical development.²⁹

A prominent example for non-classical platinum drugs is satraplatin, **7**, an octahedral platinum(IV) complex, which is inert and lipophilic enough to be administered orally. It is believed that it undergoes reduction in the hypoxic environment of the cancer tissue, after which it loses its axial carboxylate ligands and forms an active species similar to cisplatin. This principle is called activation by reduction.³⁰ However, satraplatin failed in clinical phase three due to the lack of activity in comparison with standard therapy. However, studies are going on.^{31–34}

ⁱⁱⁱ Out of simplicity reasons the term “drug” is also used for compounds without clinical approval, although this notation might not be 100% correct.

Another example is BBR 3464, **8**, a positively charged tri-nuclear platinum(II) complex, which causes three dimensional DNA changes distinct from cisplatin. BBR3464 is 100 to 1000 fold as cytotoxic as cisplatin and active also in cisplatin resistant cancer cell lines. Unfortunately clinical trials revealed a too high systemic toxicity for this compound.^{35,31}

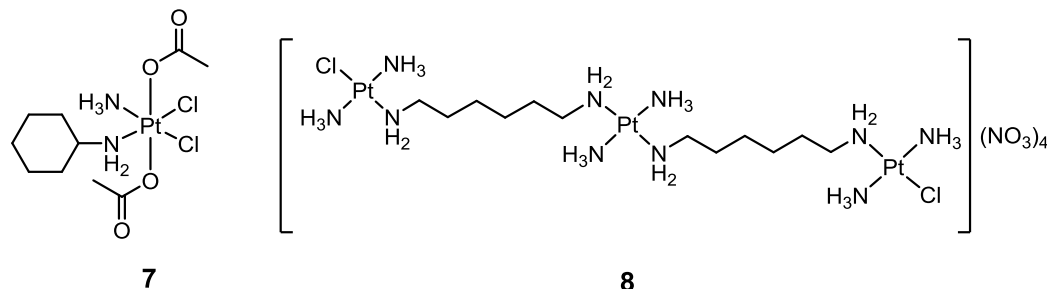


Chart 3: Two examples for non-classical platinum drugs: **7**, satraplatin; **8**, BBR3464.

1.3 Ruthenium based anticancer drugs

Ruthenium complexes offer several advantages in comparison with platinum(II)-based complexes:

- (i) Different coordination modes (i. e. octahedral, and the so called “piano stool” conformation).³⁶
- (ii) The oxidation states +II, +III and +IV are available in the physiological range. Certain ruthenium(III) complexes are possibly reduced within the hypoxic environment of cancer tissues leading to less stable anticancer active ruthenium(II) complexes (activation by reduction).³⁷
- (iii) Ruthenium stands in the 8th group of the periodic table like iron and has some common features, like the oxidation states +II and +III. It is assumed that ruthenium uses similar transport pathways as iron. This might be an advantage since it is known that tumors need an enhanced iron supply due to their fast growth. In addition, it was shown that ruthenium complexes bind to human serum albumin (HSA), a transport protein which is also largely responsible for iron transport.^{38,39}

Until now, three ruthenium complexes have entered clinical trials, namely NAMI-A, **9**, KP1019, **10**, and its sodium salt analogue KP1339, **11**.

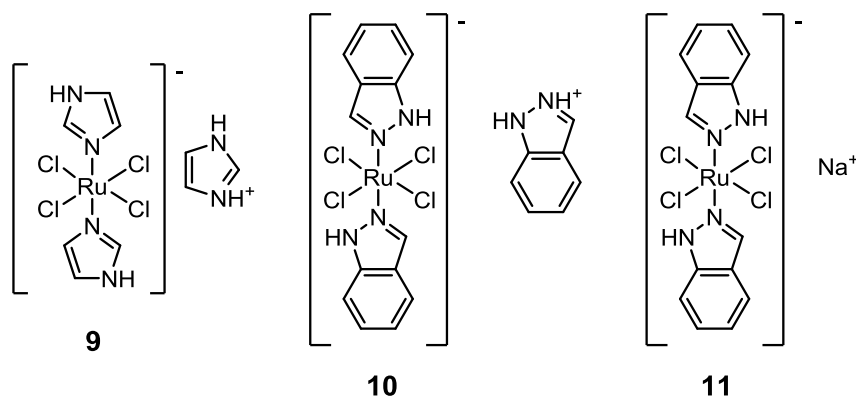


Chart 4: Ruthenium drugs that have entered clinical trials: **9**, NAMI-A; **10**, KP1019; **11**, KP1339.

NAMI-A, **9** is an antimetastatic agent without pronounced activity against primary tumors,⁴⁰ in a clinical phase I trial one out of 24 patients reached stable disease, the dose-limiting toxicity was painful blister formation.⁴¹ A clinical phase I trial with KP1019 resulted in disease stabilization for five out of six patients, dose limiting toxicities have not been found in this study, because the low solubility of this compound would have led to intolerably high infusion volumes.⁴² These dose limitations were the reason why clinical investigations shifted to the better soluble sodium salt analogue of KP1019, named KP1339, **11**. A recent phase I study including 34 patients revealed partial response in one patient and stable disease in seven patients. KP1339 was generally well tolerated; grade 2-3 nausea was the dose-limiting toxicity. This is a promising result and KP1339 might be a good candidate for clinical application.⁴³ A comprehensive review about recent developments in the field of ruthenium-based anticancer drugs, including the current knowledge about possible action mechanisms is included in the “results” section of this thesis.

1.4 Ribonucleotide reductase

Ribonucleotide reductases (RNRs) are essential for all life forms for the synthesis of DNA out of RNA by the reduction of ribonucleotides to the related deoxyribonucleotides.⁴⁴ A too high or too low concentration of deoxyribonucleoside triphosphates within the cell leads to genomic instability and RNR is largely responsible for the tight maintenance of this balance.⁴⁵ Three groups of RNRs are known today, they differ by their metal cofactor, which is needed for the generation of a cysteine originated thiyl radical at the enzymes active site.^{46,47}

Class I RNRs are oxygen dependent and can be grouped into three subclasses according to their metal cofactor identity.⁴⁸ They consist of tetrameric $\alpha_2\beta_2$ or oligomeric $\alpha_n(\beta_2)_m$ ($n = 4$ or 6 , $m = 1, 2$ or 3) structures.^{49,50} The α_2 homodimer (also named R1) contains the substrate binding site and the active center. R1 also regulates the enzyme activity, on one hand by the use of ATP (ATP = activator, dATP = down-regulator) and on the other hand by a unique allosteric mechanism.^{51,52} The β_2 subunit (also named R2) is significantly smaller than R1 and houses the metal cofactor which is necessary to form a tyrosyl radical ($Y^\bullet$). This radical needs then to be transported via a complicated multistep long range electron transfer mechanism, over a distance of about 35 Å to the active site in the α_2 subunit to generate a transient thiyl radical there.⁵³ Class Ia RNR requires two iron atoms,⁵⁴ class Ib contains two manganese atoms⁵⁵ and class Ic contains one iron and one manganese atom.⁵⁶

Class II RNRs are oxygen independent. They are composed only of mono- or dimeric α subunits, which contain a cobalt containing adenosylcobalamin cofactor (coenzyme B12) which is located near the enzymes active center.^{57,58} The radical is generated via homolytic cleavage of a Co-C bond, and then transported by a mechanism, that is not fully understood, over a distance of 6.5 Å to the active center.^{59,60}

Class III RNRs work only under anaerobic conditions. They are consisting of α_2 and β_2 subunits. The latter bear the metal cofactor which is a 4Fe-4S cluster, needed to form an oxygen sensitive glycyl radical ($G^\bullet$).^{61,62} The reductant needed for substrate turnover is formate, which is oxidized to CO_2 ,⁶³ or thioredoxin in special cases,⁶⁴ in contrast to class I and II RNRs, which use exclusively thiol dependent reductants that get oxidized upon the formation of disulfide bridges.⁴⁶

Since in humans and other mammals, as well as in most eukaryotes only RNR of the type Ia is present, the further discussion will touch this type only.

1.4.1 Mechanism of action

The overall reaction mechanism for the synthesis of 2'-deoxyribonucleotides out of ribonucleotides starts with the formation of a stable tyrosyl radical in the R2 subunit of the RNR enzyme. Figure 3 gives a simplified scheme of this process, a more complete possible mechanism, including different intermediates, is given in ref. 46.

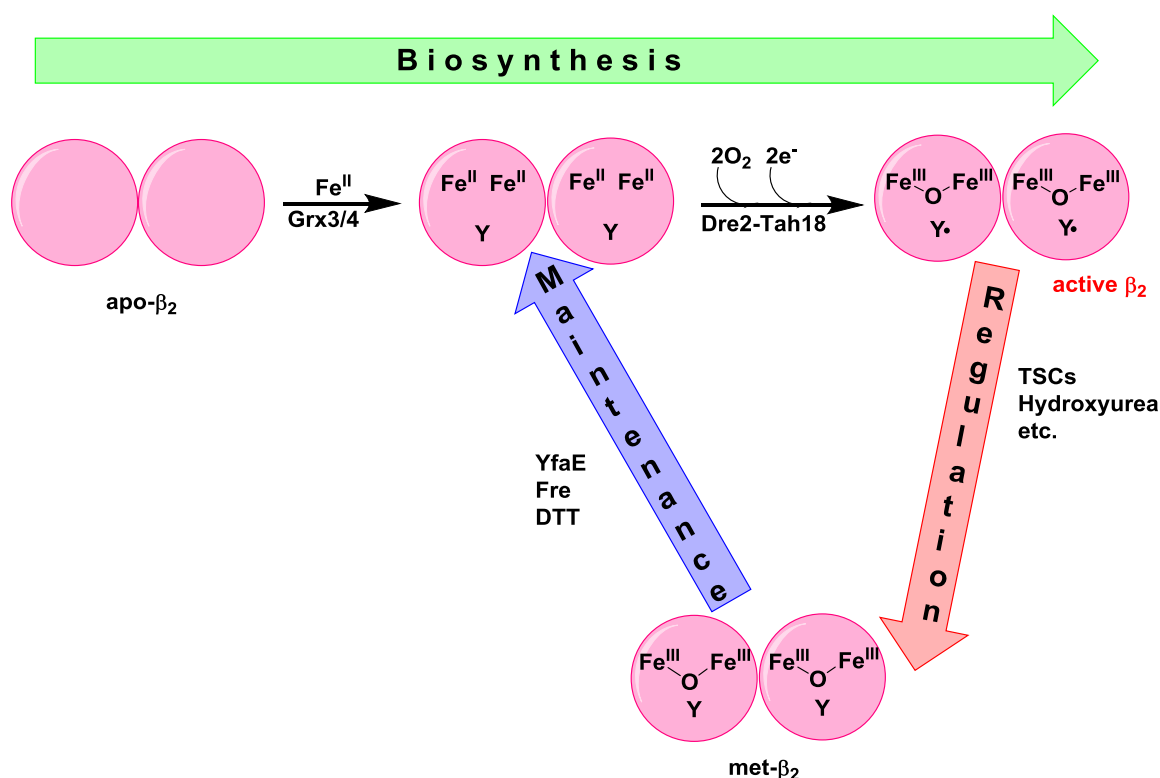


Figure 3: Schematic depiction of the radical generation in R2 of RNR.

The biosynthesis of $\text{Y}_{122}^\bullet$ in β_2 starts with iron(II) loading of the apo- β_2 protein. Two cytosolic monothiol glutaredoxins, bearing a Fe_2S_2 cluster, namely Grx3 and Grx4 are needed to successfully deliver iron(II) to the coordinating side chains in β_2 .⁶⁵ One oxygen molecule and one electron are then needed for the generation of each diferric tyrosyl radical. A recent study suggests that the electron is delivered by the NADPH/NADP⁺ dependent Dre2-Tah18 protein complex (Dre2 is a Fe-S cluster protein, while Tah18 is an oxidoreductase).⁶⁶ Hydroxyurea, Triapine and other small molecules, which will be discussed in chapter 1.4.2, are able to selectively reduce the active β_2 to a stable diferric state without radical, named met- β_2 . The enzyme is not able to perform catalysis in this state.⁶⁷ To regain its function the enzyme has to undergo a maintenance pathway, in which the iron(III) is reduced to iron(II) again. *In vitro* this can be performed by iron(II) and dithiothreitol (DTT).⁶⁸ *In vivo* the issue is more complex and not understood in every detail. However, it seems likely that the ferredoxin YfaE, containing an Fe_2S_2 cluster, together with the ferredoxin reductase Fre is involved and largely responsible for this process.⁶⁹

In the next step the substrate is bound to the R1 subunit of the enzyme, which leads to a conformational change within the enzyme. This alteration is the rate-determining step in the substrate turnover⁷⁰ and allows the radical in R2 to be transported via proton-coupled

long range electron transfer involving redox active thyrosins, to generate a thiyl radical in the active center 35 Å apart from the iron cluster in β_2 .⁷¹

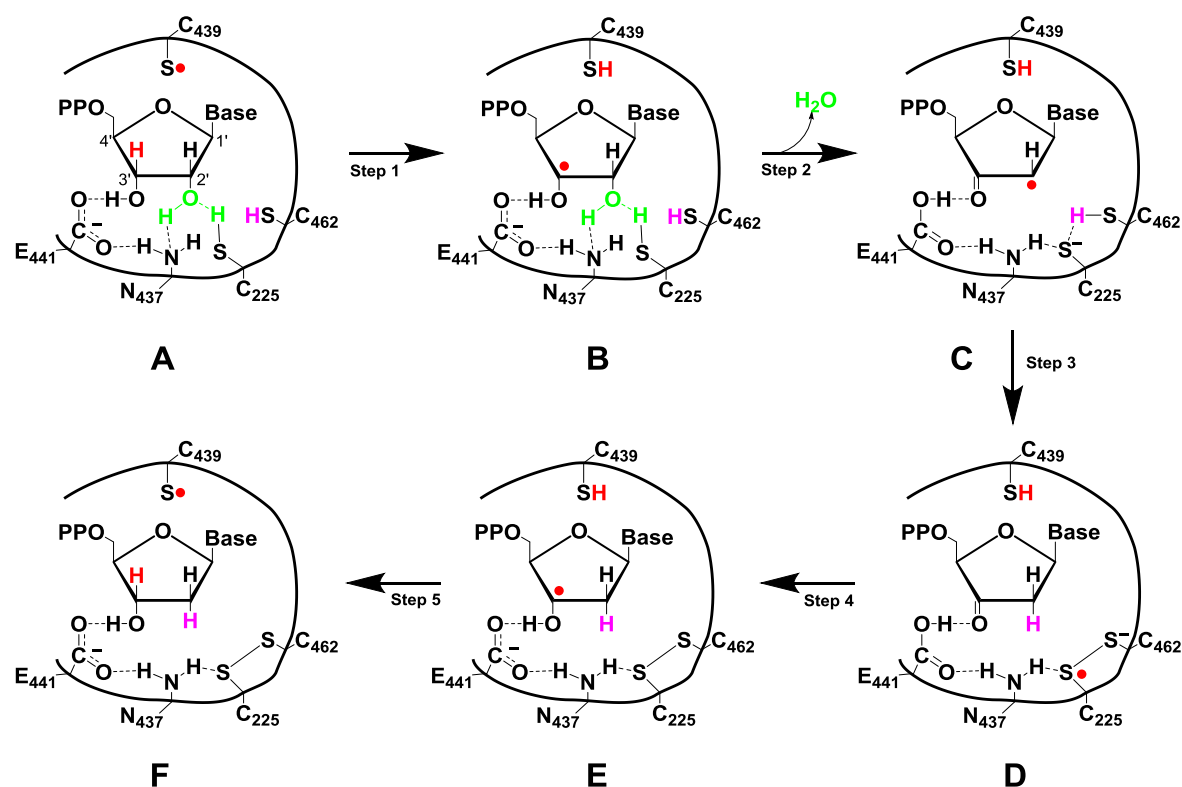


Figure 4: Postulated mechanism for the reduction of Ribonucleotides by class I RNR.

The actual substrate turnover on the enzymes active site is illustrated in Figure 4. The thiyl radical formed in R1 (C₄₃₉•) is able to abstract a hydrogen from the 3' position of the ribose ring from bound ribonucleotides and thereby generate the substrate radical **B**.^{72,73} In step 2 the 3' hydroxyl group gets deprotonated by glutamate₄₄₁ and the one at the 2' position gets protonated by cysteine₄₆₂. Both processes are facilitated by the 3' radical. Finally the 3' hydroxyl group leaves the molecule as water and the radical shifts to the 2' position, while a keto group is formed at the 3' position. The role of asparagine₄₃₇ has remained unclear for quite some time, as it never reacts with the substrate, although mutations at this site showed that it is crucial for the enzymes activity.⁷⁴ Computational methods using a large model, comprising the whole R1 unit of the RNR enzyme, revealed that N₄₃₇ is needed in the second step of catalysis. It essentially directs the 2' hydroxyl group of the substrate in the right orientation towards the thiol group of C₂₂₅ by hydrogen bond interaction with the former. Only this pinpoint orientation allows the elimination of water, otherwise the radical would return to C₄₃₉ and from there reform the thyrosyl radical in R2.⁷⁵ In the third step the substrate radical **C** abstracts a hydrogen from cysteine₄₆₂. The 2' position of the sugar gets thereby reduced and the disulfide

radical anion intermediate **D** evolves, which has been observed using high frequency EPR spectroscopy and a E₄₄₁ mutated RNR.⁷⁶ In the next step the disulfide radical anion gets oxidized and the radical migrates back via N₄₃₇ and E₄₄₁ to the 3'-position of the ribose ring. E₄₄₁ acts as acid in this step by protonating the 3' oxygen.⁷⁷ The fifth and last step is the reverse of the first step, the radical on the substrates 3'-position abstracts the thiol hydrogen from C₄₃₉ completing the substrate reduction. The resulting thiyl radical is then transferred back to the enzymes R2 unit reforming the tyrosyl radical there. For a complete turnover the disulfide bridge between C₂₂₅ and C₄₆₂ has to be reduced, this is performed via an intramolecular mechanism that involves the oxidation of two cysteins on the enzymes C-terminus.⁷⁸ In turn the C-terminal disulfide bridge has to be reduced. This is realized by the two NADPH dependent redox systems thioredoxin reductase and thioredoxin, or glutathione reductase, glutathione (GSH) and glutaredoxin. In mammalian cells thioredoxin is the main electron donor during DNA synthesis in the S-phase, while glutaredoxin is predominantly utilized for DNA repair and mitochondrial DNA synthesis.⁷⁹ The inevitable reduction of the disulfide bridge in the RNRs active center is the reason why the radical is stored in a remote subunit in the form of Fe^{III}₂-Y•. If C₄₃₉• would still be present in the active center during the mentioned reduction step this radical would be reduced much faster than the disulfide bridge, resulting in a quenched enzyme activity.⁴⁶

1.4.2 RNR inhibitors

Human RNR is especially active during S phase, when 3.2 billion base pairs need to be synthesized.⁸⁰ In the late M phase the R2 protein gets degraded⁸¹ while the R1 subunit is present during the whole cell cycle.⁸² During G₀/G₁ phase the R2 subunit is represented by the p53-inducible p53R2 protein,⁸³ which might supply the cell with nucleotides for DNA repair and mitochondrial DNA synthesis. However, the p53R2 protein is present only in a much smaller cellular concentration than R2 during S phase.⁸⁴ RNR inhibition prevents both DNA repair and cell division which makes it an attractive target for anticancer therapy.

RNR inhibitors can be roughly divided into three groups, (i) compounds that prevent the formation of the RNR enzyme, (ii) inhibitors of the R1 subunit and (iii) inhibitors of the R2 subunit.

(i) The first class can be divided into two subclasses, translational inhibitors are oligonucleotides, designed to interact with mRNA or DNA in order to prevent the expression of certain genes. One example is GTI 2501, a 20-mer oligonucleotide, which interacts with the mRNA of the R1 protein and thereby down-regulates its expression. GTI 2501 has shown *in vitro* and *in vivo* activity against a variety of cancers, for example

colon, lung, breast, renal, brain and other tumors.⁸⁵ The other subclass consists of compounds which prevent the dimerization of the R1 and R2 subunit. These so called dimerization inhibitors are mostly peptides or peptidomimetics that resemble the flexible C-terminus of the R2 protein and strongly interact with the R1 subunit, thus preventing the binding of R2. The advantage of these compounds is that the R2 subunits C-terminus is not conserved between different species, this means that it is possible to target the RNR of certain parasites or viruses without affecting the host.⁸⁶

(ii) R1 inhibitors are mainly ribonucleotide analogues designed to bind at the enzyme's active site and, thereby, interrupt its function. The two best studied R1 inhibiting anticancer drugs are clofarabine, **12**⁸⁷ and gemcitabine, **13**.⁸⁸

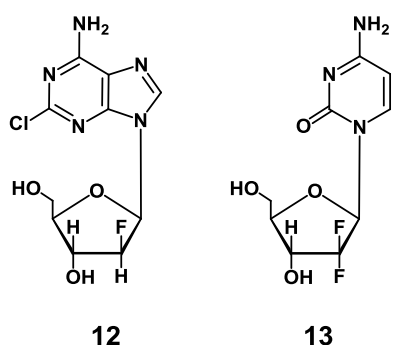


Chart 5: Inhibitors of the RNRs R1 subunit: **12**, clofarabine, 2-chloro-2'-arabino-fluoro-2'-deoxyadenosine, CAFdA; **13**, gemcitabine, 2',2'-difluoro-2'-deoxycytidine, dFdC.

The purine analog clofarabine is currently used in the treatment of pediatric leukemias.⁸⁹ It is an allosteric RNR inhibitor, which changes the quaternary structure of the R1 protein. It resembles dATP (desoxyadenosine triphosphate) after endogenous phosphorylation and consequently binds to the enzyme's allosteric site, which down-regulates the RNR activity. Clofarabine is not an irreversible inhibitor of RNR. It was shown that the enzyme is able to recover 50% of its initial activity after undergoing some conformational transitions.⁹⁰ RNR inhibition is not the only mechanism responsible for its anticancer activity. Clofarabine triphosphate competes with dATP and is incorporated into the DNA by certain DNA polymerases, which impairs DNA repair and synthesis. Moreover, it has also been shown that clofarabine is able to directly induce apoptosis by down-regulation of death suppressor proteins.⁹¹

Gemcitabine is a pyrimidine analog and a standard chemotherapeutic used against a variety of malignancies such as breast, pancreatic, lung and ovarian cancer.^{92–95} Gemcitabine gets phosphorylated within the organism, its di- and triphosphate are believed to be the active species and enter the enzymes catalytic site. The exact

mechanism explaining enzyme inactivation is complex and seems to involve more than one pathway. First computational models predicted that gemcitabine is processed in a way similar to normal ribonucleotides, at least in the first steps of catalysis. After abstraction of the C3' hydrogen by C₄₃₉• a C3' radical is formed which is transferred to the 2' position of the ribose ring once one fluorine got protonated by C₂₂₅ and left the molecule as HF. This happens in analogy to the 2' hydroxyl group of natural ribonucleotides, which leaves the molecule as water. The following steps are similar to the normal catalysis pathway. Until the interaction of the C₄₃₉ thiole hydrogen with the second fluorine, which also is liberated as HF and a C₄₃₉ thiolate gets formed. This prevents radical formation at C₄₃₉, instead a 2' substrate radical is formed. In the normal catalysis the C₄₃₉ hydrogen would be donated to the 3' radical of the ribose ring under reformation of the initial thiyl radical (step 5 in Figure 4).⁹⁶ A later model proposed covalent bond formation between substrate and protein under reductive conditions as a consequence off the above described mechanism.⁹⁷ Biochemical studies could in part support this model, namely the liberation of two fluorines per gemcitabine bound and covalent bond formation between enzyme and ribose ring. Although the initial model was modified and other, new pathways have been suggested.^{98–100} It also has been shown that gemcitabine affects the quaternary structure of the RNR enzyme by the formation of a tight $\alpha_6\beta_6$ complex, explaining complete RNR inhibition even at substoichiometric amounts.¹⁰¹ However, a recent study challenges even the radical transfer from 3' to 2' position of gemcitabines ribose ring in the above mentioned mechanism, which leads to a new set of open questions regarding its mechanism of RNR inhibition.¹⁰² Like clofarabine also gemcitabine has more than one way of anticancer action, its incorporation into DNA strands stops DNA synthesis, furthermore gemcitabine is able to induce apoptosis through caspase signaling.¹⁰³ It is noteworthy that besides clofarabine and gemcitabine there is a variety of other nucleoside analogues that are used in the clinic or are in clinical development.^{50,86,104}

(iii) Inhibitors of the RNRs small subunit are either radical scavengers destroying its tyrosyl radical, or iron chelators which prevent iron loading of the apo- β_2 protein. The three best studied R2 inhibitors are hydroxyurea (HU), **14**, deferoxamine (DFO), **15** and the thiosemicarbazone Triapine, **16**.

Hydroxyurea¹⁰⁵ is an orally active anticancer compound, which is used against a variety of cancers, such as certain blood malignancies, ovary, cervical, head and neck carcinoma, as well as melanoma and meningioma.¹⁰⁶ The exact mechanism of R2 inhibition is not clear yet. However, it was shown that HU is able to react with the di-iron center of mouse RNR.¹⁰⁷ Another proposed mechanism implies the formation of nitroxyl radicals after *in vivo* metabolism of HU, which act as scavengers for the free tyrosyl radical in R2.¹⁰⁸ The use of hydroxyurea against cancer is controversial because of its low *in vivo* RNR selectivity, which makes the administration of large doses necessary. The high applied doses lead to severe side effects, such as gastrointestinal symptoms and bone marrow depression, leading to leucopenia and anemia, which is dose-limiting.¹⁰⁶ Also a possible HU induced transformation of myeloproliferative neoplasms into leukemia is under debate.¹⁰⁹ Hydroxyurea is also applied in sickle-cell anemia¹¹⁰ and AIDS.¹¹¹

Deferoxamine (DFO) is a potent iron chelator, which is standardly used in the treatment of iron overload disease.¹¹² However, it also shows potential against cancer.^{113,114} DFO is not acting as radical scavenger, rather it depletes intracellular iron pools and prevents thereby iron loading of the apo- β_2 enzyme.^{115,116}

1.5 Thiosemicarbazones

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antibacterial, antifungal, antimalarial, antiviral and anticancer.^{119–124} α -Pyridyl thiosemicarbazones (HCTs) are especially active against cancer. The anticancer activity of HCTs was discovered in the 1950s, when 2-formylpyridine TSC, **17** was able to significantly increase the life-span of leukemia bearing mice.¹²⁵ The best studied HCT is Triapine, **16**,^{126,127} which has already been evaluated in several clinical phase I and II studies.^{128–136} It can be stated that triapine is widely ineffective against solid tumors, but shows promising activity against hematologic malignancies such as leukemia. This was supported by a recent phase II study including patients bearing myeloproliferative neoplasms, revealing an encouraging complete remission rate of 24% in a heavily pretreated patient population.¹³⁷ Until recently the accepted mechanism was that an iron(II)bis(triapine) complex gets formed within the cell, which is able to generate reactive oxygen species (ROS) when oxidized to the corresponding iron(III) complex. The ROS, in turn, destroy the tyrosyl radical in the R2 subunit of the RNR enzyme (see chapter 1.4).^{138,139} It has been shown that the $\text{Fe}^{\text{III/II}}$ redox potential in TSC complexes is accessible under physiological conditions, which allows the endogenous formation of the active iron(II) species from the respective iron(III) complex.^{140,141} However, recently it was shown that the tyrosyl radical can be quenched without involvement of ROS, suggesting that the radical might be reduced directly by the iron(II)bis(triapine) complex.¹⁴²

Besides RNR, TSCs are also inhibitors of the enzyme Topoisomerase II α (Topo II α), which controls the DNA topology during cell division. The DNA double helix has to be unwound for transcription and replication processes to occur, which is a problem since unwinding at a certain place of the DNA would result in over-winding at another place. Topo II α solves this issue by introducing transient double strand breaks within DNA double strands.^{143,144} The enzyme ATP binding site has been suggested as the possible target for TSCs.¹⁴⁵ It was shown that square-planar copper(II)-TSC complexes are often much more potent Topo II α inhibitors compared to the free ligands.¹⁴⁶ Nuclear labeling experiments have shown that copper(II) complexes are Topo II α inhibitors *in vivo*.¹⁴⁷

1.5.1 Structure activity relationships

A N,N,S donor set is crucial for the anticancer activity of formylpyridine based thiosemicarbazones.¹⁴⁸ This has been shown already more than 50 years ago when 2-formylpyridine TSC, **17** was found to be active in a mice model while its isomers 3- and 4-formylpyridine (**18** and **19**) were lacking activity.¹²⁵

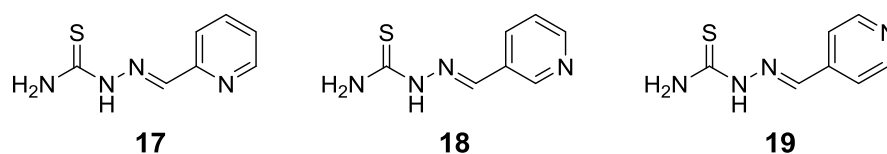


Chart 7: **17**, 2-formylpyridine TSC; **18**, 3-formylpyridine TSC; **19**, 4-formylpyridine TSC.

The nature of the chalcogen donor atom has also significant impact on the antiproliferative activity of TSCs. The substitution of sulfur by oxygen leads to a distinct drop in cytotoxicity, while the substitution by selenium has no marked impact, or leads to a slightly higher activity.¹⁴⁹

Another important factor for the development of new anticancer agents is their lipophilicity. Very hydrophilic compounds often show a strongly diminished anticancer activity, because they have difficulties in passing the double lipid layer of cell membranes, which widely prevents target interaction. Finding the right balance between lipophilicity and hydrophilicity is of utmost importance in the design of new TSCs. Dimethylation of the terminal nitrogen, for example, leads to a higher lipophilicity and consequently to an enhancement in cytotoxicity.¹⁵⁰ However, low hydrophilicity combined with high general toxicity and low aqueous solubility is a general problem of TSCs. The attachment of hydrophilic groups on the TSC scaffold allows studies in aqueous solution which are of great importance in gaining a deeper insight in the mode of action of these compounds.¹⁵¹

Reaction with different metals has also a distinct effect on the cytotoxicity of TSCs. Copper(II), for example, is capable of enhancing the Topo II α inhibiting effect of certain TSCs as mentioned before.¹⁴⁶ However, reaction with copper(II) has also an effect on TSCs that do not target Topo II α . For example it has been shown that the presence of copper(II) ions diminishes the *in vitro* anticancer activity of Triapine, **16**, while the activity of its dimethylated derivative Dp44mT, **20**¹⁵² increased under the same conditions. 2-Formylpyridine, **17** and its dimethylated counterpart, **21** behaved similarly, the activity of the dimethylated compound was enhanced in the presence of copper(II) while that of 2-formylpyridine TSC was decreased.¹⁵³

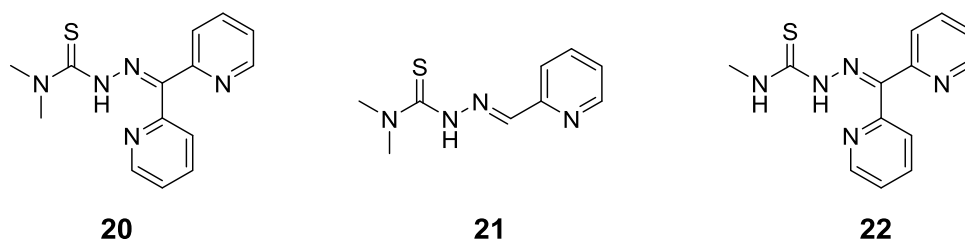


Chart 8: **20**, Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; **21**, 2-formylpyridine 4,4-dimethyl-3-thiosemicarbazone; **22**, Dp4mT, di-2-pyridylketone 4-methyl-3-thiosemicarbazone.

This observation might be explainable by a possible copper catalyzed desulfurization reaction that requires at least one proton at the terminal nitrogen of the TSC scaffold,^{149,154} although this reaction has not been observed in a later study with Triapine, which was performed in a DMSO/water mixture.¹⁵⁵ Dp44mT is about two orders of magnitude more cytotoxic than Triapine (IC_{50} = 0.0027 vs. 0.29 μ M, respectively in a HL60 leukemia cell line), while its monomethylated analogue Dp4mT, **22** shows a similar activity to Triapine ($\approx$ 0.3 μ M).^{153,156} There exist contradictory reports about Dp44mT being a Topo II α inhibitor.^{157,158} However, the above mentioned results suggest that in the case of Dp44mT (and possibly other terminal dimethylated TSCs) not the iron(II) but the copper(II) species is the active one *in vivo*, especially when taking into account the high stability constant of the copper(II) complex, together with its high cytotoxicity.¹⁵⁹

Taken together one can conclude that thiosemicarbazones and their metal complexes are a hot topic in the field of anticancer research. Many questions regarding their SARs remain to be answered, for example, (i) which is the right metal for the respective ligand? (ii) What are the structural elements making a TSC a strong Topo II α or RNR inhibitor, or an inhibitor of both enzymes? (iii) Where is the perfect balance between lipophilicity and hydrophilicity. Studies in aqueous solution are required to answer these questions and the results reported herein be regarded as a solid basis for the development of novel thiosemicarbazones with clinical potential.

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2 Results

This Ph.D. thesis is based on the following papers and manuscripts, which are presented in the original format or as the submitted manuscripts:

Effects of Terminal Dimethylation and Metal Coordination of Proline- 2-formylpyridine Thiosemicarbazone Hybrids on Lipophilicity, Antiproliferative Activity, and hR2 RNR Inhibition

Bacher, F.; Dömötör, O.; Kaltenbrunner, M.; Mojović, M.; Popović-Bijelić, A.; Gräslund, A.; Ozarovski, A.; Filipović, L.; Radulović, S.; Enyedy, É. A.; Arion, V. B.
Inorg. Chem. **2014**, 53, 12595–12609.

Strong Effect of Copper(II) Coordination on Antiproliferative Activity of Thiosemicarbazone-Piperazine and Thiosemicarbazone-Morpholine Hybrids

Bacher, F.; Dömötör, O.; Chugunova, A.; Nagy, N. V.; Filipović, L.; Radulović, S.; Enyedy, É. A.; Arion, V. B.
Dalton Trans., accepted.

Ruthenium Compounds as Antitumor Agents: New Developments

Bacher, F.; Arion, V. B.
Reference Module in Chemistry, Molecular Sciences and Chemical Engineering, Elsevier: **2014**.

2.1 Effects of Terminal Dimethylation and Metal Coordination of Proline- 2-formylpyridine Thiosemicarbazone Hybrids on Lipophilicity, Antiproliferative Activity, and hR2 RNR Inhibition

Bacher, F.; Dömötör, O.; Kaltenbrunner, M.; Mojović, M.; Popović-Bijelić, A.; Gräslund, A.; Ozarovski, A.; Filipović, L.; Radulović, S.; Enyedy, É. A.; Arion, V. B.
Inorg. Chem. **2014**, *53*, 12595–12609.

Effects of Terminal Dimethylation and Metal Coordination of Proline-2-formylpyridine Thiosemicarbazone Hybrids on Lipophilicity, Antiproliferative Activity, and hR2 RNR Inhibition

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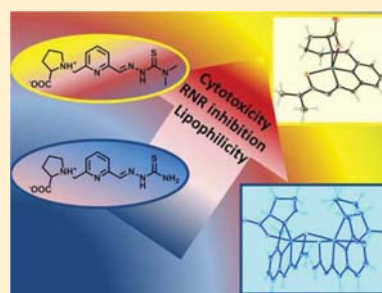
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Supporting Information

ABSTRACT: The nickel(II), copper(II), and zinc(II) complexes of the proline-thiosemicarbazone hybrids 3-methyl-(S)-pyrrolidine-2-carboxylate-2-formylpyridine thiosemicarbazone (L-Pro-FTSC or (S)-H₂L¹) and 3-methyl-(R)-pyrrolidine-2-carboxylate-2-formylpyridine thiosemicarbazone (D-Pro-FTSC or (R)-H₂L¹), as well as 3-methyl-(S)-pyrrolidine-2-carboxylate-2-formylpyridine 4,4-dimethyl-thiosemicarbazone (dm-L-Pro-FTSC or (S)-H₂L²), namely, [Ni(L-Pro-FTSC-2H)]₂ (1), [Ni(D-Pro-FTSC-2H)]₂ (2), [Ni(dm-L-Pro-FTSC-2H)]₂ (3), [Cu(dm-L-Pro-FTSC-2H)] (6), [Zn(L-Pro-FTSC-2H)] (7), and [Zn(D-Pro-FTSC-2H)] (8), in addition to two previously reported, [Cu(L-Pro-FTSC-2H)] (4), [Cu(D-Pro-FTSC-2H)] (5), were synthesized and characterized by elemental analysis, one- and two-dimensional ¹H and ¹³C NMR spectroscopy, circular dichroism, UV-vis, and electrospray ionization mass spectrometry. Compounds 1–3, 6, and 7 were also studied by single-crystal X-ray diffraction. Magnetic properties and solid-state high-field electron paramagnetic resonance spectra of 2 over the range of 50–420 GHz were investigated. The complex formation processes of L-Pro-FTSC with nickel(II) and zinc(II) were studied in aqueous solution due to the excellent water solubility of the complexes via pH potentiometry, UV-vis, and ¹H NMR spectroscopy. The results of the antiproliferative activity *in vitro* showed that dimethylation improves the cytotoxicity and hR2 RNR inhibition. Therefore, introduction of more lipophilic groups into thiosemicarbazone-proline backbone becomes an option for the synthesis of more efficient cytotoxic agents of this family of compounds.



INTRODUCTION

Thiosemicarbazones (TSCs) are known as versatile ligands for various metal ions.¹ Especially their first row transition metal coordination chemistry is well-developed.² A specific feature of TSCs and their metal complexes is their broad spectrum of biological properties including antiviral, antibacterial, antimalarial, antifungal, and anticancer activity.^{3–6} α -N-Heterocyclic TSCs (HCTs) have been known for their anticancer activity since the 1950s when 2-formylpyridine thiosemicarbazone was discovered to possess *in vivo* antileukemic activity in a mice model.⁷ The best-studied HCT to date is 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP), also referred to as Triapine, which has already been examined in several clinical phase I and II trials.^{8–10} Triapine was found to be safe and

effective against hematologic malignancies, for example, leukemia.^{11,12} A recent clinical phase II study including 37 patients with aggressive myeloproliferative neoplasms is of particular note since a response rate of 49% and complete remission in 24% of all patients has been documented.¹³ However, other clinical phase II studies showed that Triapine is ineffective against a variety of solid tumors including pancreatic, adeno-, lung, and renal carcinoma.^{14–17} In the 1970s it was discovered that HCTs are inhibitors of the enzyme ribonucleotide reductase (RNR),^{18,19} which catalyzes the rate-determining step of DNA synthesis, namely, the reduction of

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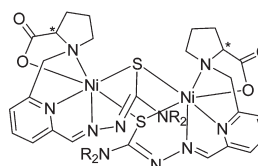
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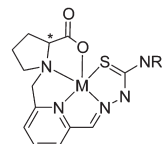
We reported recently on the first proline-TSC hybrids (L- and D-Pro-FTSC) and their copper(II) complexes.⁴⁹ These

The compounds were characterized by analytical and spectroscopic methods, magnetic susceptibility, electron paramagnetic resonance (EPR) measurements (2) and X-ray diffraction (1–3, 6, and 7). Solution equilibria of the nickel(II) and zinc(II) complexes formed with L-Pro-FTSC were studied in detail by the combination of various methods such as pH-

R = H, * = S, L-Pro-FTSC ((S)-H₂L¹)
 R = H, * = R, D-Pro-FTSC ((R)-H₂L¹)
 R = CH₃, * = S, dm-L-Pro-FTSC ((S)-H₂L²)



- $$\begin{aligned} \text{R} = \text{H}, * = \text{S}, [\text{Ni}(\text{L-Pro-FTSC-2H})]_2 & \quad (1) \\ \text{R} = \text{H}, * = \text{R}, [\text{Ni}(\text{D-Pro-FTSC-2H})]_2 & \quad (2) \\ \text{R} = \text{CH}_3, * = \text{S}, [\text{Ni}(\text{dm-L-Pro-FTSC-2H})]_2 & \quad (3) \end{aligned}$$



- M = Cu, R = H, * = S, [Cu(L-Pro-FTSC-2H)] (4)
M = Cu, R = H, * = R, [Cu(D-Pro-FTSC-2H)] (5)
M = Cu, R = CH₃, * = S, [Cu(dm-L-Pro-FTSC-2H)] (6)
M = Zn, R = H, * = S, [Zn(L-Pro-FTSC-2H)] (7)
M = Zn, R = H, * = R, [Zn(D-Pro-FTSC-2H)] (8)

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potentiometry, UV–vis, and ^1H NMR spectroscopy to determine the stoichiometry and the thermodynamic stability of complexes formed in aqueous solution. Complexation of these bivalent metal ions is compared to that of copper(II). Finally, the antiproliferative activity and hR2 RNR inhibiting activity of new compounds was assayed and discussed.

EXPERIMENTAL SECTION

Chemicals. 2,6-Dihydroxymethylpyridine and L-proline methyl ester hydrochloride were purchased from Alfa Aesar, while D-proline methyl ester hydrochloride was purchased from Acros Organics. Solvents were dried using standard procedures if needed.⁵⁵ 2-Hydroxymethyl-6-chloromethylpyridine and 6-chloromethylpyridine-2-carboxaldehyde were synthesized according to published procedures.⁵⁶ KOH and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were Sigma-Aldrich products, and HCl, KCl, NiCl_2 , and ZnCl_2 were Reanal products. Nickel(II) and zinc(II) stock solutions were prepared by dissolving the appropriate amount of the metal chlorides in known amount of HCl, and their concentrations were determined by complexometry via the ethylenediaminetetraacetic acid (EDTA) complexes. Accurate strong acid content of the metal stock solutions were calculated on the basis of pH-potentiometric titrations.

Synthesis of Ligands. D- and L-Pro-FTSC were synthesized as described elsewhere.⁴⁹

dm-L-Pro-FTSC- H_2O -0.2EtOH. (S)-1-[(6-Formylpyridin-2-yl)methyl]pyrrolidine-2-carboxylic acid (0.30 g, 1.28 mmol) and 4,4-dimethyl-3-thiosemicarbazide (0.15 g, 1.28 mmol) were dissolved in dry ethanol (6 mL), and the reaction mixture was stirred at room temperature for 24 h. The white precipitate formed was filtered off under inert atmosphere, washed with dry ethanol, and dried in vacuo. Yield: 0.17 g, 39%. Anal. Calcd for $\text{C}_{15}\text{H}_{21}\text{N}_5\text{O}_2\text{S}\cdot\text{H}_2\text{O}\cdot 0.2\text{C}_2\text{H}_5\text{OH}$ ($M = 362.65$ g/mol): C, 51.00; H, 6.73; N, 19.31; S, 8.84. Found: C, 50.84; H, 6.56; N, 19.01; S, 8.68%. E-isomer: ^1H NMR (500 MHz, deuterated dimethyl sulfoxide ($\text{DMSO}-d_6$)) δ 11.20 (s, 1H, H^a), 8.21 (s, 1H, H^{13}), 7.88–7.77 (m, 2H, H^5 , H^6), 7.45 (d, $J = 7.0$ Hz, 1H, H^4), 4.16 (d, $J = 14.2$ Hz, 1H, H^7), 3.90 (d, $J = 14.2$ Hz, 1H, H^7), 3.49 (dd, $J = 8.9$, 5.3 Hz, 1H, H^8), 3.13–3.06 (m, 1H, H^{11}), 2.66–2.58 (m, 1H, H^{11}), 2.19–2.05 (m, 1H, H^9), 1.96–1.67 (m, 3H, H^9 , H^{10}). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 181.06 (Cq, C^{14}), 173.76 (Cq, C^{12}), 157.59 (Cq, C^3), 153.46 (Cq, C^1), 144.17 (CH, C^{13}), 137.81 (CH, C^5), 123.55 (CH, C^4), 118.64 (CH, C^6), 66.23 (CH, C^8), 59.13 (CH₂, C^7), 53.54 (CH₂, C^{11}), 42.76 (2CH₃, C^{15} , C^{16}), 29.28 (CH₂, C^9), 23.71 (CH₂, C^{10}). Z-isomer: ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 14.75 (s, 1H, H^2), 8.05 (t, $J = 7.8$ Hz, 1H, H^5), 7.62 (m, 2H, H^4 , H^6), 7.58 (s, 1H, H^{13}), 4.10 (d, $J = 14.1$ Hz, 1H, H^7), 3.79 (d, $J = 14.0$ Hz, 1H, H^7), 3.51–3.42 (m, 1H, H^8), 2.96–2.90 (m, 1H, H^{11}), 2.56–2.43 (m, 1H, H^{11} , overlapped with residual DMSO signal), 2.19–2.05 (m, 1H, H^9), 1.96–1.67 (m, 3H, H^9 , H^{10}). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 180.61 (Cq, C^{14}), 174.92 (Cq, C^{12}), 158.33 (Cp, C_3), 151.60 (Cq, C^1), 139.26 (CH, C^5), 136.65 (CH, C^{13}), 124.96 (CH, C^6), 124.03 (CH, C^4), 65.29 (CH, C^8), 59.41 (CH₂, C^7), 53.32 (CH₂, C^{11}), 40.61 (2CH₃, C^{15} , C^{16} , overlapped with residual DMSO signal), 29.33 (CH₂, C^9), 23.43 (CH₂, C^{10}). For atom numbering and structures of E and Z isomers see Supporting Information, Scheme S2. Solubility in water ≥ 413 mg/mL. Electrospray ionization mass spectrometry (ESI-MS) (methanol), positive: m/z 336 ($[\text{M} + \text{H}]^+$). IR (attenuated total reflectance (ATR), selected bands, ν_{max}): 3156, 2963, 1643, 1579, 1547, 1517, 1445, 1346, 1266, 1080, 831, 683, 592 cm^{-1} .

Synthesis of Metal Complexes. $[\text{Ni}(\text{L-Pro-FTSC}-2\text{H})]_2\cdot 2\text{H}_2\text{O}\cdot \text{MeOH}$ (1-2H₂O-MeOH). To a solution of L-Pro-FTSC (0.10 g, 0.33 mmol) in water (15 mL) was added a solution of nickel(II) acetate tetrahydrate (0.16 g, 0.66 mmol) in water (5 mL). The mixture was heated at 343 K for 1 h and then stirred at room temperature overnight. The resulting brown solution of the raw product in water was subjected to preparative high-performance liquid chromatography (HPLC) (MeOH/ H_2O). Slow diffusion of diethyl ether into a methanolic solution of the purified product gave brown crystals, which were filtered off, washed with a diethyl ether/methanol mixture (5:1), and dried in vacuo. Yield: 0.07 g, 54%. Anal. Calcd for

$\text{C}_{26}\text{H}_{30}\text{Ni}_2\text{N}_{10}\text{O}_4\text{S}_2\cdot 2\text{H}_2\text{O}\cdot \text{CH}_3\text{OH}$ ($M = 796.17$ g/mol): C, 40.73; H, 4.81; N, 17.59; S, 8.05. Found: C, 40.49; H, 4.51; N, 17.35; S, 7.77%. Solubility in water ≥ 10.3 mg/mL. ESI-MS (methanol), positive: m/z 364 ($[(1/2 \text{M} + \text{H})^+]$), 386 ($[(1/2 \text{M} + \text{Na})^+]$). IR (ATR, selected bands, ν_{max}): 3294, 1588, 1459, 1162, 785, 668, 605 cm^{-1} .

$[\text{Ni}(\text{D-Pro-FTSC}-2\text{H})]_2\cdot 3.7\text{H}_2\text{O}\cdot \text{MeOH}$ (2-3.7H₂O-MeOH). To a solution of D-Pro-FTSC (0.17 g, 0.55 mmol) in water (30 mL) was added a solution of nickel(II) acetate tetrahydrate (0.28 g, 1.11 mmol) in water (10 mL). The mixture was heated at 343 K for 1 h and then stirred at room temperature overnight. The resulting brown solution of the raw product in water was subjected to preparative HPLC (MeOH/ H_2O). Slow diffusion of diethyl ether into a methanolic solution of the purified product gave brown crystals, which were filtered off, washed with a diethyl ether/methanol mixture (5:1), and dried in vacuo. Yield: 0.13 g, 65%. Anal. Calcd for $\text{C}_{26}\text{H}_{30}\text{Ni}_2\text{N}_{10}\text{O}_4\text{S}_2\cdot 3.7\text{H}_2\text{O}\cdot \text{CH}_3\text{OH}$ ($M = 826.78$ g/mol): C, 39.22; H, 5.04; N, 16.94; S, 7.75. Found: C, 39.00; H, 4.65; N, 17.08; S, 7.37%. Solubility in water ≥ 10.3 mg/mL. ESI-MS (methanol), positive: m/z 364 ($[(1/2 \text{M} + \text{H})^+]$), 386 ($[(1/2 \text{M} + \text{Na})^+]$). IR (ATR, selected bands, ν_{max}): 3294, 1588, 1459, 1162, 785, 668, 605 cm^{-1} .

$[\text{Ni}(\text{dm-L-Pro-FTSC}-2\text{H})]_2\cdot 0.2\text{H}_2\text{O}\cdot 3.6\text{MeOH}$ (3-0.2H₂O-3.6MeOH). (S)-1-[(6-Formylpyridin-2-yl)methyl]pyrrolidine-2-carboxylic acid (0.19 g, 0.81 mmol), 4,4-dimethyl-3-thiosemicarbazide (0.10 g, 0.81 mmol), and nickel(II) acetate tetrahydrate (0.21 g, 0.86 mmol) were dissolved in dry ethanol (12 mL) in a 25 mL Schlenk tube. The mixture was stirred at 343 K overnight. The next day a gray precipitate of $[\text{Ni}(4,4\text{-dimethyl-3-thiosemicarbazide})_2]$ was filtered off. The filtrate was subjected to preparative HPLC (MeOH/ H_2O). Slow diffusion of diethyl ether into a methanolic solution of the purified product gave dark brown crystals, which were filtered off, washed with a diethyl ether/methanol mixture (5:1), and dried in vacuo. Yield: 0.09 g, 27%. Anal. Calcd for $\text{C}_{30}\text{H}_{38}\text{Ni}_2\text{N}_{10}\text{O}_4\text{S}_2\cdot 0.2\text{H}_2\text{O}\cdot 3.6\text{CH}_3\text{OH}$ ($M = 903.16$ g/mol): C, 44.69; H, 5.89; N, 15.51; S, 7.10. Found: C, 44, 35; H, 5.65; N, 15.85; S, 6.79%. Solubility in water ≥ 15.3 mg/mL. ESI-MS (methanol), positive: m/z 392 ($[(1/2 \text{M} + \text{H})^+]$). IR (ATR, selected bands, ν_{max}): 3384, 2922, 1593, 1507, 1362, 1253, 1129, 909, 805, 677 cm^{-1} .

Complexes $[\text{Cu}(\text{L-Pro-FTSC}-2\text{H})]$ (4) and $[\text{Cu}(\text{D-Pro-FTSC}-2\text{H})]$ (5) were prepared by following a recently published protocol.⁴⁹

$[\text{Cu}(\text{dm-L-Pro-FTSC}-2\text{H})]\cdot \text{H}_2\text{O}\cdot 0.2\text{MeOH}$ (6- $\text{H}_2\text{O}\cdot 0.2\text{MeOH}$). (S)-1-[(6-Formylpyridin-2-yl)methyl]pyrrolidine-2-carboxylic acid (0.30 g, 1.28 mmol), 4,4-dimethyl-3-thiosemicarbazide (0.15 g, 1.28 mmol), and copper(II) acetate monohydrate (0.27 g, 1.36 mmol) were dissolved in dry ethanol (10 mL) in a 50 mL Schlenk tube. The mixture was stirred at 343 K overnight. The resulting green solution of the raw product in water was subjected to preparative HPLC (MeOH/ H_2O). Slow diffusion of diethyl ether into a methanolic solution of the purified product gave green crystals, which were filtered off, washed with a diethyl ether/methanol mixture (5:1), and dried in vacuo. Yield: 0.19 g, 38%. Anal. Calcd for $\text{C}_{15}\text{H}_{19}\text{CuN}_5\text{O}_2\text{S}\cdot \text{H}_2\text{O}\cdot 0.2\text{CH}_3\text{OH}$ ($M = 421.38$ g/mol): C, 43.32; H, 5.21; N, 16.62; S, 7.60. Found: C, 43.46; H, 5.00; N, 16.46; S, 7.54. Solubility in water ≥ 8.5 mg/mL. ESI-MS (methanol), positive: m/z 795 ($[2\text{M} + \text{H}]^+$), 817 ($[2\text{M} + \text{Na}]^+$). IR (ATR, selected bands, ν_{max}): 3542, 3152, 2923, 1596, 1503, 1361, 1247, 1131, 910, 792, 625 cm^{-1} .

$[\text{Zn}(\text{L-Pro-FTSC}-2\text{H})]\cdot 1.9\text{H}_2\text{O}$ (7-1.9H₂O). To a solution of L-Pro-FTSC (0.12 g, 0.39 mmol) in water (15 mL) was added a solution of zinc(II) acetate dihydrate (0.17 g, 0.78 mmol) in water (5 mL). The mixture was heated at 343 K for 1 h. After it cooled, the solution was concentrated under reduced pressure to ~ 5 mL and allowed to stand at 277 K overnight. Yellow, needlelike crystals were filtered off, washed with cold water, and dried in vacuo. Yield: 0.13 g, 65%. Anal. Calcd for $\text{C}_{13}\text{H}_{15}\text{N}_5\text{O}_2\text{SZn}\cdot 1.9\text{H}_2\text{O}$ ($M = 404.98$ g/mol): C, 38.56; H, 4.68; N, 17.29; S, 7.92%. Found: C, 38.75; H, 4.29; N, 16.98; S, 8.04%. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 8.22 (s, 1H, H^{12}), 8.08 (t, $J = 7.7$ Hz, 1H, H^4), 7.62 (d, $J = 7.5$ Hz, 1H, H^5), 7.57 (s, 2H, H^{11}), 7.42 (d, $J = 7.6$ Hz, 1H, H^3), 4.34 (d, $J = 16.8$ Hz, 1H, H^9), 4.08 (d, $J = 16.9$ Hz, 1H, H^9), 3.50 (m, 1H, H^{10}), 3.25–3.12 (m, 1H, H^7), 2.99–2.86 (m, 1H, H^{10}), 2.32 (m, 1H, H^8), 1.95 (m, 1H, H^9), 1.79 (m, 2H, H^8 , H^9) ppm. ^{13}C NMR (125 MHz, $\text{DMSO}-d_6$) δ 183.96 (Cq, C^{13}), 177.11

Table 1. Crystal Data and Details of Data Collection for 1·3.25CH₃OH, 2·4CH₃OH, 3·2.88CH₃OH, 6·H₂O, and 7·CH₃OH

	1·3.25CH ₃ OH	2·4CH ₃ OH	3·2.88CH ₃ OH	6·H ₂ O	7·CH ₃ OH
empirical formula	C _{29.25} H _{43.5} N ₁₀ Ni ₂ O _{7.75} S ₂	C ₃₀ H ₄₆ N ₁₀ Ni ₂ O ₈ S ₂	C _{32.88} H _{49.5} N ₁₀ Ni ₂ O _{6.88} S ₂	C ₁₅ H ₂₁ CuN ₅ O ₃ S	C ₁₄ H ₁₉ N ₅ O ₃ SZn
Fw	840.78	856.31	677.09	414.97	725.05
space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁
A [Å]	13.1049(7)	13.2489(4)	11.7885(13)	6.6987(7)	8.2454(4)
B [Å]	15.1240(13)	15.2821(5)	11.8487(13)	13.3713(18)	11.8012(6)
C [Å]	18.0808(16)	17.9458(7)	55.473(6)	18.755(2)	16.8293(7)
α [deg]					
β [deg]					93.338(3)
γ [deg]					
V [Å ³]	3583.6(5)	3633.5(2)	7748.4(15)	1679.9(4)	1634.81(13)
Z	4	4	8	4	4
λ [Å]	0.710 73	0.710 73	0.710 73	0.710 73	0.710 73
ρ _{calc} [g cm ⁻³]	1.558	1.565	1.502	1.641	1.636
crystal size, mm	0.10 × 0.02 × 0.01	0.22 × 0.18 × 0.11	0.37 × 0.28 × 0.05	0.46 × 0.25 × 0.23	0.07 × 0.07 × 0.04
T [K]	120(2)	100(2)	100(2)	100(2)	120(2)
μ [mm ⁻¹]	1.230	1.215	1.139	1.451	1.654
R ₁ ^a	0.0603	0.0316	0.0405	0.0189	0.0408
wR ₂ ^b	0.1373	0.0661	0.1081	0.0522	0.0647
GOF ^c	1.010	1.034	1.092	1.010	1.019
Flack parameter	−0.04(2)	0.001(7)	0.03(1)	−0.009(6)	0.008(9)

^aR₁ = Σ||F_o| − |F_c||/Σ|F_o|. ^bwR₂ = {Σ[w(F_o² − F_c²)²]/Σ[w(F_o²)²]}^{1/2}. ^cGOF = {Σ[w(F_o² − F_c²)²]/(n − p)}^{1/2}, where n is the number of reflections, and p is the total number of parameters refined.

(Cq, C¹¹), 156.16 (Cq, C¹), 149.78 (Cq, C²), 143.04 (CH, C⁴), 135.15 (CH, C¹²), 123.05 (CH, C³), 123.01 (CH, C⁵), 72.48 (CH, C⁷), 60.82 (CH₂, C⁶), 58.57 (CH₂, C¹⁰), 31.38 (CH₂, C⁸), 24.98 (CH₂, C⁹) ppm. Solubility in water ≥ 1.4 mg/mL. ESI-MS (methanol), positive: m/z 370 ([M + H]⁺), 392 ([M + Na]⁺). IR (ATR, selected bands, ν_{max}): 3287, 1596, 1454, 1155, 1076, 1009, 904, 781, 656, 605 cm⁻¹.

[Zn(o-Pro-FTSC-2H)]·2.6H₂O (8·2.6H₂O). To a solution of o-Pro-FTSC (0.15 g, 0.49 mmol) in water (20 mL) was added a solution of zinc(II) acetate dihydrate (0.22 g, 0.98 mmol) in water (5 mL). The mixture was heated at 343 K for 1 h. After it cooled, the solution was concentrated under reduced pressure to ~5 mL and allowed to stand at 277 K overnight. Yellow, needlelike crystals were filtered off, washed with cold water, and dried in vacuo. Yield: 0.06 g, 33%. Anal. Calcd for C₁₃H₁₅N₅O₂SZn·2.6H₂O (M = 417.59 g/mol): C, 37.39; H, 4.88; N, 16.77; S, 7.68. Found: C, 37.64; H, 4.87; N, 16.41; S, 7.30%. ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.22 (s, 1H, H¹²), 8.08 (t, J = 7.7 Hz, 1H, H⁴), 7.62 (d, J = 7.5 Hz, 1H, H⁵), 7.57 (s, 2H, H³), 7.42 (d, J = 7.6 Hz, 1H, H³), 4.34 (d, J = 16.8 Hz, 1H, H⁶), 4.08 (d, J = 16.9 Hz, 1H, H⁶), 3.50 (m, 1H, H¹⁰), 3.25–3.12 (m, 1H, H⁷), 2.99–2.86 (m, 1H, H¹⁰), 2.32 (m, 1H, H⁸), 1.95 (m, 1H, H⁹), 1.79 (m, 2H, H⁸, H⁹) ppm. ¹³C NMR (125 MHz, DMSO-*d*₆) δ 183.96 (Cq, C¹³), 177.11 (Cq, C¹¹), 156.16 (Cq, C¹), 149.78 (Cq, C²), 143.04 (CH, C⁴), 135.15 (CH, C¹²), 123.05 (CH, C³), 123.01 (CH, C⁵), 72.48 (CH, C⁷), 60.82 (CH₂, C⁶), 58.57 (CH₂, C¹⁰), 31.38 (CH₂, C⁸), 24.98 (CH₂, C⁹) ppm. Solubility in water ≥ 1.4 mg/mL. ESI-MS (methanol), positive: m/z 370 ([M + H]⁺), 392 ([M + Na]⁺). IR (ATR, selected bands, ν_{max}): 3287, 1596, 1454, 1155, 1076, 1009, 904, 781, 656, 605 cm⁻¹.

pH-Potentiometric Measurements. The exact concentration of the stock solutions of the L-Pro-FTSC was determined from pH-potentiometric titrations by using the computer program HYPERQUAD.⁵⁷ The pH-metric measurements for the determination of the proton dissociation constants of the L-Pro-FTSC and the overall stability constants of the metal complexes were carried out at 298.0 ± 0.1 K in water and at an ionic strength of 0.10 M KCl to keep the activity coefficients constant. The titrations were performed with carbonate-free KOH solution of known concentration (0.10 M). The concentrations of the base and the HCl 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 buret were used for the titrations. The electrode system was calibrated to the pH = −log[H⁺] scale according to the method

suggested by Irving et al.⁵⁸ The average water ionization constant pK_w is 13.76 ± 0.01, which corresponds well to the literature data.⁵⁹ The reproducibility of the titration points included in the calculations was within 0.005 pH. The pH-metric titrations were performed in the pH range of 2.0–11.5. The initial volume of the samples was 5.0 mL. The ligand concentration was 2 mM, and metal ion-to-ligand ratios of 1:1–1:4 were used. The accepted fitting of the titration curves was always less than 0.01 mL. Samples were deoxygenated by bubbling purified argon through them for approximately 10 min prior to the measurements. Argon was also passed over the solutions during the titrations.

The protonation constants of the L-Pro-FTSC compounds were determined with the computer program HYPERQUAD.⁵⁷ PSE-QUAD⁶⁰ was utilized to establish the stoichiometry of the complexes and to calculate the stability constants (logβ(M_nL_qH_r)). β(M_nL_qH_r) is defined for the general equilibrium pM + qL + rH ⇌ M_nL_qH_r as β(M_nL_qH_r) = [M_nL_qH_r]/[M]ⁿ[L]^q[H]^r, where M denotes the metal ion, and L denotes the completely deprotonated ligand. In all calculations exclusively titration data were used from experiments in which no precipitate was visible in the reaction mixture.

UV-vis Spectrophotometric, Circular Dichroism, and ¹H NMR Measurements. A Hewlett-Packard 8452A diode array spectrophotometer was used to record the UV-vis spectra in the 200 to 800 nm window. The path length was 0.5 or 1.0 cm. The spectrophotometric titrations were performed on samples of the L-Pro-FTSC alone or with nickel(II) ions; the concentration of the ligand was ~100 μM, and the metal-to-ligand ratios were 1:1 and 1:2 over the pH range between 2 and 11.5 at an ionic strength of 0.10 M (KCl) in water at 298.0 ± 0.1 K. Measurements for the nickel(II)-L-Pro-FTSC systems at metal-to-ligand ratio 1:1 were also carried out at various concentrations (1 μM–0.8 mM) and at pH 7.4 (20 mM HEPES buffer).

One-dimensional ¹H and ¹³C NMR and two-dimensional ¹H–¹H COSY, ¹H–¹H TOCSY, ¹H–¹H TOCSY, ¹H–¹H ROESY or ¹H–¹H NOESY, ¹H–¹³C HSQC, and ¹H–¹³C HMQC NMR spectra were recorded on two Bruker Avance III instruments. DMSO-*d*₆ or CDCl₃ were used as solvent. ¹H or ¹³C chemical shifts were measured relative to the residual solvent peaks. The pH-dependent ¹H NMR studies were carried out on a Bruker Ultrashield 500 Plus instrument. 4,4-Dimethyl-4-silapentane-1-sulfonic acid was used as an internal NMR standard, and the WATERGATE method was used to suppress the

solvent resonance. L-Pro-FTSC was dissolved in a 10% (v/v) D₂O/H₂O mixture in a concentration of 1 mM, the zinc(II)-to-ligand ratios were 1:1 and 1:2, and the nickel(II)-to-ligand ratio was 1:1 at 298 K. PSEQUAD⁶⁰ was used to calculate the pK value of the complex [ZnLH]⁺. ¹H NMR spectra were recorded for the zinc(II)-L-Pro-FTSC (1:1) system at 1 mM concentration in the presence of 630 μM human serum albumin (HSA) at pH 7.40 (20 mM HEPES buffer, 0.1 M KCl) or in minimum Eagle's essential medium (MEM) at 298 K after 24 h incubation.

Circular dichroism (CD) spectra for the nickel(II) and zinc(II) complexes of L-Pro-FTSC and D-Pro-FTSC were recorded on a Jasco J-815 spectrometer in the wavelength interval from 230 to 600 nm. Samples contained 0.500 mM nickel(II) or 0.025 mM zinc(II) complex at pH 7.4 (20 mM HEPES buffer, 0.1 M KCl) using optical cells of 0.2 or 0.5 cm path lengths, respectively. CD data are given as the differences in molar absorptivities between left and right circularly polarized light, based on the concentration of the ligand ($\Delta\epsilon = \Delta A/l \cdot c_{\text{ligand or complex}}$).

Determination of the Distribution Coefficient D . $D_{7.4}$ values of the zinc(II) and nickel(II) complexes of L- and D-Pro-FTSC, as well as of dm-L-Pro-FTSC (3, 6–8) were determined by the traditional shake-flask method in *n*-octanol/buffered aqueous solution at pH 7.4 (20 mM HEPES buffer) at 298.0 ± 0.2 K, as described previously,⁴⁹ and compared to those of metal-free hybrids. Two parallel experiments were performed for each sample. The complexes were dissolved in 0.1 mM in the *n*-octanol presaturated aqueous solution of the buffer at constant ionic strength (0.10 M KCl). The aqueous solutions and *n*-octanol with 1:1 phase ratio were gently mixed with 360° vertical rotation for 3 h to avoid the emulsion formation, and the mixtures were centrifuged at 5000 rpm for 3 min by a temperature-controlled centrifuge (Sanyo) at 298 K. After separation UV-vis spectra of the complexes in the aqueous phase were compared to those of the original aqueous solutions. $D_{7.4}$ values of the metal complexes were calculated as the mean of [Absorbance (original solution)/Absorbance (aqueous phase after separation) – 1] obtained at the λ_{max} values (360 nm for the zinc(II), 375 or 380 nm for the nickel(II), and 396 nm for the copper(II) complexes). When no measurable amount of the complexes was found in the *n*-octanol phase, $D_{7.4}$ values for those compounds were merely estimated.

Crystallographic Structure Determination. X-ray diffraction measurements were performed on Bruker X8 APEXII CCD and Bruker D8 VENTURE diffractometers. Single crystals were positioned at 40, 35, 100, 35, and 40 mm from the detector, and 561, 772, 2147, 2797, and 815 frames were measured, each for 100, 4, 24, 4, and 30 s over 1, 0.4, 0.4, 0.4, and 1° scan width for 1:3.25CH₃OH, 2:4CH₃OH, 3:2.88CH₃OH, 6:H₂O, and 7:CH₃OH, correspondingly. The data were processed using SAINT software.⁶¹ Crystal data, data collection parameters, and structure refinement details are given in Table 1. The structures were solved by direct methods and refined by full-matrix least-squares techniques. Non-H atoms were refined with anisotropic displacement parameters. H atoms were inserted in calculated positions and refined with a riding model. Co-crystallized methanol in 3:2.88CH₃OH was found in part disordered. The disorder was solved by using SADI instructions implemented in SHELXL-97, while the atoms involved were refined with isotropic displacement parameters, and the positions of H atoms were not calculated. One methanol molecule from two crystallographically independent in the structure of 7:CH₃OH was found disordered over two positions with s.o.f. 0.6:0.4. The following computer programs were used: structure solution, SHELXS-97; refinement, SHELXL-97;⁶² molecular diagrams, ORTEP.⁶³

Magnetic Measurements and EPR Spectroscopy. Magnetic susceptibility data (2–300 K) were collected on powdered samples using a SQUID magnetometer (Quantum Design MPMS-XL) at 0.1 T. The temperature dependence of the magnetic susceptibility for dinuclear nickel(II) cluster 2 was analyzed according to the Hamiltonian (eq 1),⁶⁴

$$\hat{H} = -2J\hat{S}_1\hat{S}_2 + \mu_B\mathbf{B}\{g_1\}\hat{S}_1 + \hat{S}_1\{D_1\}\hat{S}_1 + \mu_B\mathbf{B}\{g_2\}\hat{S}_2 + \hat{S}_2\{D_2\}\hat{S}_2 + \hat{S}_1\{D_{12}\}\hat{S}_2 \quad (1)$$

in which $\{g_1\}$ and $\{g_2\}$ represent the local *g* matrices, $\{D_1\}$ and $\{D_2\}$ are the local single-ion zero-field splitting (zfs) tensors, and the $\{D_{12}\}$ term contains the magnetic dipolar interactions along with the anisotropic exchange interactions between the metal ions. The more commonly used zfs parameters *D* and *E* are related to the tensor components by eq 2.

$$D = (2D_{zz} - D_{xx} - D_{yy})/2, E = (D_{xx} - D_{yy})/2 \quad (2)$$

These formulas are valid for each of the $\{D\}$ quantities in eq 1.

To obtain a manageable model it was assumed that the two Ni ions are equivalent, which is not strictly correct. Different orientation of the $\{D_1\}$ and $\{D_2\}$ tensors was taken into account, as the *Z* axes of the two $\{D\}$ tensors are tilted by 26 deg from each other according to the DFT calculations (see the DFT section below), but the corresponding components of $\{D_1\}$ and $\{D_2\}$ were assumed equal. These $\{D\}$ tensor components were not fitted but were calculated from the *D* and *E* values in the *S* = 2 state found from EPR (below). The spin Hamiltonian matrix was diagonalized to find the energy levels and the magnetic susceptibility per mole of dimer was calculated from eq 3.

$$\chi_d = -\frac{N \sum_i \frac{\partial E_i}{\partial B} \exp(-E_i/kT)}{B \sum_i \exp(-E_i/kT)} \quad (3)$$

The derivatives $\partial E_i/\partial B$ were evaluated numerically by calculating the energy levels slightly below and slightly above (± 5 G) the operational magnetic field of a SQUID magnetometer (1000 G in our case).

The terms $\{D_1\}$ and $\{D_2\}$ represent the usual zfs of the ³A₂ ground state of Ni(II).⁶⁵

This and similar models have been widely used to characterize magnetic interaction in different dinuclear nickel(II) complexes.^{65–67} We also allowed an interdimer exchange term (*z'*) in the molecular field approximation⁶⁸ according to eq 4.

$$\chi' = \frac{\chi}{1 - \left(\frac{2z'}{N g^2 \mu_B^2} \right) \chi} \quad (4)$$

The X-band (9.5 GHz) EPR spectra were recorded at 20 K on a Bruker Elexsys-II EPR spectrometer with an Oxford Instruments ESR900 helium cryostat under the following conditions: microwave power 3.2 mW, modulation amplitude 5 G, modulation frequency 100 kHz, conversion time 29.3 ms. The concentration of the tyrosyl radical was determined by double integration of EPR spectra recorded at nonsaturating microwave power levels and compared with the copper standard (1 mM CuSO₄ in 10 mM EDTA). The calculated radical concentration was normalized and expressed in percent of the control sample. High-field, high-frequency EPR spectra at temperatures ranging from ca. 3 to 10 K were recorded on a home-built spectrometer at the EMR facility of the NHMFL.⁶⁹ The instrument is a transmission-type device in which microwaves are propagated in cylindrical lightpipes. The microwaves were generated by a phase-locked Virginia Diodes source generating frequency of 13 ± 1 GHz, which was multiplied by a cascade of frequency doublers and/or triplers. A superconducting magnet (Oxford Instruments) capable of reaching a field of 17 T was employed.

We simulated EPR spectra of 2 using the “giant spin” approach, in which the spectra of the coupled states (*S* = 1 and *S* = 2) were analyzed separately in terms of the coupled-spin Hamiltonian (eq 5),

$$\hat{H}_S = \mu_B\mathbf{B}\cdot\{g_S\}\hat{S} + D[\hat{S}_x^2 - S(S+1)/3] + E(\hat{S}_x^2 - \hat{S}_y^2) \quad (5)$$

in which each of the spin states has its own zfs parameters *D*, *E*, and *g* matrices, which can be related to the single-ion parameters^{64,70} corresponding to the spin Hamiltonian (1).

Cell Lines and Culture Conditions. Human cervical carcinoma (HeLa), human melanoma (FemX), human alveolar basal adenocarci-

noma (A549), human breast cancer (MDA-MB-453) cell lines, and normal human fetal lung fibroblast cell line (MRC-5) were maintained as monolayer culture in the Roswell Park Memorial Institute (RPMI) 1640 nutrient medium (Sigma Chemicals Co, USA). RPMI 1640 nutrient medium was prepared in sterile deionized water, supplemented with penicillin (192 U/mL), streptomycin (200 mg/mL), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (25 mM), L-glutamine (3 mM), and 10% of heat-inactivated fetal calf serum (FCS) (pH 7.2). The cells were grown at 310 K in 5% CO₂ in a humidified air atmosphere.

MTT Assay. Antiproliferative activity of the investigated compounds was determined using 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay.⁷¹ Cells were seeded into 96-well cell culture plates (Thermo Scientific Nunc) at a cell density of 3000 c/w (HeLa), 6000 c/w (A549), 4000 c/w (MDA-MB-453), and 5000 c/w (FemX, MRC-5) in 100 μ L of culture medium. After 24 h of growth, cells were exposed to the serial dilutions of the tested complexes. The investigated compounds were dissolved in DMSO at a concentration of 30 mM as stock solution, and prior to use they were diluted with nutrient medium to the desired final concentrations (in range from 18.75 to 300 μ M). Each concentration was tested in triplicate. Serial dilutions were made in culture medium so that the final concentration of DMSO per well was less than 1% (v/v) in all experiments. After incubation periods of 48 h, a 20 μ L aliquot of MTT solution (5 mg/mL in phosphate buffer solution, pH 7.2) was added to each well. Samples were incubated for 4 h at 310 K, with 5% CO₂ in a humidified atmosphere. Formazan crystals were dissolved in 100 μ L of 10% sodium dodecyl sulfate (SDS). Absorbances were recorded after 24 h, on an enzyme-linked immunosorbent assay (ELISA) reader (ThermoLabsystems Multiskan EX 200–240 V), at the wavelength of 570 nm. IC₅₀ values (μ M), were determined from the cell survival diagrams. The percentages of surviving cells relative to untreated controls were determined. The IC₅₀ value, defined as the concentration of the compound causing 50% cell growth inhibition, was estimated from the dose–response curves.

Ribonucleotide Reductase Inhibition. Sample Preparation for EPR Measurements. The tyrosyl radical reduction kinetics in human R2 RNR protein (hR2) by L-Pro-FTSC and dm-L-Pro-FTSC, as well as complexes 4, 6, and 7, was monitored by EPR spectroscopy. Purified recombinant hR2 was obtained from the Department of Medical Biochemistry and Biophysics, Karolinska Institute, Sweden. The iron-reconstituted R2 protein contained two iron ions and 0.38 tyrosyl radicals per polypeptide. Samples containing 20 μ M hR2 in Tris buffer, pH 7.60/100 mM KCl/5% glycerol, 20 μ M compound (1% (w/w) DMSO/H₂O), and 2 mM dithiothreitol (DTT) (only for experiments done in the presence of the reductant) were incubated at room temperature for designated times and quickly frozen in cold isopentane. The same sample was used for repeated incubations and was refrozen before each EPR measurement. The intrinsic decay of the tyrosyl radical, obtained from the control sample containing 20 μ M hR2 in Tris buffer, pH 7.60/100 mM KCl/5% glycerol, was subtracted at each time point.

RESULTS AND DISCUSSION

Synthesis and Characterization of dm-L-Pro-FTSC. dm-L-Pro-FTSC was synthesized in six steps as shown in Supporting Information, Scheme S1. The first five steps to the key aldehyde **F** were described in detail previously.⁴⁹ Aldehyde **F** was then condensed in a Schiff-base reaction with 4,4-dimethyl-3-thiosemicarbazide in dry ethanol at room temperature. One-dimensional ¹H and ¹³C NMR and two-dimensional ¹H–¹H COSY, ¹H–¹H TOCSY, ¹H–¹H NOESY, ¹H–¹³C HSQC, and ¹H–¹³C HMBC NMR spectra were in agreement with the expected structure, enabling the assignment of all ¹H and ¹³C resonances (for atom numbering see Scheme S2 in the Supporting Information). dm-L-Pro-FTSC exists as a mixture of *E* and *Z* isomers in DMSO with an *E/Z* ratio of 1:0.39. The proton at the hydrazinic nitrogen N², in the *Z*

configuration, is involved in a hydrogen bond to the pyridine nitrogen leading to a strong downfield shift of its resonance signal from 11.20 to 14.75 ppm. The solvent-dependent formation of *E* and *Z* isomers is well-documented in the literature, and our data are in good agreement with those reported for other α -pyridyl-TSCs.⁷² The purity of the compound was further confirmed by elemental analysis. The ESI mass spectra recorded in a positive ion mode showed a strong peak at *m/z* 336 due to the [M + H]⁺ ion.

Synthesis and Characterization of the Metal(II) Complexes. The zinc(II) and nickel(II) complexes of D- and L-Pro-FTSC were prepared similarly to the previously reported copper(II) complexes, by reaction of the metal(II) acetate hydrates with the ligand in aqueous solution. The nickel(II) and copper(II) complexes of dm-L-Pro-FTSC were prepared *in situ* by reaction of the aldehyde **F** with 4,4-dimethyl-3-thiosemicarbazide in the presence of the corresponding metal(II) acetate in dry ethanol. The structure of all metal(II) complexes (except [Zn(D-Pro-FTSC–2H)]) in the solid state was confirmed by single-crystal X-ray diffraction. ESI-MS spectra of **1** and **2** recorded in a positive ion mode showed peaks at *m/z* 364 and 386 attributed to [Ni(L-Pro-FTSC–2H) + H]⁺ or [Ni(D-Pro-FTSC–2H) + H]⁺ and to [Ni(L-Pro-FTSC–2H) + Na]⁺ or [Ni(D-Pro-FTSC–2H) + Na]⁺, respectively, while that of **3** at *m/z* 392 was due to [Ni(dm-L-Pro-FTSC–2H) + H]⁺. Strong signals at *m/z* 795 and 817 in the mass spectrum of **6** were assigned to [[Cu(dm-L-Pro-FTSC–2H)]₂ + H]⁺ and [[Cu(dm-L-Pro-FTSC–2H)]₂ + Na]⁺, respectively, while those at *m/z* 370 and 392 in mass spectra of **7** and **8** were assigned to [M + H]⁺ and [M + Na]⁺ ions, respectively. The purity of the compounds was confirmed by elemental analyses. CD spectra recorded for the aqueous solutions of nickel(II) and zinc(II) complexes of L-Pro-FTSC and D-Pro-FTSC (**1**, **2** and **7**, **8**) at physiological pH reveal that all of them are optically active and show Cotton effects (see Figures S1 and S2 in the Supporting Information). As expected, they are mirror images over the 230–350 nm region of the CD spectra, while their UV–vis spectra are identical.

X-ray Crystallography. The results of X-ray diffraction studies of **1**–**3**, **6**, and **7** are shown in Figure S3 (see Supporting Information) and Figures 1–4. The complexes **1**–**3** and **6** crystallized in the noncentrosymmetric orthorhombic space group *P*2₁2₁2₁, while **7** crystallized in the noncentrosymmetric monoclinic space group *P*2₁. The proline-thiosemicarbazone hybrids L-Pro-FTSC, D-Pro-FTSC, and/or dm-L-Pro-FTSC in **1**–**3**, **6**, and **7** act as pentadentate doubly deprotonated ligands binding to nickel(II), copper(II), or zinc(II) via pyridine nitrogen atom, imine nitrogen, thiolato S atom, tertiary proline nitrogen, and proline carboxylato oxygen atom. While in copper(II) and zinc(II) complexes **6** and **7** (Figures 3 and 4) the coordination number (CN) of the central metal ion is 5, and the coordination polyhedron can be described as a square-pyramid ($\tau = 0.09$ and 0.07, respectively),⁷³ the CN of the nickel(II) in compounds **1**–**3** is increased to six by coordination of an adjacent Ni(L-Pro-FTSC–2H), Ni(D-Pro-FTSC–2H), or Ni(dm-L-Pro-FTSC–2H) complex via thiolato S atom, which acts as a bridging ligand between two nickel(II) ions associating the two monomeric complexes in a dimer (Supporting Information, Figure S3 and Figures 1 and 2). One precedence of a related thiolato-bridged dimer with a central Ni(μ -S)₂Ni core, in which each nickel(II) atom is surrounded by O, N, S donor atoms of a tridentate doubly deprotonated 5-nitrobenzaldehyde 4N-

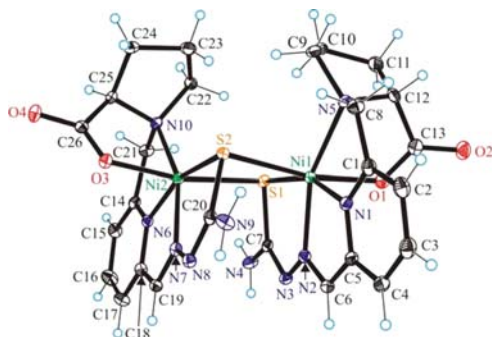


Figure 1. ORTEP view of **2** with thermal displacement ellipsoids drawn at the 50% probability level. Selected bond distances (Å) and bond angles (deg): Ni1–N1 1.9944(17), Ni1–N2 2.0515(17), Ni1–N5 2.2247(18), Ni1–S1 2.3946(5), Ni1–S2 2.4995(5), Ni1–O1 2.0766(15), N2–N3 1.363(2), C7–S1 1.762(2); N1–Ni1–N2 78.71(7), N1–Ni1–N5 78.08(7), N5–Ni1–O1 81.40(6), N1–Ni1–O1 87.41(6), N2–Ni1–O1 88.37(6), S1–Ni1–O1 96.00(4), S1–Ni1–S2 86.845(18), Ni1–S1–Ni2 92.065(18), Ni1–S2–Ni2 92.648(18), Ni2–N6 1.9894(17), Ni2–N7 2.0496(17), Ni2–N10 2.2107(16), Ni2–S2 2.4042(5), Ni2–S1 2.5321(6), Ni2–O3 2.0417(14), N7–N8 1.367(2), C20–S2 1.770(2); N6–Ni2–N7 78.83(7), N6–Ni2–N10 79.09(7), N10–Ni2–O3 81.55(6), N6–Ni2–O3 89.72(6), N7–Ni2–O3 89.52(6), S2–Ni2–O3 93.26(4), S1–Ni2–S2 85.905(18).

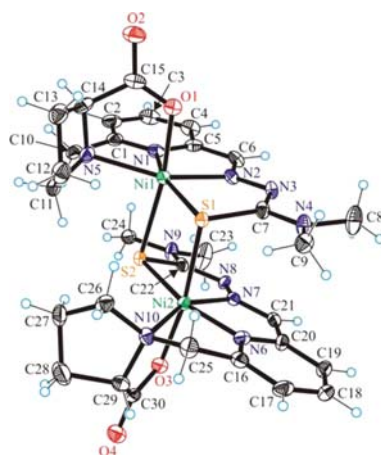


Figure 2. ORTEP view of **3** with thermal displacement ellipsoids drawn at the 50% probability level. Selected bond distances (Å) and bond angles (deg): Ni1–N1 1.975(3), Ni1–N2 2.027(3), Ni1–N5 2.199(4), Ni1–S1 2.3896(11), Ni1–S2 2.5239(11), Ni1–O1 2.059(3), N2–N3 1.359(5), C7–S1 1.766(4); N1–Ni1–N2 79.10(14), N1–Ni1–N5 79.17(14), N5–Ni1–O1 80.98(13), N1–Ni1–O1 89.41(13), N2–Ni1–O1 89.49(13), S1–Ni1–O1 95.82(9), S1–Ni1–S2 86.12(4), Ni1–S1–Ni2 93.22(4), Ni1–S2–Ni2 93.86(4), Ni2–N6 1.989(3), Ni2–N7 2.038(3), Ni2–N10 2.198(3), Ni2–S2 2.3758(11), Ni2–S1 2.5360(11), Ni2–O3 2.032(3), N7–N8 1.355(5), C22–S2 1.767(4); N6–Ni2–N7 79.08(14), N6–Ni2–N10 79.01(14), N10–Ni2–O3 81.18(12), N6–Ni2–O3 91.80(13), N7–Ni2–O3 89.03(12), S2–Ni2–O3 90.76(9), S1–Ni2–S2 86.14(4).

methylthiosemicarbazone (H_2L) and two nitrogen atoms of a bipyridine ligand, characterized by X-ray diffraction, has been

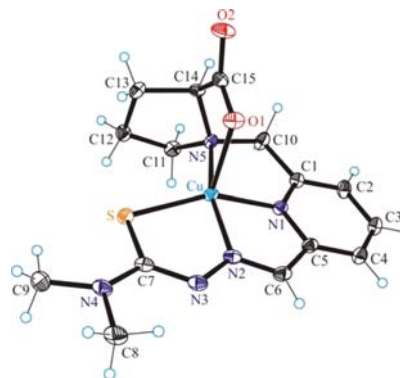


Figure 3. ORTEP view of **6** with thermal displacement ellipsoids drawn at the 50% probability level. Selected bond distances (Å) and bond angles (deg): Cu–N1 1.9335(11), Cu–N2 1.9887(11), Cu–S 2.2668(4), Cu–N5 2.1343(11), Cu–O1 2.1655(10), N2–N3 1.3511(15), C7–S 1.7636(13); N1–Cu–N2 80.36(5), N1–Cu–N5 81.75(4), N5–Cu–O1 79.14(4), N1–Cu–O1 94.63(4), N2–Cu–O1 106.20(4), S–Cu–O1 106.69(3).

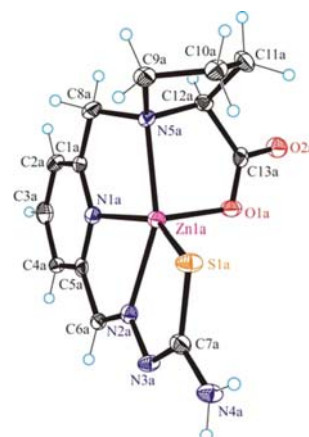


Figure 4. ORTEP view of **7** with thermal displacement ellipsoids drawn at the 50% probability level. Selected bond distances (Å) and bond angles (deg): Zn1a–N1a 2.066(3), Zn1a–N2a 2.140(3), Zn1a–S1a 2.2913(9), Zn1a–N5a 2.231(3), Zn1a–O1a 1.989(2), N2a–N3a 1.354(4), C7a–S1a 1.748(3); N1a–Zn1a–N2a 75.00(12), N1a–Zn1a–N5a 77.48(11), N5a–Zn1a–O1a 82.97(10), N1a–Zn1a–O1a 99.77(11), N2a–Zn1a–O1 105.14(10), S1a–Zn1a–O1a 107.26(8).

found in CSD database.⁷⁴ Upon coordination of *L*-proline moiety to metal ion via the nitrogen atom, the latter, in addition to C12 (or C14 in **3** and **6**), becomes a chiral center. In contrast to the literature reports,⁷⁵ where in most cases the nitrogen atom adopts the same configuration as the asymmetric proline carbon ($S_C S_N$ or $R_C R_N$), in complexes **1–3**, **6**, and **7** the nitrogen and the asymmetric carbon of the proline moiety adopt opposite configurations by coordination to metal ($S_C R_N$ or $R_C S_N$). Documented opposite configurations that resulted from coordination to metal ion or protonation of the proline nitrogen atom are rare.⁷⁶ Of note is the formation of four five-membered chelate cycles upon coordination of the ligand to

metal ion. Three of these are essentially planar, while the fourth prolinic moiety adopts a half-chair conformation.

The complexes are involved in intermolecular hydrogen bonding interactions. In particular, the nitrogen atom N4 of one terminal amino group in the dimer **2** acts as a proton donor in hydrogen bonds to oxygen atoms O6 and O7 of the two cocrystallized methanol molecules (see Supporting Information, Figure S4), while the nitrogen atom N9 of the second terminal amino group also forms two hydrogen bonds, one to carboxylate oxygen atom O4ⁱ and the second to O8ⁱ of the third cocrystallized methanol of the adjacent complex. The hydrazinic nitrogen N3, and carboxylate oxygens O1, O2, and O4 are proton acceptors in strong hydrogen bonds with O8ⁱⁱ, O5ⁱⁱ, O6ⁱⁱ, and O7ⁱ of the neighboring methanol molecules. (Atoms marked with (i) were generated via symmetry transformation $x - 0.5, -y + 0.5, -z + 1$, while those marked with (ii) were generated via symmetry transformation $-x + 0.5, -y + 1, z - 0.5$.)

Dimethylation of the terminal amino group reduces strongly the involvement of complexes in intermolecular hydrogen bonding interactions, as can be seen in the crystal structures of **3** and **6**. Detail of H-bonding in **3** is not specified because of severe disorder of cocrystallized methanol molecules in the crystal. The cocrystallized water molecule in **6** acts as a proton donor to carboxylate oxygen atom O2ⁱ and thiolato atom Sⁱⁱ forming two strong hydrogen bonds (Supporting Information, Figure S5). Atoms marked with (i) were generated via symmetry transformation $x + 1, y, z$, while those marked with (ii) were generated via symmetry transformation $-x + 0.5, -y + 1, z + 0.5$.

The terminal amino group of both crystallographically independent molecules A and B of the complex [Zn(l-Pro-FTSC)] (**7**) are involved as proton donors in hydrogen bonding to N3b (molecule B) and O2aⁱ (molecule A) and to N3a and O3b, respectively (Supporting Information, Figure S6). The carboxylate oxygen atoms O2a and O2b act as proton acceptors in hydrogen bonds with O3aⁱⁱ and O3bⁱⁱ, which play a role of proton donors. Atoms marked with (i), (ii), and (iii) were generated via symmetry transformations $x + 1, y, z, -x, y + 0.5, -z + 1$ and $-x + 1, y + 0.5, -z + 1$, respectively.

Magnetic Properties. The magnetic properties of **2** were investigated in the temperature range of 2–300 K. At 300 K the $\chi_M T$ product (at 0.1 T) is $2.43 \text{ cm}^3 \text{ K mol}^{-1}$, and it increases when the temperature is lowered indicating the presence of ferromagnetic exchange interactions in the Ni₂ dimer (Figure 5). The room-temperature magnitude of $\chi_M T$ imposes the upper limit of 2.2 on the g value. The $\chi_M T$ product increases

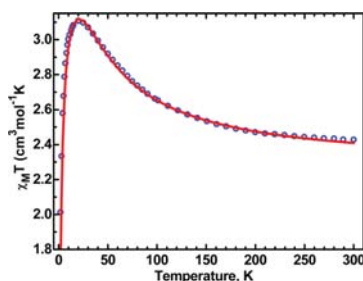


Figure 5. Plots of $\chi_M T$ and χ_M vs T for **2**. The red trace corresponds to the best fit with model parameters indicated in the text.

continuously to reach a maximum of $3.16 \text{ cm}^3 \text{ K mol}^{-1}$ at $\sim 20 \text{ K}$ (Figure 5), and subsequently drops to $2.01 \text{ cm}^3 \text{ K mol}^{-1}$ at 2 K as a result of zfs or/and intermolecular interactions.

The fitting procedure led to $J = +10.5(3) \text{ cm}^{-1}$, $g = 2.13(2)$, $D_{\text{zfs}} = +7.8 \text{ cm}^{-1}$ (fixed), $E = 2.2 \text{ cm}^{-1}$ (fixed) $z' = -0.29 \text{ cm}^{-1}$ in reasonable agreement with the experimental data ($R = 6 \times 10^{-4}$). When the restrictions on D and E are released, much better fit is possible even without the z' term, but the resulting D and E are in disagreement with the EPR data.

DFT Calculations. “Broken-symmetry”⁷⁷ (BS) density functional theory (DFT) calculations were performed using the software package ORCA⁷⁸ to get more insight into the exchange interactions. The X-ray coordinates were used in the calculations. The TZVPP function base was used for nickel and all coordinated atoms, while VDZ functions were used for other atoms.⁷⁹ The B3LYP functional was employed.⁸⁰ In the BS procedure a self-consistent field (SCF) calculation is first performed on a molecule in the high-spin state (HS), which in our case is an $S = 2$ state. Next, a (BS) state is set up in which two unpaired electrons on one nickel(II) are spin-up and two electrons on another nickel(II) are spin-down, and a second SCF calculation is performed. The exchange integral is then evaluated using $J = -(E_{\text{HS}} - E_{\text{BS}})/(\langle S^2 \rangle_{\text{HS}} - \langle S^2 \rangle_{\text{BS}})$. $J = 15 \text{ cm}^{-1}$ was calculated, in a reasonable agreement with the result of the magnetic data fitting.

Ferromagnetic interactions in the present dimer are reflected in weak overlap between the magnetic orbitals of the two nickel ions. The overlap integrals calculated from DFT for two pairs of the magnetic orbitals (Figure 6) are 0.0065 and 0.0002,

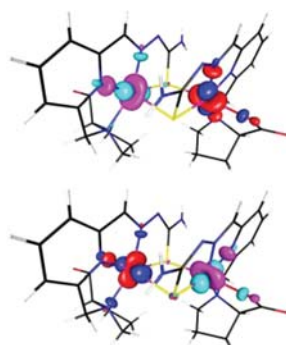


Figure 6. Two pairs of the magnetic orbitals of Ni(II)-derived from the broken symmetry DFT calculations. Small overlap integrals (0.0065 and 0.0002 for the upper and lower pair, respectively) favor the ferromagnetic exchange.

which favors the ferromagnetic exchange. For comparison, in an antiferromagnetic dinuclear Ni(II) complex with $J = -19 \text{ cm}^{-1}$ (converted to the notation used in this paper), the overlap integrals of 0.042 and 0.075 were found in an analogous calculation.⁸¹

EPR Spectra. Well-resolved high-field spectra were observed at frequencies over the range of 50–420 GHz at 3 K —the lowest temperature that can be reached on our EPR instrument (Figure 7). At this temperature the spectra are expected to exhibit mainly transitions within the ground quintet state ($S = 2$). No resonances due to the excited $S = 1$ state were observed at higher temperatures as the spectra quality very quickly deteriorated with the temperature. The highest-field

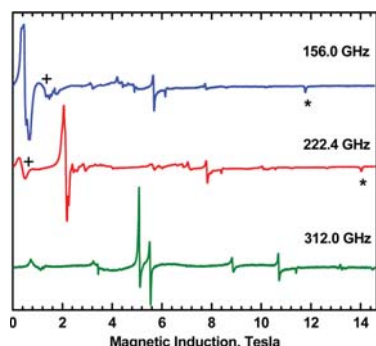


Figure 7. EPR spectra of **2** recorded at 3 K with the microwave frequencies as indicated. The resonance marked with * was identified as the $|{-2}\rangle \leftrightarrow |{-1}\rangle$ transition and was used to determine $D = +2.11 \text{ cm}^{-1}$ and $g_z = 2.10$ within the giant spin formalism. That resonance would be frozen out at 3 K if D were negative. The line marked with + also appears to be a $|{-2}\rangle \leftrightarrow |{-1}\rangle$ transition. When the frequency is lowered below 222 GHz, that transition moves to a higher field showing that the M_S levels involved are split in zero magnetic field by slightly more than 7.41 cm^{-1} (corresponding to the 222.4 GHz quantum energy). The E parameter of 0.52 cm^{-1} could be estimated.

resonance in the 156 and 222 GHz spectra in Figure 7, which moves beyond the available magnetic field at higher frequencies, is the “Z” resonance in the $S = 2$ state.

The giant spin method requires that the coupled spin states are well separated from each other, which is not fulfilled in the present case where the exchange interactions are comparable to the zfs causing their mixing. Simulation of the powder spectra was thus not successful, but the observation of the high-field Z resonance over a wide range of frequencies (Supporting Information, Figure S7) allowed determination of the D parameter in the $S = 2$ state of $+2.11 \text{ cm}^{-1}$ and $g_z = 2.10$. If the zfs tensors of two ions were collinear, the D found above would imply that D on single ions is $+6.33 \text{ cm}^{-1}$, neglecting the D_{12} term in eq 1.⁶⁴ However, there are complicating factors. The two nickel ions in our system are nonequivalent; thus, their g matrices and the zfs tensors must be different. The molecular structure indicates that neither the g tensors of two ions nor their zfs tensors can be coaxial. In the EPR spectra of the dinuclear systems, the coupled g is observed, and the zfs in the coupled states is affected by the zfs on the separate ions and by the anisotropic metal–metal interactions. The zfs of the coupled states depends not only on the $\{D_1\}$, $\{D_2\}$ and $\{D_{12}\}$ component magnitudes but also on their orientation. This information is not available experimentally. Although DFT is not expected to provide correct magnitudes of the zfs parameters, calculations were performed to gain insight into the orientation of the $\{D_1\}$ and $\{D_2\}$ tensors and g matrices of eq 1. The calculations, again performed by using ORCA, indicate that the z axis of the $\{D_1\}$ tensor forms an angle of 26° with the $\{D_2\}$ tensor z axis. With this arrangement it was found that $D = +7.8 \text{ cm}^{-1}$ and $E = +2.2 \text{ cm}^{-1}$ on each Ni ion are required to calculate the resonances marked with * and + in Figure 8 at right positions, assuming that the Ni ions are equivalent and neglecting the $\{D_{12}\}$ term in eq 1. It does not seem that we can extract more from our EPR data because of the lack of symmetry in the molecule. An additional aspect is that in the absence of the inversion center the antisymmetric exchange

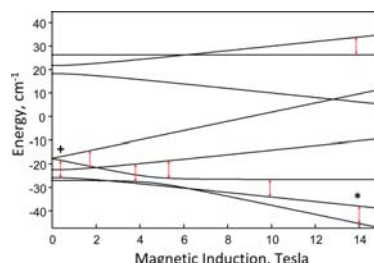


Figure 8. Energy levels calculated using the spin Hamiltonian (4) with $D_1 = D_2 = 7.8 \text{ cm}^{-1}$, $E_1 = E_2 = 2.2 \text{ cm}^{-1}$, $J = 11 \text{ cm}^{-1}$, and $g_z = 2.10$. The magnetic field is along the Z axis of the coupled system, that is, along the bisector of the Z_1 and Z_2 axes of the Ni(II) ions. Transitions expected at 222.4 GHz are shown as vertical red lines. * and + mark the same transitions as in Figure 7

(Dzialoshinskii–Moriya interaction)⁸² cannot be ruled out. High-field EPR studies on more symmetric systems were considerably more successful.⁷⁰

Solution Chemistry. Lipophilicity. The hydro-lipophilic character of dm-L-Pro-FTSC, its nickel(II) and copper(II) complexes **3** and **6**, as well as of both zinc(II) complexes of L-Pro-FTSC and D-Pro-FTSC (**7** and **8**) was studied at pH 7.4 via the partitioning between *n*-octanol and water. The data are summarized in Supporting Information, Table S1. The enantiomers of the zinc(II) complexes of Pro-FTSC have similar $\log D_{7.4}$ values (L: $-1.41(9)$; D: $-1.56(5)$) indicating rather hydrophilic character. However, they are slightly more lipophilic than the ligands alone ($\log D_{7.4} < -1.7$).⁴⁹

The nickel(II) complexes with L-Pro-FTSC and D-Pro-FTSC (**1** and **2**) were found to be very hydrophilic, and practically no metal complex could be detected in the organic phase after partitioning. Therefore, only a threshold limit could be estimated for their distribution coefficients ($\log D_{7.4} < -1.7$) as in case of the ligands alone.⁴⁹ The dimethyl derivatives as expected were still hydrophilic, but appreciably more lipophilic than nonmethylated compounds.

Proton Dissociation Processes of the Ligand L-Pro-FTSC. pK_a values of L-Pro-FTSC (see Chart 1) were determined in aqueous solution by the combined approach of pH-potentiometric, UV–vis, and ^1H NMR titrations in our previous work⁴⁹, and data obtained here were found to be identical with them. Although this organic compound consists of four dissociable protons (COOH , $\text{N}_{\text{Pro}}\text{H}^+$, $\text{N}_{\text{pyridine}}\text{H}^+$, and $\text{N}_{\text{hydrazinic}}\text{H}$) only three pK_a values can be determined in the studied pH range. pK_1 (1.86) most probably belongs to the deprotonation of the COOH moiety (partly overlapped with the deprotonation of $\text{N}_{\text{pyridine}}\text{H}^+$) and pK_2 (8.78) and pK_3 (11.08) to $\text{N}_{\text{Pro}}\text{H}^+$ and the hydrazinic-NH functional groups, respectively. It is noteworthy that the ligand is mainly present in its neutral form at pH 7.4 adopting a zwitterionic structure, which results in excellent water-solubility and a fairly hydrophilic character.⁴⁹

Complex Formation of L-Pro-FTSC with Zinc(II) and Nickel(II) in Aqueous Solution. Complex equilibria in water were investigated by pH-potentiometry in all cases, and stoichiometries and cumulative stability constants of the metal complexes furnishing the best fits to the experimental data are listed in Table 2. The recorded titration curves indicate that L-Pro-FTSC is an efficient metal-ion chelator in a wide pH range for nickel(II) and zinc(II) ions. Representative titration

Table 2. Cumulative ($\log \beta(M_p L_q H_r)$) and Derived Stability Constants of the Nickel(II)-, Copper(II)-, and Zinc(II)-L-Pro-FTSC Complexes^a [$T = 298$ K and $I = 0.10$ M (KCl)]

	$\log \beta$ [MLH] ⁺	$\log \beta$ [ML]	$\log \beta$ [MLH ₁] [−]	pK [MLH] ⁺	pM^b
nickel(II)	20.73(2)	15.78(3)	5.04(4)	4.95	11.7
copper(II) ^c	24.03	21.64	9.59	2.39	17.5
zinc(II)	18.28(3)	13.31(2)		4.97 ^d	9.2

^aThe numbers in parentheses are standard deviations of the quoted $\log \beta$ values determined by pH-potentiometry. Proton dissociation constants of the ligand: $pK_1 = 1.86$, $pK_2 = 8.78$, and $pK_3 = 11.08$ taken from ref 49. ^b $pM = -\log[M]$ at pH 7.40; $c_L/c_M = 10$; $c_M = 0.001$ mM. ^cData are taken from ref 49. ^d $pK = 4.98(1)$ calculated from the ¹H NMR δ values of the CH=N protons of the bound ligand.

curves (Figure 9) reveal that complex formation processes start at lowest pH in the case of nickel(II), while curves registered in the presence of zinc(II) are superimposed with that of the free ligand up to pH ≈ 3.5 . Fairly simple equilibrium model was obtained for all systems as formation of monoligand complexes ([MLH]⁺, [ML], and [MLH₁][−]) is the most probable (Table 2). In the protonated complexes [MLH]⁺, the proton can presumably be attributed to the noncoordinating hydrazinic N² atom, while [ML] contains the completely deprotonated ligand. pK values of complexes [MLH]⁺ are significantly lower than pK_2 (N_{Pro}H⁺) and pK_3 (hydrazinic-NH) of the ligand in all cases, which strongly suggests the involvement of the Pro nitrogen into the coordination beside the thiosemicarbazide moiety in the complexes [ML]. Species [MLH₁][−] are regarded as mixed hydroxido complexes, that is, [ML(OH)][−].

Direct comparison of the overall stability constants ($\log \beta$) represents well the stability rank order of the metal complexes formed with the studied ligand: nickel(II) > zinc(II). It is noteworthy that copper(II) forms complexes with L-Pro-FTSC with unquestionably higher stability compared with these bivalent metal ions. First of all pM values were computed to compare the extent of complex formation at physiological pH (Table 2). The higher pM value represents stronger chelating ability. Calculated pM values show that the ligand effectiveness is increased in the following order: zinc(II) < nickel(II) < copper(II). Additionally the distribution of the mono complexes of zinc(II) and nickel(II) was calculated based on the determined stability constants at physiological pH at various total concentrations and depicted in Figure 10. Complexes [ML] predominate at this pH in all cases, and as expected, the partial dissociation resulting in free ligand and metal ion is more pronounced with decreasing total

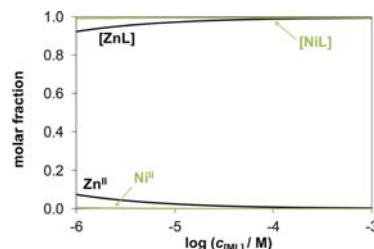


Figure 10. Concentration distribution curves for the metal(II)-L-Pro-FTSC systems at various total concentrations of the [ML] complexes at pH 7.40 [$T = 298$ K and $I = 0.10$ M (KCl)].

concentrations. Nickel(II) is able to preserve the original entity more efficiently owing to the higher stability of its complex; however, even in the case of zinc(II) the decomposition of [ZnL] is only 8% at 1 μ M concentration at pH 7.4.

To confirm the speciation obtained by pH-potentiometry for the zinc(II)-L-Pro-FTSC system ¹H NMR spectroscopic measurements were applied. Slow ligand-exchange processes are seen with respect to the NMR time scale as the chemical shifts of the protons of the free and Zn-bound ligand are observed separately (Figure 11 and Supporting Information, Figure S8).

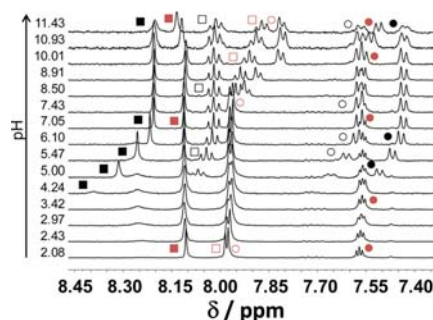


Figure 11. Low-field region of the ¹H NMR spectra recorded for the zinc(II)-L-Pro-FTSC system at the indicated pH values. Black symbols are shown for the bound ligand, and red symbols are shown for the nonbound ligand: ●: CH_{Ar}(6); ○: CH_{Ar}(4); □: CH_{Ar}(5); ■: CH=N; [$c_L = 1.0$ mM; Zn:L = 1:2; $T = 298$ K and $I = 0.10$ M (KCl); 10% D₂O].

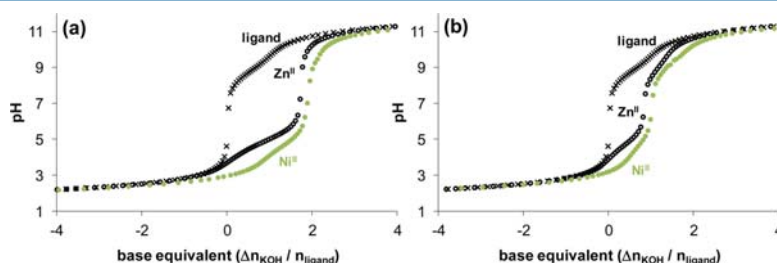


Figure 9. Representative pH-potentiometric titration curves for ligand L-Pro-FTSC (x), zinc(II)-ligand (o), and nickel(II)-ligand (●) systems [$c_L = 0.0019$ M; $M/L = 1:1.1$ (a) and $1:2.3$ (b); $T = 298$ K and $I = 0.10$ M (KCl) in water]. Negative base equivalent values mean an excess amount of acid.

Peaks belonging only to the nonbound ligand are seen at $\text{pH} < \sim 4$, and a new set of signals appears additionally with increasing pH , which belongs, most probably, to the protonated complex $[\text{ZnLH}]^+$. Its deprotonation is accompanied by significant electronic shielding effects, namely, an upfield shift of the peaks in the low-field region of the spectra. pK value of $[\text{ZnLH}]^+$ calculated on the basis of the change of the signals of the $\text{CH}=\text{N}$ protons of the bound ligand (Table 2) is in good agreement with that obtained from the pH-metric titration data. The peaks of species $[\text{ZnL}]$ have constant positions at pH 7–11.5, which strongly suggests that the formation of a mixed-hydroxido complex $[\text{ZnL}(\text{OH})]^-$ is not probable in this pH range. The integrated areas of the corresponding $\text{CH}=\text{N}$ peaks of the bound and nonbound ligand could be calculated and converted to molar fractions, which were also computed under the same conditions based on the stability constants obtained by pH-potentiometry (Figure 12). The strong correlation between the data of the two independent methods supports the accuracy of the stability constants determined.

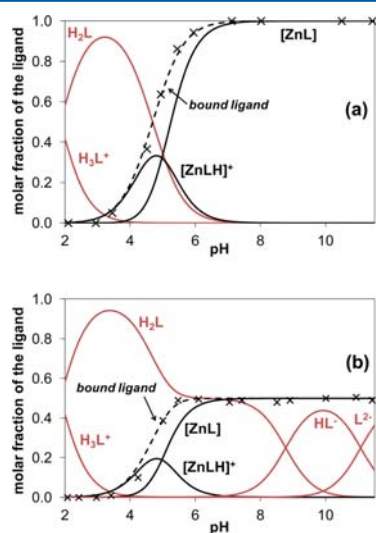


Figure 12. Concentration distribution curves of the zinc(II)-L-Pro-FTSC system at 1:1 (a) and 1:2 (b) metal-to-ligand ratios obtained with the help of the stability constants (solid lines) together with the summed molar fraction of the bound ligand calculated (dashed line) and estimated from the integrated area of the signals of the $\text{CH}=\text{N}$ proton (x) [$c_L = 1.0 \text{ mM}$; $T = 298 \text{ K}$ and $I = 0.10 \text{ M}$ (KCl)].

In the case of the outstanding high stability $[\text{CuL}]$ complex of L-Pro-FTSC the coordination of the COO^- and proline-N functionalities of the Pro moiety in addition to the ($\text{N}_{\text{pyr}}, \text{N}, \text{S}^-$) donor set of thiosemicarbazide moiety could be proved both in solution and in solid phase in our previous work.⁴⁹ The relatively high stability of the $[\text{ZnL}]$ complex, which predominates at neutral pH even at micromolar concentrations, supports a similar binding pattern in solution. Moreover, the pentadentate ($\text{N}_{\text{pyr}}, \text{N}, \text{S}^-, \text{COO}^-, \text{N}_{\text{Pro}}$) coordination mode of L-Pro-FTSC to zinc(II) was also confirmed by single-crystal X-ray diffraction (Figure 4).

The pentadentate binding of the ligand was also found in the case of nickel(II) in the solid phase, although the

thiosemicarbazone sulfur atom acts as a bridging ligand in the dinuclear species crystallized out from the solution (Supporting Information, Figure S3 and Figures 1 and 2). The pH-potentiometric titration curves for the nickel(II)-L-Pro-FTSC system could be fitted by the assumption of monomer complexes (Table 2); however, this method has limitations in distinguishing between the formation of mononuclear and dinuclear species with the same metal-to-ligand ratio. Therefore, clarifying the actual coordination mode of the nickel(II) complexes in solution is more difficult. To confirm the speciation model obtained by pH-potentiometry and to get an insight into the geometry of the complexes, UV-vis spectrophotometric and ^1H NMR measurements were performed. According to the electronic absorption spectra (Figure 13), formation of octahedral nickel(II) complexes is

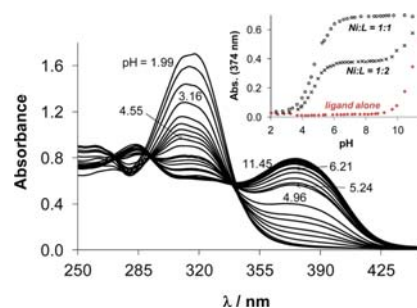


Figure 13. UV-vis spectra recorded for the nickel(II)-L-Pro-FTSC system at various pH values; [$c_L = 0.160 \text{ mM}$; $\text{Ni}/\text{L} = 1:1$; $l = 0.5 \text{ cm}$; $T = 298 \text{ K}$ and $I = 0.10 \text{ M}$ (KCl)]. (inset) The pH dependence of the absorbance values at 374 nm for the same system (○) together with those recorded for the ligand alone (—) and at 1:2 metal-to-ligand ratio (x) for comparison.

probable with this ligand in the measured pH range due to the lack of the characteristic bands of the square-planar complexes in the visible region. Additionally, the pH -dependent ^1H NMR spectra (Figure 14a) represent broad signals accompanying the formation of the nickel(II) complexes (Figure 14b), which strongly suggests the presence of high-spin paramagnetic species with octahedral geometry in solution.

On the other hand, UV-vis spectra at 1:1 metal-to-ligand ratio were measured in a wide concentration range ($1 \mu\text{M}$ – 0.8 mM) at pH 7.4. A clearly linear correlation was found between the absorbance and concentration (Supporting Information, Figure S9) representing the unchanged molar absorptivity (ϵ) and thus unchanged species (ratio) in the studied concentration range. Dilution generally can shift the monomer/dimer equilibrium to the direction of dissociation affecting the ϵ values as the ratio of the mononuclear and dinuclear species would change. Thus, the formation of only monomer $[\text{NiL}]$ species in solution is more probable due to the constant ϵ values.

Stability of the Zinc(II) Complex of L-Pro-FTSC in Minimum Essential Medium and Its Interaction with HSA. MEM is usually used for the *in vitro* cytotoxicity studies and contains various amino acids, which can potentially compete for the metal ion with the original ligand. To assess the stability of the $[\text{ZnL}]$ complex of L-Pro-FTSC in the MEM, ^1H NMR spectra of the complex were measured in this medium and in aqueous solution at pH 7.40 for comparison (Supporting Information,

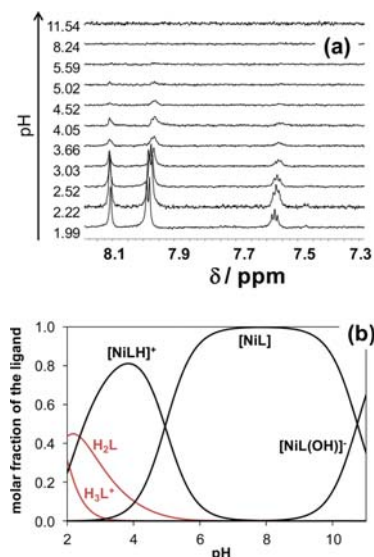


Figure 14. Low-field region of the ^1H NMR spectra recorded for the nickel(II)-L-Pro-FTSC system at the indicated pH values (a) [$c_L = 1.0$ mM; $\text{Ni}/L = 1:1$; $T = 298$ K and $I = 0.10$ M (KCl); 10% D_2O] and concentration distribution curves for the same system (b).

Figure S10). The peaks of $[\text{ZnL}]$ in MEM seem to remain unchanged compared to those detected in water, and no free ligand is liberated due to a possible ligand displacement by the amino acids. This provides strong evidence that the complex is stable in MEM. (Since nickel(II) complexes possess $\log \beta$ values by 2 orders of magnitude higher than those of zinc(II), no ligand-displacement reactions are expected in MEM.)

Competition reaction for the zinc(II) ion between L-Pro-FTSC and the most abundant blood serum protein, HSA, was followed by ^1H NMR spectroscopy. HSA serves as a transport vehicle for a wide variety of endogenous species such as copper(II) and zinc(II) ions and exogenous compounds and various pharmaceuticals. At the proposed Zn-binding site of HSA (*Multi-Metal Binding Site*) two histidine N atoms, an asparagine carboxylate, an aspartic acid carboxylate, and a water molecule are coordinated to the metal ion.⁸³ The conditional binding constants of HSA for zinc(II) under physiological conditions are given in the literature as $\log K' \approx 7.1$ – 7.9 .⁸⁴ The ^1H NMR spectrum recorded in the presence of HSA (Figure 15) clearly shows the partial displacement of L-Pro-FTSC by the protein ($\sim 30\%$) under the given condition. Beside HSA, human serum transferrin (Tf) has also zinc(II) binding ability at the iron-binding sites;⁸⁵ however, the concentration of Tf is approximately 17 times lower compared to that of HSA. Concentration distribution curves were computed for the zinc(II) complex of L-Pro-FTSC in the presence of these serum proteins at pH 7.4 at various total concentrations taking into consideration the stability constants of the zinc(II) complexes of the ligand, HSA, and Tf (Supporting Information, Figure S11a). These calculations reveal that $\sim 30\%$ of zinc(II) is bound to HSA at mM concentrations (as the ^1H NMR spectrum also showed in Figure 15), but this number is more than 80% at $10\ \mu\text{M}$. The role of Tf seems to be almost negligible in this concentration range. On the basis of similar computational

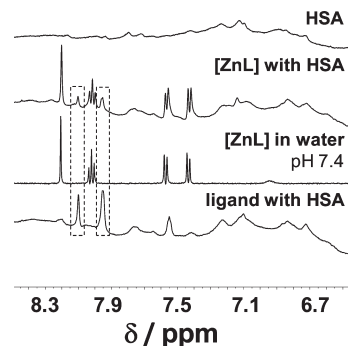


Figure 15. Low-field region of the ^1H NMR spectra of HSA, complex $[\text{ZnL}]$ of L-Pro-FTSC with HSA and in HEPES buffer (20 mM) at pH 7.40, and for L-Pro-FTSC with HSA [$c_L = 1.0$ mM; $c_{\text{HSA}} = 0.63$ mM; $T = 298$ K and $I = 0.10$ M (KCl); 10% D_2O]. Peaks framed up correspond exclusively to the nonbound ligand.

modelings, it can be concluded that the higher stability nickel(II) and copper(II) complexes are able to preserve their original composition more strongly in the presence of the serum proteins (Supporting Information, Figure S11b,c).⁸⁶

Cytotoxicity in Cancer Cell Lines. The antiproliferative activity of the investigated compounds (1–3 and 6–8) was evaluated for 48 h of continuous drug action, using colorimetric MTT assay. The study was performed in several human neoplastic cell lines, namely, HeLa, FemX, A549, MDA-MB-453, and one human fetal lung fibroblast cell line (MRC-5), which was used as a noncancerous model for the *in vitro* toxicity evaluation of 1, 2 and 7, 8, and in HeLa, A549, and MRC-5 for testing of the metal-free ligands and complexes 3 and 6. The obtained cytotoxicity results for the nonmethylated metal-free hybrids L-Pro-FTSC and D-Pro-FTSC and their nickel(II), copper(II), and zinc(II) complexes revealed that all compounds possessed poor activity ($\text{IC}_{50} > 300\ \mu\text{M}$) against all cell lines used (HeLa, FemX, MDA-MB-453). Complex formation of the FTSC-proline hybrids with nickel(II), copper(II), and zinc(II) (1, 2, 4, 7, and 8) does not enhance antiproliferative effects *in vitro*.

We further investigated the antiproliferative activity of the dm-L-Pro-FTSC hybrid and its copper(II) and nickel(II) complexes. The results shown in Table 3 indicate that dimethylation improves the cytotoxicity of the hybrid ($\text{IC}_{50}(\text{dm-L-Pro-FTSC}) < \text{IC}_{50}(\text{D-Pro-FTSC})$ and L-Pro-FTSC). Complex formation of dm-L-Pro-FTSC with copper(II) (complex $[\text{Cu}(\text{dm-L-Pro-FTSC})]$ (6)) significantly increases cytotoxicity toward HeLa and MRC5 cell lines compared to dm-L-Pro-FTSC hybrid alone (IC_{50} values of 98.3 ± 5.5 and $69.4 \pm 4.7\ \mu\text{M}$, compared to 224.6 ± 6.4 and $178.4 \pm 1.5\ \mu\text{M}$, respectively). These results, showing that dimethylation has a favorable impact on cytotoxicity, are in accordance with the previously reported studies.^{54,87}

Ribonucleotide Reductase Inhibition Capacity. The reduction of the tyrosyl radical content in human R2 RNR protein by L-Pro-FTSC and dm-L-Pro-FTSC hybrids, and complexes 4, 6, and 7 was monitored by EPR spectroscopy. The hR2 protein exhibits an EPR spectrum that arises from the tyrosyl radical associated with enzyme activity (Figure 16b inset).⁸⁹ It was observed that in the nonreducing conditions, the compounds are not active in tyrosyl radical destruction

Table 3. Results of MTT Assay Presented as IC_{50} (μM) Values after 48 h of Incubation Time

compound/cell line	IC_{50}^a , μM (mean $\pm$ SD)		
	HeLa	A549	MRC5
L-Pro-FTSC	>300 ^b	>300 ^b	>300 ^b
dm-L-Pro-FTSC	224.6 $\pm$ 6.4	204.3 $\pm$ 4.8	178.4 $\pm$ 1.5
[Ni(dm-L-Pro-FTSC-2H)] ₂ (3)	>300 ^b	>300 ^b	>300 ^b
[Cu(dm-L-Pro-FTSC-2H)] (6)	98.3 $\pm$ 5.5	176.8 $\pm$ 1.7	69.4 $\pm$ 4.7
CDDP ^c	7.8 $\pm$ 2.3	17.2 $\pm$ 0.7	30.3 $\pm$ 3.0

^a IC_{50} values were calculated as mean values obtained from two to three independent experiments and quoted with their standard deviations. ^bThe sign > (in front of the maximum value of the concentration) indicates that IC_{50} value is not reached in the examined range of concentrations. Compounds contain cocrystallized solvent (see Experimental Section). ^cTaken from ref 88.

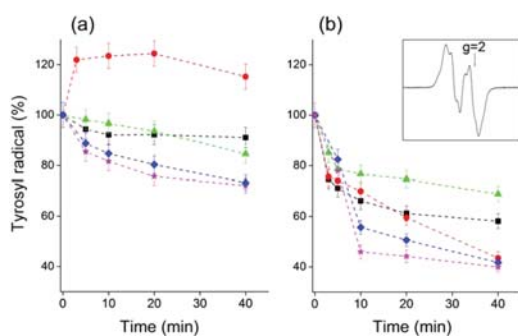


Figure 16. Tyrosyl radical reduction in human R2 RNR protein in (a) absence and (b) presence of the external reductant DTT, by ligands L-Pro-FTSC (black trace) and dm-L-Pro-FTSC (blue trace) and complexes 4 (red trace), 6 (magenta trace), and 7 (green trace). The samples contained 20 μM hR2 protein in Tris buffer, pH 7.60/100 mM KCl/5% glycerol, 20 μM compound (1% (w/w) DMSO/ H_2O), and 2 mM DTT (only b). Error bars are standard deviation from two independent experiments. (inset) EPR spectrum of the tyrosyl radical in hR2 RNR protein at 20 K. Experimental conditions are given in the Experimental Section.

(Figure 16a). Moreover, complex 4 caused a small increase in tyrosyl radical content, an effect that was previously observed when mouse R2 protein (mR2) was incubated with only DTT.^{24,90} An increase in radical content is caused by the continued radical reconstitution process, which requires the presence of oxygen. This result indicates that in the absence of a reductant, complex 4 may have a protective role for the tyrosyl radical in hR2.

In the presence of an external reductant (DTT), all compounds showed appreciable radical destruction capacity compared to the nonreducing conditions (Figure 16b). The reduction by complex 4 was more efficient than by the ligand L-Pro-FTSC alone, indicating that the presence of the redox active copper(II) ion influences the inhibitory potential. Inhibition by complex 7 did not change markedly upon addition of DTT, as this compound contains zinc(II), which is redox inactive. Among the five tested compounds, dm-L-Pro-FTSC and complex 6 proved to be the best inhibitors, 6 being slightly more potent due to the presence of the copper(II) ion. The difference in the extent of the radical reduction observed

between ligands L-Pro-FTSC and dm-L-Pro-FTSC (and their corresponding copper(II) complexes, 4 and 6) indicates that the presence of two methyl groups increases the potency of the ligand.

It is interesting to compare these compounds with Triapine, which is an analogue of 2-formylpyridine thiosemicarbazone. Triapine was shown to be a good mR2²⁴ and hR2²⁵ inhibitor. In both proteins, Triapine exhibited 70% radical reduction after 20 min in the absence of the reductant and reduced the radical completely after 5 min in the presence of DTT. This suggests that the proline moiety decreases the inhibitory potential of the ligand.

CONCLUSION

Nickel(II) and zinc(II) complexes with chiral proline-thiosemicarbazone hybrids L- and D-Pro-FTSC have been synthesized and characterized by standard analytical methods and in the case of complexes 1–3 also by single-crystal X-ray diffraction. The temperature dependence of magnetic susceptibility, high-field EPR spectra, and DFT calculations indicate ferromagnetic interaction between paramagnetic Ni(II) ions in 2 with the total spin ground state $S_T = 2$. As these complexes possess excellent water solubility, the solution speciation of nickel(II) and zinc(II) complexes of L-Pro-FTSC has been characterized in pure aqueous solution via a combined approach using pH-potentiometry, ¹H NMR spectroscopy, and UV-vis spectrophotometry. Stability of the species formed is compared to that of copper(II) complexes. Exclusive formation of the monoligand complexes such as [MLH]⁺, [ML], and [ML(OH)][−] was detected. L-Pro-FTSC was found to act as a pentadentate ligand in solution coordinating the metal ions via the (N_{pyr} , N, S[−], COO[−], N_{pro}) donor atoms. This binding mode was confirmed by X-ray crystallography in the case of the [ZnL] complex. On the other hand, nickel(II) forms a dinuclear complex with a central Ni(μ -S)₂Ni core in solid state, and the two metal ions are chelated by the (N_{pyr} , N, S[−], COO[−], N_{pro}) donor set; however, the presence of monomeric species in solution is somewhat more probable. In the protonated [MLH]⁺ complexes the pentadentate (N_{pyr} , N, S, COO[−], N_{pro}) binding mode with a protonated noncoordinating hydrazine N² atom is suggested. On the basis of the determined stability constants the effectiveness of L-Pro-FTSC to chelate the metal ions is in the rank order zinc(II) < nickel(II) < copper(II). The predominant species at pH = 7.4 are [ML] complexes in all cases. Complexes of zinc(II), nickel(II), and copper(II) possess such high stability that they remain intact during dilution at physiological pH in the biologically relevant micromolar concentration range. Additionally, [ZnL] remains unaltered in MEM, while partial displacement of L-Pro-FTSC by HSA in the serum is probable, especially at lower concentrations of the complex. The compound L-Pro-FTSC was N-terminally dimethylated, and the structures of its nickel(II) and copper(II) complexes were determined by X-ray crystallography. Compounds prepared in this work were tested for antiproliferative activity in different human cancer cell lines. It was shown that dimethylation of terminal aminogroup in L- and D-Pro-FTSC resulted in antiproliferative activity of the hybrids. Coordination to copper(II) further enhances the cytotoxicity of the dimethylated hybrid. hR2 RNR inhibition capacity of selected compounds was also assayed. It was found that they are not active in tyrosyl radical destruction under nonreducing conditions, while they exhibit much stronger radical quenching capacity in the presence of the

applied reductant DTT. The N-terminal dimethylation resulted in higher inhibitory potential of the ligand and its copper(II) complex. Further increase of the lipophilicity of proline-thiosemicarbazone hybrids via esterification of the proline moiety, and attachment of naphthyl, trimethylsilyl groups at the terminal nitrogen atom, which are underway in our laboratory, should result in more effective antiproliferative agents suitable for further development as potential anticancer drugs.

■ ASSOCIATED CONTENT

■ Supporting Information

Illustration of synthesis of dm-L-Pro-FTSC, structures of *E* and *Z* isomers of dm-L-Pro-FTSC, additional structural illustrations, UV–vis molar absorbance spectra of Ni(II) and Zn(II) complexes of dm-L-Pro-FTSC, plot showing frequency dependence of EPR resonances, selected NMR spectra, Table of log $D_{7.4}$ values for dm-L-Pro-FTSC and seven other compounds, additional references. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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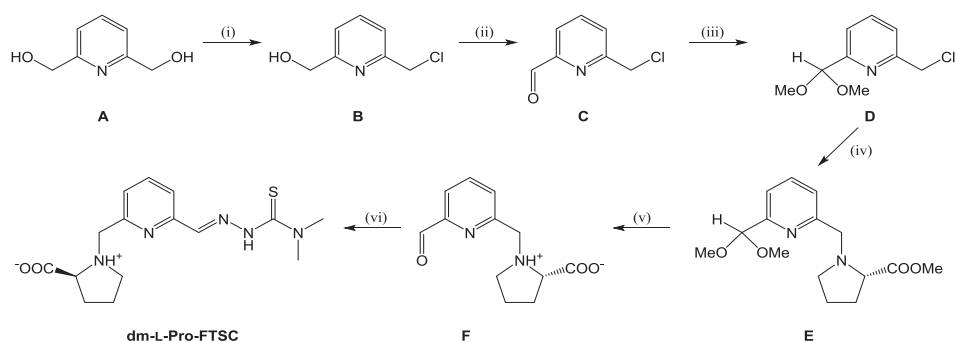
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Supporting Information
for
**Effects of Terminal Dimethylation and Metal Coordination of Proline-2-
 Formylpyridine Thiosemicarbazone Hybrids on Lipophilicity, Anti-
 proliferative Activity and hR2 RNR inhibition**

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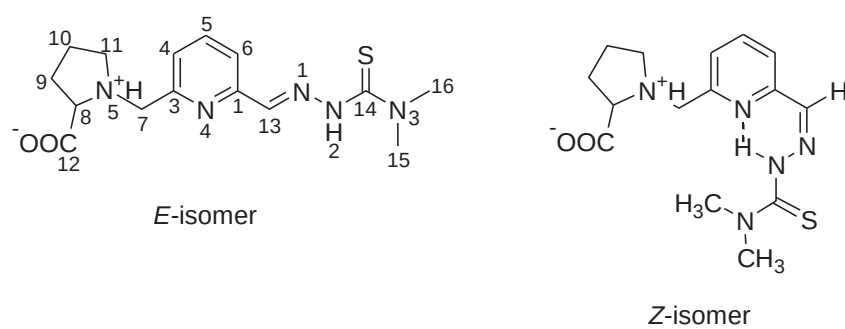
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Keywords: Proline-thiosemicarbazone hybrids, Solution equilibrium, Stability constants, Antitumor activity, hR2 RNR



Scheme S1. Synthesis of dm-L-Pro-FTSC ^a

^aReagents and conditions: (i) and (ii) see ref. 1; (iii) trimethyl orthoformate, methanesulfonic acid, methanol, 351 K, 3 h; (iv) L- proline methylester hydrochloride, triethylamine, THF/CH₂Cl₂ 1.5:1, 313 K, 12 h, purification by column chromatography; (v) water, reflux, 48 h; (vi) 4,4-dimethyl-3-thiosemicarbazide, EtOH abs., room temperature, 24 h.



Scheme S2. *E* and *Z* isomers of dm-L-Pro-FTSC and atom numbering scheme for ^1H and ^{13}C NMR data.

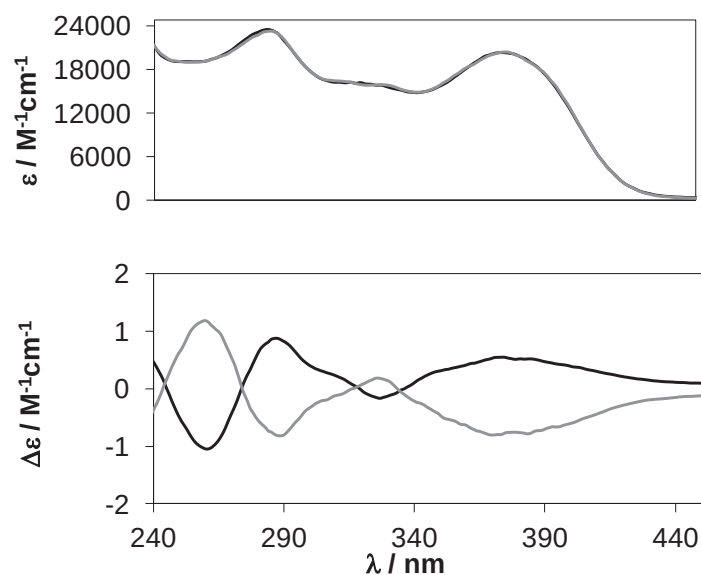


Figure S1. UV-vis molar absorbance (top) and CD spectra (bottom) of the nickel(II) complexes of L-Pro-FTSC (black traces) (1) and D-Pro-FTSC (grey traces) (2) recorded at pH 7.40 ($T = 298$ K; $I = 0.10$ M (KCl); 20 mM HEPES buffer).

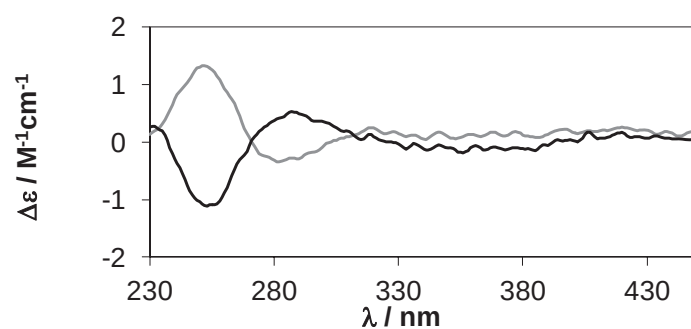


Figure S2 CD spectra of the zinc(II) complexes of L-Pro-FTSC (black traces) (7) and D-Pro-FTSC (grey traces) (8) recorded at pH 7.40 ($T = 298$ K; $I = 0.10$ M (KCl); 20 mM HEPES buffer).

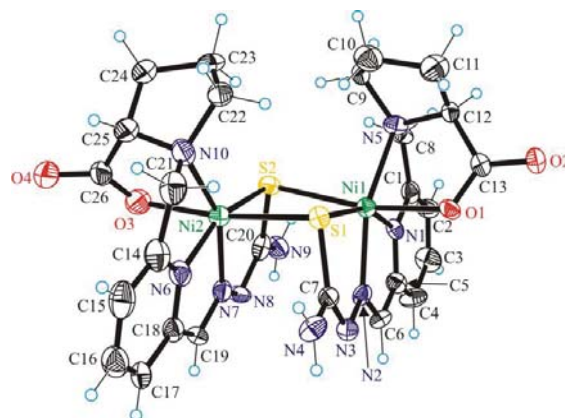


Figure S3. ORTEP view of **1** with thermal displacement ellipsoids drawn at the 50% probability level. Selected bond distances (Å) and bond angles (deg): Ni1–N1 1.974(6), Ni1–N2 2.042(6), Ni1–N5 2.211(6), Ni1–S1 2.4057(19), Ni1–S2 2.522(2), Ni1–O1 2.041(5), N2–N3 1.383(7), C7–S1 1.767(7); N1–Ni1–N2 78.3(2), N1–Ni1–N5 79.5(2), N5–Ni1–O1 81.4(2), N1–Ni1–O1 89.8(2), N2–Ni1–O1 90.2(2), S1–Ni1–O1 93.74(14), S1–Ni1–S2 86.07(7), Ni1–S1–Ni2 91.99(7), Ni1–S2–Ni2 92.15(7), Ni2–N6 1.990(6), Ni2–N7 2.028(6), Ni2–N10 2.212(7), Ni2–S2 2.375(2), Ni2–S1 2.500(2), Ni2–O3 2.054(6), N7–N8 1.351(8), C20–S2 1.748(9); N6–Ni2–N7 78.9(3), N6–Ni2–N10 77.9(3), N10–Ni2–O3 81.5(2), N6–Ni2–O3 87.9(2), N7–Ni2–O3 88.2(2), S2–Ni2–O3 95.52(16), S1–Ni2–S2 87.22(7).

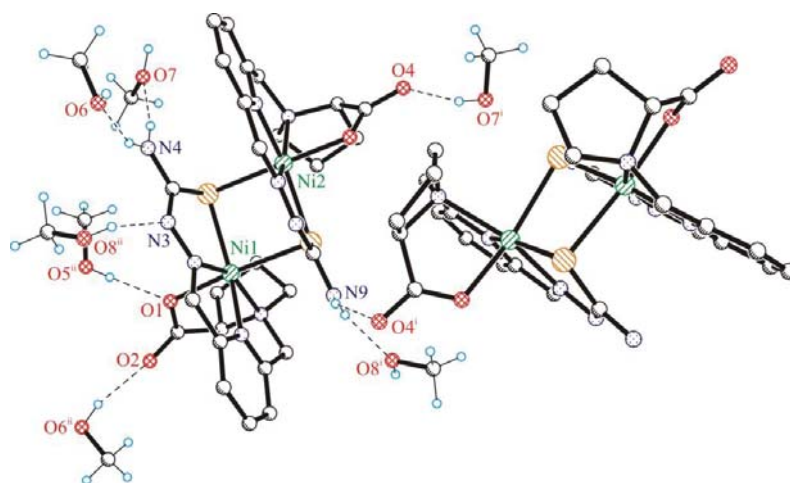


Figure S4. Portion of the crystal structure of 2,4-CH₃OH showing the intermolecular hydrogen bonding interactions. Many hydrogen atoms are omitted for clarity. Atoms marked with i have been generated via symmetry transformation $x - 0.5, -y + 0.5, -z + 1$, while those with ii via symmetry transformation $-x + 0.5, -y + 1, z - 0.5$. Main hydrogen bonding parameters: N4...O6 2.895(2) Å, N4-H...O6 156.3°; N4...O7 3.009(2) Å, N4-H...O7 169.8°; N9...O4ⁱ 2.966(2) Å, N9-H...O4ⁱ 151.7°; N9...O8ⁱ 2.877(2) Å, N9-H...O8ⁱ 155.9°; O1...O5ⁱⁱ 2.838(2) Å, O1-H...O5ⁱⁱ 174.3°; N3...O8ⁱⁱ 2.810(2) Å, N3-H...O8ⁱⁱ 153.5°; O2...O6ⁱⁱ 2.774(2) Å, O2-H...O6ⁱⁱ 158.93°; O4...O7ⁱ 2.756(2) Å, O4-H...O7ⁱ 164.5°.

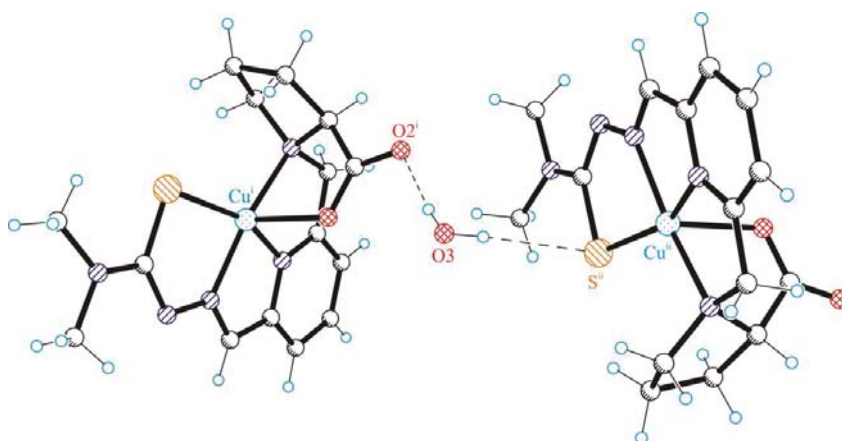


Figure S5. Portion of the crystal structure of **6**·H₂O showing the intermolecular hydrogen bonding interactions. Atoms marked with i have been generated via symmetry transformation $x + 1, y, z$, while those with ii via symmetry transformation $-x + 0.5, -y + 1, z + 0.5$. Main hydrogen bonding parameters: O3···O2ⁱ 2.737(2) Å, O3–H···O2ⁱ 172.9°; O3···Sⁱⁱ 3.495(1) Å, O3–H···Sⁱⁱ 173.4°.

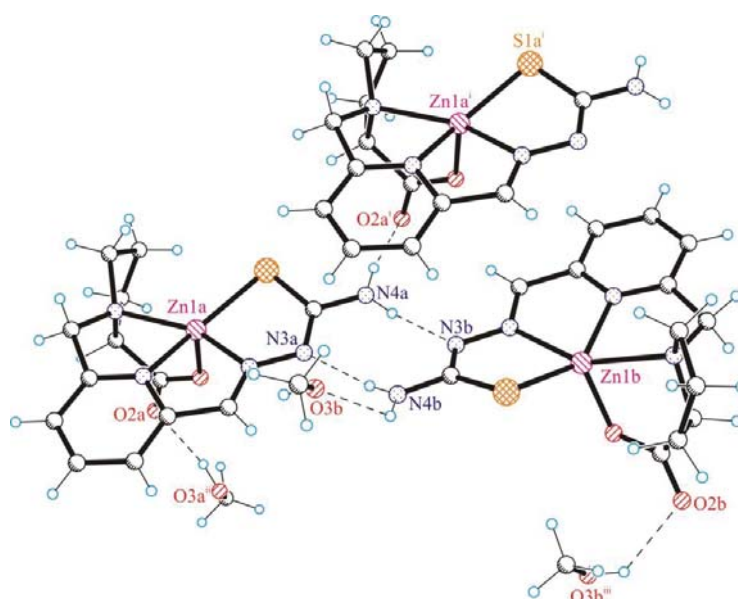


Figure S6. Portion of the crystal structure of 7-CH₃OH showing the intermolecular hydrogen bonding interactions. Atoms marked with i have been generated via symmetry transformation $x + 1, y, z$, while those with ii and iii via symmetry transformation $-x, y - 0.5, -z + 1$ and $-x + 1, y - 0.5, -z + 1$, respectively. Main hydrogen bonding parameters: N4a $\cdots$ N3b 2.911(4) Å, N4a-H $\cdots$ N3b 170.1°; N4b $\cdots$ N3a 3.076(4) Å, N4b-H $\cdots$ N3a 171.5°; N4b $\cdots$ O3b 2.988(7) Å, N4b-H $\cdots$ O3b 122.7°; N4a $\cdots$ O2aⁱ 3.015(4) Å, N4a-H $\cdots$ O2aⁱ 166.7°; O2a $\cdots$ O3aⁱⁱ 2.750(4) Å, O2a $\cdots$ H-O3aⁱⁱ 169.3°; O2b $\cdots$ O3bⁱⁱⁱ 2.806(6) Å, O2b $\cdots$ H-O3bⁱⁱⁱ 134.2°.

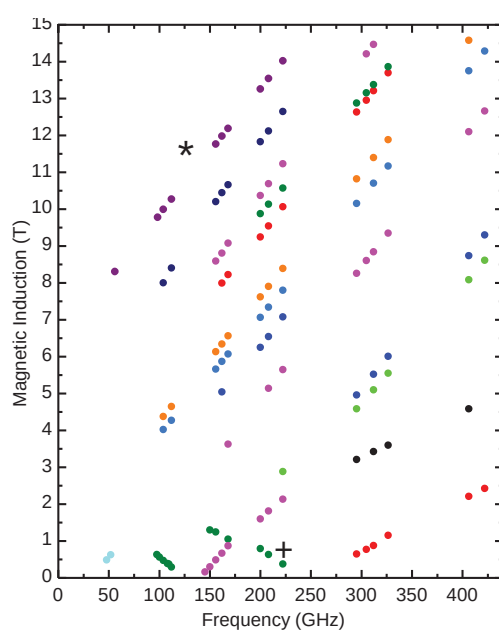


Figure S7. The frequency dependence of the prominent resonances observed in the EPR spectra (see Figure 7). The * and + marks correspond to those in Figure 7. The experimental points which appear to belong to the same transitions are plotted with the same color.

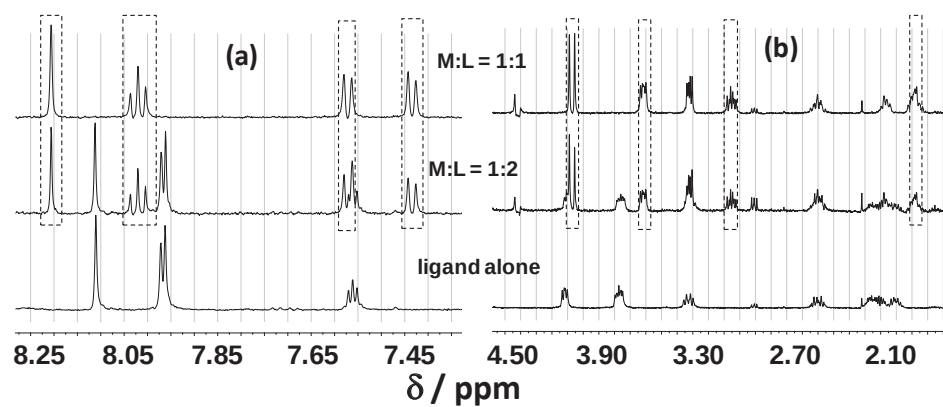


Figure S8. Low- (a) and high-field (b) regions of the ^1H NMR spectra recorded for the L-Pro-FTSC alone and for the zinc(II) – L-Pro-FTSC system at 1:1 and 1:2 metal-to-ligand ratio at pH 7.40 [$c_{\text{L}} = 1.0$ mM; $T = 298$ K and $I = 0.10$ M (KCl); 10% D_2O]. Peaks framed up correspond exclusively to the complex $[\text{ZnL}]$.

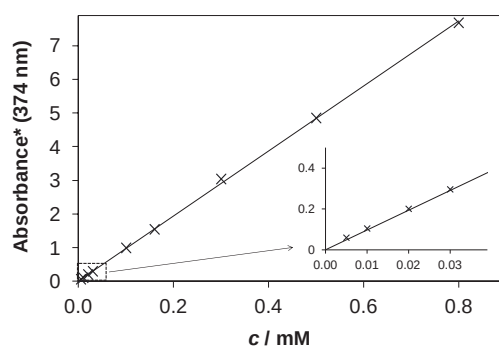


Figure S9. Absorbance values relating to 1 cm path length at 374 nm recorded for the nickel(II) – L-Pro-FTSC (1:1) system at pH 7.40 at various concentrations using cuvettes with different path lengths ($l = 0.2, 0.5, 1.0$ or 2.0 cm) in order to get well-measurable absorbance values (Abs. = 0.10–1.54); [$T = 298$ K and $I = 0.10$ M (KCl)].

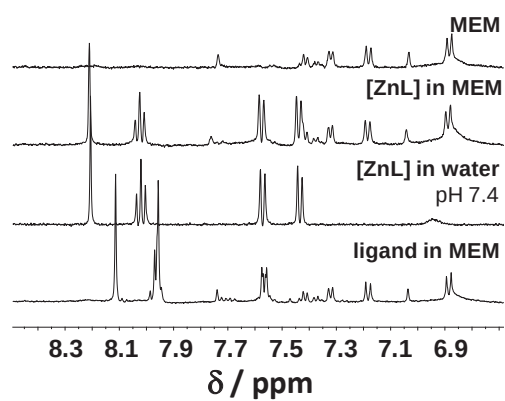


Figure S10. Low-field region of the ^1H NMR spectra of MEM, complex [ZnL] of L-Pro-FTSC in MEM and in HEPES buffer (20 mM) at pH 7.40 and for L-Pro-FTSC in MEM [$c_L = 0.83$ mM; $T = 298$ K and $I = 0.10$ M (KCl); 10% D_2O].

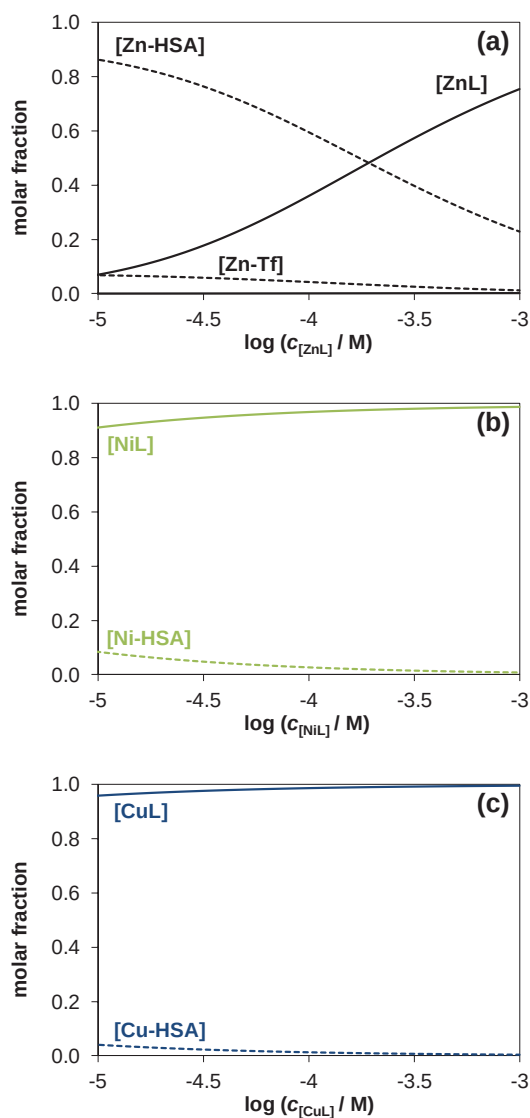


Figure S11. Estimated concentration distribution curves for the metal(II) – L-Pro-FTSC systems at various total concentrations of the [ML] complexes at pH 7.40 in the presence of human serum albumin (630 μM) and transferrin (37 μM) [$T = 298 \text{ K}$ and $I = 0.10 \text{ M}$ (KCl)]. Stability constants used for the calculations were determined herein for the L-Pro-FTSC complexes and are literature data for the M-protein complexes: $\log K' [\text{Zn-HSA}] = 7.5$ taken from ref. 2, $\log K' [\text{Zn-Tf}] = 7.8$ and $\log K' [\text{Zn}_2\text{-Tf}] = 6.4$

taken from ref. 3; $\log K'$ [Ni-HSA] = 6.8 and $\log K'$ [Ni₂-HSA] = 4.9 taken from ref. 4; $\log K'$ [Cu-HSA] = 12.0 and $\log K'$ [Cu₂-HSA] = 8.0 taken from ref. 4.

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2.2 Strong Effect of Copper(II) Coordination on Antiproliferative Activity of Thiosemicarbazone-Piperazine and Thiosemicarbazone-Morpholine Hybrids

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Strong Effect of Copper(II) Coordination on Antiproliferative Activity of Thiosemicarbazone-Piperazine and Thiosemicarbazone-Morpholine Hybrids†

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Abstract. In this study, 2-formylpyridine thiosemicarbazones and three different heterocyclic pharmacophores were combined to prepare thiosemicarbazone-piperazine mPip-FTSC (**HL**¹) and mPip-dm-FTSC (**HL**²), thiosemicarbazone-morpholine Morph-FTSC (**HL**³) and Morph-dm-FTSC (**HL**⁴), thiosemicarbazone-methylpyrrole-2-carboxylate hybrids mPyr-FTSC (**HL**⁵) and mPyr-dm-FTSC (**HL**⁶) as well as their copper(II) complexes [CuCl(mPipH-FTSC-H)]Cl (**1+H**)Cl, [CuCl(mPipH-dm-FTSC-H)]Cl (**2+H**)Cl, [CuCl(Morph-FTSC-H)] (**3**), [CuCl(Morph-dm-FTSC-H)] (**4**), [CuCl(mPyr-FTSC-H)(H₂O)] (**5**) and [CuCl(mPyr-dm-FTSC-H)(H₂O)] (**6**). The substances were characterized by elemental analysis, one- and two-dimensional NMR spectroscopy (**HL**¹–**HL**⁶), ESI mass spectrometry, IR and UV–vis spectroscopy and single crystal X-ray diffraction (**1**–**5**). All compounds were prepared in an effort to generate potential antitumor agents with an improved therapeutic index. In addition, the effect of structural alterations with organic hybrids on aqueous solubility and copper(II) coordination ability was investigated. Complexation of ligands **HL**² and **HL**⁴ with copper(II) was studied in aqueous solution by pH-potentiometry, UV–vis spectrophotometry and EPR spectroscopy. Proton dissociation processes of **HL**² and **HL**⁴ were also characterized in detail and microscopic constants for the *Z/E* isomers were determined. While the hybrids **HL**⁵, **HL**⁶ and their copper(II) complexes **5** and **6** proved to be insoluble in aqueous solution, precluding antiproliferative activity studies, the thiosemicarbazone-piperazine and thiosemicarbazone-morpholine hybrids **HL**¹–**HL**⁴, as well as copper(II) complexes **1**–**4** were soluble in water enabling cytotoxicity assays. Interestingly, the metal-free hybrids showed very low or even a lack of cytotoxicity (IC₅₀ values > 300 μM) in two human cancer cell lines HeLa (cervical carcinoma) and A549 (alveolar basal adenocarcinoma), whereas their copper(II) complexes were cytotoxic showing IC₅₀ values from 25.5 to 65.1 μM and 42.8 to 208.0 μM, respectively in the same human cancer cell lines after 48 h of incubation. However, the most sensitive for **HL**⁴ and complexes **1**–**4** proved to be the human cancer cell line LS174 (colon carcinoma) as indicated by the calculated IC₅₀ values varying from 13.1 to 17.5 μM.

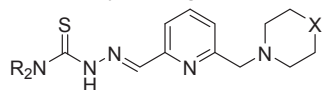
Introduction

Thiosemicarbazones (TSCs) are known as potent metal chelators with high affinity for first row transition metals.^{1, 2} TSCs and their metal complexes possess a variety of biological activities, such as antifungal, antiviral, antibacterial, antimalarial and anticancer.³⁻⁸ The anticancer activity of α -N-heterocyclic TSCs (HCTs) has been known since the 1950s when 2-formylpyridine thiosemicarbazone (FTSC) showed antileukemic activity in a mice model.⁹ To date, the best-studied HCT is 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP or Triapine). Several clinical phase I and II trials revealed that Triapine is ineffective against a variety of solid tumors but very promising against hematologic malignancies such as leukemia.¹⁰⁻¹⁸ The outcome of a recent clinical phase II study including 37 patients with aggressive myeloproliferative neoplasms, with a response rate of 49% and complete remission in 24% of all patients, has recently been reported.¹⁹ Ribonucleotide reductase (RNR),^{20, 21} an enzyme catalyzing the reduction of ribonucleotides to the corresponding 2'-deoxyribonucleotides, which is the rate determining step in DNA synthesis,²² and topoisomerase II α (Topo II α), an enzyme that controls the DNA topology during cell division by introducing temporary double strand breaks have been considered as possible targets for this class of compounds.²³⁻²⁶ New insights into the mechanism of action for RNR inhibiting HCTs and especially Triapine were recently reported.²⁷⁻³⁰ The enzymes ATP binding pocket was suggested as major target for Topo II α inhibiting HCTs.³¹ The reaction of copper(II) with HCTs leading to square-planar complexes markedly enhances the Topo II α inhibition rate.³²

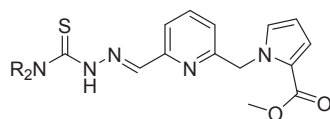
2-Acetylpyridine thiosemicarbazones possess very high cytotoxicity in human cancer cell lines with IC₅₀ values in the nanomolar concentration range and the ability to destroy the tyrosyl radical of the mammalian RNR R2 protein under the slightly reducing conditions typical for tumors.^{33, 34} However, high general toxicity and, consequently, the low therapeutic index along with low aqueous solubility for these and other related thiosemicarbazones prompted us to design hybrid systems, based on thiosemicarbazones and other pharmacophores. Recently, we prepared proline-TSC hybrids (3-methyl-(*S*)-pyrrolidine-2-carboxylate-2-formylpyridine thiosemicarbazone (L-Pro-FTSC) and 3-methyl-(*R*)-pyrrolidine-2-carboxylate-2-formylpyridine thiosemicarbazone (D-Pro-FTSC)) and their copper(II) complexes.³⁵ These new compounds are highly water soluble but exhibit very low cytotoxicity, most probably because of their very low lipophilicity. We decided to extend our work and use other pharmacophoric groups for attachment at the 6-position of the TSCs pyridine ring, in order to increase the lipophilicity and modulate the antiproliferative activity. We attached the six-membered rings methylpiperazine and morpholine as well as methylpyrrole-2-carboxylate containing a five-membered planar heterocycle. It is well-known that the attachment of a piperazine moiety on a

hydrophobic scaffold has a favourable effect on its water solubility,³⁶⁻³⁹ moreover the piperazine heterocycle is found in a broad variety of biologically active compounds, some of which are currently used in clinical therapy.⁴⁰⁻⁴⁹ Biologically active metal-based compounds containing a piperazine ring have also been reported.⁵⁰⁻⁵⁴ Morpholine is another well-known water-solubilizing unit incorporated in structures of biologically active compounds, showing often favorable pharmacologic effects.⁵⁵⁻⁵⁷ In particular, a morpholine moiety is also present in the approved anticancer drugs Gefitinib (against certain breast, lung and other cancers) and Carfilzomib (against multiple myeloma).^{58, 59} A series of TSCs with different substituents at position 4 of the pyridine ring was tested on mice bearing sarcoma 180 ascites cells. Intriguingly, the 4-morpholino-2-formylpyridine thiosemicarbazone was the most effective compound, increasing the average survival time of tumor bearing mice from 13.8 to 38 days.⁶⁰ The methylpyrrole-2-carboxylate ring was chosen as third possible option since it resembles proline.

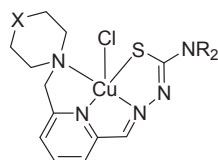
Herein, we report the synthesis of six new organic compounds, namely **HL**¹⁻⁶, representing three types of potential hybrid ligands for transition metals, as well as six copper(II) complexes all shown in Chart 1. The compounds were characterized by analytical and spectroscopic methods (¹H and ¹³C NMR, UV-vis, IR) and X-ray diffraction (**1-5**). Solution equilibria of the copper(II) complexes formed with **HL**² and **HL**⁴ were studied by pH-potentiometry, UV-vis and EPR spectroscopy and the thermodynamic stability data were compared to those for other related hybrid and non-hybrid systems. The antiproliferative activity of four ligands and four copper(II) complexes has been assayed. The cytotoxicity of **1-4** is markedly lower than that of the parent 2-acetylpyridine and 2-formylpyridine thiosemicarbazones, but significantly higher than that of thiosemicarbazone-proline hybrids and their copper(II) complexes making them pertinent for further development as potential anticancer drugs.

Chart 1. Hybrid Ligands and Their Copper(II) Complexes Studied in This Work.^a

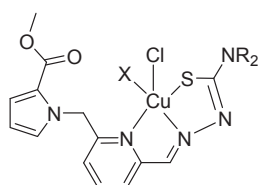
R = H, X = NCH₃, mPip-FTSC (**HL**¹)
 R = CH₃, X = NCH₃, mPip-dm-FTSC (**HL**²)
 R = H, X = O, Morph-FTSC (**HL**³)
 R = CH₃, X = O, Morph-dm-FTSC (**HL**⁴)



R = H, mPyr-FTSC (**HL**⁵)
 R = CH₃, mPyr-dm-FTSC (**HL**⁶)



R = H, X = NCH₃, [CuCl(mPipH-FTSC-H)]Cl (**1**+H)Cl^b
 R = CH₃, X = NCH₃, [CuCl(mPipH-dm-FTSC-H)]Cl (**2**+H)Cl
 R = H, X = O, [CuCl(Morph-FTSC-H)] (**3**)
 R = CH₃, X = O, [CuCl(Morph-dm-FTSC-H)] (**4**)



R = H, [CuCl(mPyr-FTSC-H)(X)] (**5**) X = H₂O (or DMF in X-ray structure)
 R = CH₃, [CuCl(mPyr-dm-FTSC-H)(X)] (**6**) X = H₂O

^aUnderlined numbers indicate complexes studied by X-ray diffraction. Co-crystallized solvent is not included in the formulas (see Experimental Section). ^bThis complex was crystallized and characterized by X-ray crystallography as [CuCl(mPip-FTSC-H)]·0.15CH₃OH.

Experimental

Chemicals. 2,6-Dihydroxymethylpyridine, 4-methylpiperazine, morpholine and methylpyrrole-2-carboxylate were purchased from Acros Organics. 2-Hydroxymethyl-6-chloromethylpyridine and 6-chloromethylpyridine-2-carboxaldehyde were synthesized according to published protocols.⁶¹ 2-(Chloromethyl)-6-(dimethoxymethyl)pyridine was prepared as described previously.³⁵ Solvents were dried using standard procedures, if required.⁶² KOH, KCl, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-(N-morpholino)ethanesulfonic acid (MES), ethylenediaminetetraacetic acid (EDTA) were Sigma-Aldrich products, while HCl and CuCl₂ were from Reanal. CuCl₂ was dissolved in a known amount of HCl in order to get the copper(II) stock solution. Its concentration was determined by complexometric titrations with EDTA.

Synthesis of ligands

1-((6-(Dimethoxymethyl)pyridin-2-yl)methyl)-4-methylpiperazine. 2-(Chloromethyl)-6-(dimethoxymethyl)pyridine (1.82 g, 9.02 mmol) was dissolved in a 1 : 1 mixture of dry THF and dry dichloromethane (40 mL) in a 100 mL Schlenk tube. Methylpiperazine (1.50 mL, 13.53 mmol) and triethylamine (3.64 mL, 27.06 mmol) were added. The reaction mixture was stirred at 46 °C overnight. The next day a white precipitate of triethylammonium chloride was filtered off and washed with THF. The filtrate was concentrated under reduced pressure to yield a brown oily raw product. This was purified on a silica column using chloroform/methanol 4:1 as eluent. The solvent was removed under reduced pressure to yield the product as a yellow oil. Yield: 1.99 g, 79%. ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.82 (t, *J* = 7.7 Hz, 1H, CH_(Ar)), 7.41 (d, *J* = 7.7 Hz, 1H, CH_(Ar)), 7.36 (d, *J* = 7.7 Hz, 1H, CH_(Ar)), 5.26 (s, 1H, CH(OCH₃)₂), 3.60 (s, 2H, CH₂), 3.30 (s, 6H, (OCH₃)₂), 2.50 – 2.35 (m, 8H, CH₂(Pip), overlapped with residual DMSO signal), 2.24 (s, 3H, CH₃(Pip)).

4-((6-(Dimethoxymethyl)pyridin-2-yl)methyl)morpholine. To 2-(Chloromethyl)-6-(dimethoxymethyl)pyridine (0.80 g, 3.97 mmol) in a 1 : 1 mixture of dry THF and dry dichloromethane (20 mL) in a 50 mL Schlenk tube were added morpholine (0.52 mL, 5.96 mmol) and triethylamine (1.60 mL, 11.91 mmol). The reaction mixture was stirred at 46 °C overnight. The next day a white precipitate of triethylammonium chloride was filtered off and washed with THF. The filtrate was concentrated under reduced pressure to yield a brown oily raw product. This was purified on a silica column using chloroform/methanol 97.5:2.5 as eluent. The solvent was removed under reduced pressure to yield the product as a yellow oil. Yield: 0.93 g, 93%. ¹H NMR (500 MHz, DMSO-*d*₆) δ 7.82 (t, *J* = 7.7 Hz, 1H, CH_(Ar)), 7.44 (d, *J* = 7.7 Hz, 1H, CH_(Ar)), 7.36 (d, *J* = 7.2 Hz,

1H, $CH_{(Ar)}$), 5.26 (s, 1H, $CH(OCH_3)_2$), 3.63 – 3.57 (m, 6H, $CH_{2(Morph)}$, CH_2), 3.30 (s, 6H, $(OCH_3)_2$), 2.44 – 2.40 (m, 4H, $CH_{2(Morph)}$).

Methyl 1-((6-(dimethoxymethyl)pyridin-2-yl)methyl)-1H-pyrrole-2-carboxylate. Sodium hydride (60 wt% dispersion in mineral oil) (0.10 g, 2.48 mmol) was suspended in dry DMF (3 mL) in a 25 mL Schlenk tube and cooled to 0 °C. A solution of methylpyrrole-2-carboxylate (0.31 g, 2.48 mmol) in dry DMF (4.5 mL) was added dropwise. Then a solution of 2-(chloromethyl)-6-(dimethoxymethyl)pyridine (0.50 g, 2.48 mmol) in dry DMF (2.5 mL) was added slowly. The reaction mixture was allowed to reach room temperature and then stirred overnight. The next day the crude mixture was poured into ice water (about 100 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic phases were dried over magnesium sulfate and the solvent was removed under reduced pressure to give a yellow, oily raw product. This was purified on a silica column using a mixture of 1:2 ethyl acetate/hexane as eluent. The product was obtained after removal of the solvent as a colorless oil. Yield: 0.42 g, 58%. ^1H NMR (500 MHz, DMSO- d_6) δ 7.76 (t, J = 7.8 Hz, 1H, $CH_{(Ar)}$), 7.35 (d, J = 7.7 Hz, 1H, $CH_{(Ar)}$), 7.31 – 7.26 (m, 1H, $CH_{(Pyr)}$), 6.97 (dd, J = 3.9, 1.8 Hz, 1H, $CH_{(Pyr)}$), 6.60 (d, J = 7.6 Hz, 1H, $CH_{(Ar)}$), 6.24 (dd, J = 3.9, 2.6 Hz, 1H, $CH_{(Pyr)}$), 5.64 (s, 2H, CH_2), 5.24 (s, 1H, $CH(OCH_3)_2$), 3.65 (s, 3H, $COOCH_3$), 3.31 (s, 6H, $(OCH_3)_2$).

6-((4-Methylpiperazin-1-yl)methyl)picolinaldehyde. A solution of 1-((6-(dimethoxymethyl)pyridin-2-yl)methyl)-4-methylpiperazine (0.93 g, 3.50 mmol) in water (35 mL) and 12M HCl (0.91 mL, 12.25 mmol) in a 100 mL round-bottom flask was stirred at 60 °C overnight. The next day the reaction mixture was combined with a saturated aqueous solution of sodium bicarbonate (about 100 mL) and extracted with dichloromethane (3 × 40 mL). The united organic phases were dried over magnesium sulfate and the solvent was removed under reduced pressure. The product was obtained as a yellow oil. Yield: 0.51 g, 67%. ^1H NMR (500 MHz, DMSO- d_6) δ 9.97 (d, J = 0.7 Hz, 1H, CHO), 8.03 (t, J = 7.7 Hz, 1H, $CH_{(Ar)}$), 7.83 (dd, J = 7.6, 0.9 Hz, 1H, $CH_{(Ar)}$), 7.75 (dd, J = 7.7, 0.9 Hz, 1H, $CH_{(Ar)}$), 3.70 (s, 2H, CH_2), 2.48 – 2.29 (m, 8H, $CH_{2(Pip)}$), 2.17 (s, 3H, $CH_3(Pip)$).

6-(Morpholinomethyl)picolinaldehyde. 4-((6-(Dimethoxymethyl)pyridin-2-yl)methyl) morpholine (0.92 g, 3.65 mmol) was mixed with water (35 mL) and 12M HCl (0.95 mL, 12.78 mmol) in a 100 mL round-bottom flask. The reaction mixture was stirred at 60 °C overnight. The next day the reaction mixture was combined with a saturated aqueous of sodium bicarbonate (about 100 mL) and extracted with dichloromethane (3 × 40 mL). The united organic phases were dried over magnesium

sulfate and the solvent was removed under reduced pressure. The product was obtained as a yellow oil. Yield: 0.62 g, 82 %. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 9.98 (d, J = 0.7 Hz, 1H, CHO), 8.04 (t, J = 7.7 Hz, 1H, $\text{CH}_{(\text{Ar})}$), 7.87 – 7.81 (m, 1H, $\text{CH}_{(\text{Ar})}$), 7.78 (dd, J = 7.7, 1.1 Hz, 1H, $\text{CH}_{(\text{Ar})}$), 3.72 (s, 2H, CH_2), 3.64 – 3.58 (m, 4H, $\text{CH}_{2(\text{Morph})}$), 2.47 – 2.42 (m, 4H, $\text{CH}_{2(\text{Morph})}$).

Methyl 1-((6-formylpyridin-2-yl)methyl)-1H-pyrrole-2-carboxylate. To a solution of methyl 1-((6-(dimethoxymethyl)pyridin-2-yl)methyl)-1H-pyrrole-2-carboxylate (0.21 g, 0.72 mmol) in acetone (5 mL) in a 100 mL round-bottom flask was added water (25 mL) and the reaction mixture was refluxed overnight. The next day the solvent was removed under reduced pressure to yield a white solid, which was further dried in vacuo. Yield: 0.18 g, 100%. ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 9.95 (d, J = 0.7 Hz, 1H, CHO), 7.99 (td, J = 7.8, 0.6 Hz, 1H, $\text{CH}_{(\text{Ar})}$), 7.82 (dd, J = 7.7, 0.9 Hz, 1H, $\text{CH}_{(\text{Ar})}$), 7.36 (dd, J = 2.5, 1.9 Hz, 1H, $\text{CH}_{(\text{Pyr})}$), 7.02 – 6.92 (m, 2H, $\text{CH}_{(\text{Pyr})}$, $\text{CH}_{(\text{Ar})}$), 6.27 (dd, J = 3.9, 2.6 Hz, 1H, $\text{CH}_{(\text{Pyr})}$), 5.76 (s, 2H, CH_2), 3.66 (s, 3H, COOCH_3).

mPip-FTSC·0.2CH₃OH (HL¹·0.2CH₃OH). A suspension of 6-((4-methylpiperazin-1-yl)methyl) picolinaldehyde (500 mg, 2.28 mmol) and thiosemicarbazide (208 mg, 2.28 mmol) in dry ethanol (6 mL) in a 25 mL Schlenk tube was stirred at 78 °C overnight. The color of the reaction mixture changed from yellow/orange to red/purple. The next day the solvent was removed under reduced pressure and the crude product was purified on preparative HPLC (water/methanol). The product was obtained as a pale-green powder after drying in vacuo. Yield: 0.51 g, 76%. Anal. Calcd for $\text{C}_{13}\text{H}_{20}\text{N}_6\text{S}\cdot 0.2\text{CH}_3\text{OH}$ (M 298.81 g/mol): C, 53.05; H, 7.01; N, 28.12; S, 10.73. Found: C, 53.13; H, 6.88; N, 28.28; S, 10.35. *E*-isomer: ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 11.63 (s, 1H, H^2), 8.33 (s, 1H, H^3), 8.19 – 8.09 (m, 2H, H^6 , H^3), 8.06 – 8.01 (m, 1H, H^{12}), 7.79 (t, J = 7.8 Hz, 1H, H^5), 7.39 (m, 1H, H^4), 3.57 (s, 2H, H^7), 2.35 (m, 8H, H^8 , H^9 , H^{10} , H^{11}), 2.15 (s, 3H, H^{14}). ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) δ 178.80 (Cq, C^{13}), 158.80 (Cq, C^3), 153.13 (Cq, C^1), 143.07 (CH, C^{12}), 137.35 (CH, C^5), 123.44 (CH, C^4), 118.97 (CH, C^6), 64.02 (CH_2 , C^7), 55.18 (2CH_2 , C^9 , C^{10}), 53.21 (2CH_2 , C^8 , C^{11}), 46.22 (CH_3 , C^{14}). *Z*-isomer: ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 14.27 (s, 1H, H^2), 8.51 (s, 1H, H^3), 8.19 – (m, 1H, H^3), 8.06 – 8.01 (m, 1H, H^5), 7.64 (d, J = 7.5 Hz, 1H, H^6), 7.55 (d, J = 7.6 Hz, 1H, H^4), 7.41 – 7.35 (m, 1H, H^{12}), 3.70 (s, 2H, H^7), 2.49 – 2.21 (m, 8H, H^8 , H^9 , H^{10} , H^{11}), 2.15 (s, 3H, H^{14}). ^{13}C NMR (126 MHz, $\text{DMSO-}d_6$) δ 179.34 (Cq, C^{13}), 157.93 (Cq, C^3), 151.62 (Cq, C^1), 139.16 (CH, C^5), 133.91 (CH, C^{12}), 125.26 (CH, C^6), 124.18 (CH, C^4), 63.79 (CH_2 , C^7), 55.18 (2CH_2 , C^9 , C^{10}), 53.08 (2CH_2 , C^8 , C^{11}), 46.22 (CH_3 , C^{14}). For atom numbering and structures of *E* and *Z* isomers see SI, Scheme S1. Solubility in water ≥ 3.3 mg/mL. Electrospray ionization mass spectrometry (ESI-MS, methanol), positive: m/z 293 ($[\text{M} + \text{H}]^+$). IR (attenuated total reflectance (ATR), selected bands,

$\tilde{\nu}_{\max}$): 3420, 3258, 3162, 2940, 2802, 1599, 1546, 1446, 1342, 1280, 1146, 983, 924, 832, 789, 732, 684 cm^{-1} . UV-vis in water (52 μM) most intense bands λ , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 315 (30681).

mPip-dm-FTSC·0.25CH₃OH (HL²·0.25CH₃OH). A suspension of 6-((4-methylpiperazin-1-yl)methyl)picolinaldehyde (405 mg, 1.85 mmol) and 4,4-dimethyl-3-thiosemicarbazide (220 mg, 1.85 mmol) in dry ethanol (6 mL) in a 25 mL Schlenk tube was stirred at room temperature overnight. The next day the solvent was removed under reduced pressure and the crude product was purified on preparative HPLC (water/methanol). The product was obtained as a yellow powder after drying in vacuo. Yield: 0.52 g, 87%. Anal. Calcd for C₁₅H₂₄N₆S·0.25CH₃OH (M 328.47 g/mol): C, 55.76; H, 7.67; N, 25.59; S, 9.76. Found: C, 55.93; H, 7.59; N, 25.90; S, 9.38. *E*-isomer: ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.16 (s, 1H, H²), 8.18 (s, 1H, H¹²), 7.84 – 7.71 (m, 2H, H⁶, H⁵), 7.39 (d, *J* = 7.3 Hz, 1H, H⁴), 3.57 (s, 2H, H⁷), 3.31 (s, 6H, H¹⁴, H¹⁵), 2.47 – 2.19 (m, 8H, H⁸, H⁹, H¹⁰, H¹¹), 2.15 (s, 3H, H¹⁶). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 181.10 (Cq, C¹³), 158.93 (Cq, C³), 153.36 (Cq, C¹), 144.32 (CH, C¹²), 137.55 (CH, C⁵), 123.22 (CH, C⁴), 118.25 (CH, C⁶), 64.04 (CH₂, C⁷), 55.19 (2CH₂, C⁹, C¹⁰), 53.20 (2CH₂, C⁸, C¹¹), 46.22 (CH₃, C¹⁶), 42.83 (2CH₃, C¹⁴, C¹⁵). *Z*-isomer: ¹H NMR (500 MHz, DMSO-*d*₆) δ 14.89 (s, 1H, H²), 8.05 (t, *J* = 7.8 Hz, 1H, H⁵), 7.65 (d, *J* = 7.7 Hz, 1H, H⁶), 7.59 (s, 1H, H¹²), 7.53 (d, *J* = 7.7 Hz, 1H, H⁴), 3.62 (s, 2H, H⁷), 3.42 (s, 6H, H¹⁴, H¹⁵), 2.47 – 2.19 (m, 8H, H⁸, H⁹, H¹⁰, H¹¹), 2.15 (s, 3H, H¹⁶). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 180.62 (Cq, C¹³), 157.65 (Cq, C³), 151.94 (Cq, C¹), 139.25 (CH, C⁵), 136.62 (CH, C¹²), 124.94 (CH, C⁶), 124.30 (CH, C⁴), 63.66 (CH₂, C⁷), 55.08 (2CH₂, C⁹, C¹⁰), 53.25 (2CH₂, C⁸, C¹¹), 46.18 (CH₃, C¹⁶), 40.59 (2CH₃, C¹⁴, C¹⁵, overlapped with residual DMSO signal). For atom numbering and structures of *E* and *Z* isomers see Supporting Information (SI), Scheme S1. Solubility in water $\geq$ 11.5 mg/mL. ESI-MS (methanol), positive: *m/z* 321 ([M + H]⁺). IR (ATR, selected bands, $\tilde{\nu}_{\max}$): 3039, 2929, 2801, 1597, 1542, 1446, 1361, 1156, 821, 711, 618 cm^{-1} . UV-vis in water (51 μM), λ , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 216 (20784), 271sh (14510), 314 (31569).

Morph-FTSC·0.3CH₃OH·0.1H₂O (HL³·0.3CH₃OH·0.1H₂O). A suspension of 6-(morpholinomethyl)picolinaldehyde (300 mg, 1.45 mmol) and thiosemicarbazide (132 mg, 1.45 mmol) in dry ethanol (5 mL) in a 25 mL Schlenk tube was stirred at 78 °C overnight. The next day the solvent was removed under reduced pressure and the crude product was recrystallized from water/methanol (5:1) to give a white powder which was filtered off, washed with water and dried in vacuo. Yield: 0.37 g, 91%. Anal. Calcd for C₁₂H₁₇N₃O₂S·0.3CH₃OH·0.1H₂O (M 306.78 g/mol): C, 48.16; H, 6.05; N, 22.83; S, 10.45. Found: C, 48.11; H, 6.43; N, 23.13; S, 10.66. *E*-isomer: ¹H NMR

(500 MHz, DMSO- d_6) δ 11.63 (s, 1H, H₂), 8.33 (s, 1H, H₃), 8.16 (m, 2H, H₆, H₃), 8.08 – 7.99 (m, 1H, H₁₂), 7.80 (t, J = 7.8 Hz, 1H, H₅), 7.42 (d, J = 7.6 Hz, 1H₄), 3.66 – 3.52 (m, 6H, H₇, H₉, H₁₀), 2.42 (m, 4H, H₈, H₁₁). ^{13}C NMR (126 MHz, DMSO- d_6) δ 178.81 (Cq, C¹³), 158.35 (Cq, C³), 153.20 (Cq, C¹), 143.03 (CH, C¹²), 137.37 (CH, C⁵), 123.58 (CH, C⁴), 119.05 (CH, C⁶), 66.66 (2CH₂, C⁹, C¹⁰), 64.37 (CH₂, C⁷), 53.79 (2CH₂, C⁹, C¹⁰). *Z*-isomer: ^1H NMR (500 MHz, DMSO- d_6) δ 14.26 (s, 1H, H²), 8.52 (s, 1H, H³), 8.16 (m, 1H, H³), 8.08 – 7.99 (m, 1H, H⁵), 7.65 (d, J = 7.5 Hz, 1H, H⁶), 7.58 (d, J = 7.8 Hz, 1H, H⁴), 7.39 (s, 1H, H¹²), 3.71 (s, 2H, H⁷), 3.66 – 3.52 (m, 4H, H⁹, H¹⁰), 2.48 (m, 4H, H⁸, H¹¹). ^{13}C NMR (126 MHz, DMSO- d_6) δ 179.37 (Cq, C¹³), 157.51 (Cq, C³), 151.68 (Cq, C¹), 139.19 (CH, C⁵), 133.91 (CH, C¹²), 125.31 (CH, C⁶), 124.30 (CH, C⁴), 66.66 (2CH₂, C⁹, C¹⁰), 64.10 (CH₂, C⁷), 53.69 (2CH₂, C⁹, C¹⁰). For atom numbering and structures of *E* and *Z* isomers see SI, Scheme S1. Solubility in water (with 1% DMSO) $\geq$ 1.4 mg/mL. ESI-MS (methanol), positive: m/z 280 ($[\text{M} + \text{H}]^+$). IR (ATR, selected bands, $\tilde{\nu}_{\text{max}}$): 3462, 3268, 3167, 2816, 1611, 1522, 1452, 1261, 1109, 1067, 850, 645 cm^{-1} . UV-vis in water (39 μM), λ , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 316 (26923).

Morph-dm-FTSC (HL⁴). A suspension of 6-(morpholinomethyl)picolinaldehyde (300 mg, 1.45 mmol) and 4,4-dimethyl-3-thiosemicarbazide (173 mg, 1.45 mmol) in dry ethanol (5 mL) in a 25 mL Schlenk tube was stirred at room temperature overnight. The next day a white precipitate was filtered off under inert conditions. The precipitate was washed with dry ethanol (1 mL) and dried in vacuo to give a white powder. Yield: 0.34 g, 76%. Anal. Calcd for C₁₄H₂₁N₅OS (M 307.42 g/mol): C, 54.70; H, 6.89; N, 22.78; S, 10.43. Found: C, 54.85; H, 6.92; N, 22.71; S, 10.28. *E*-isomer: ^1H NMR (500 MHz, DMSO- d_6) δ 11.16 (s, 1H, H²), 8.18 (s, 1H, H¹²), 7.84 – 7.74 (m, 2H, H⁵, H⁶), 7.44 – 7.39 (m, 1H, H⁴), 3.62 – 3.53 (m, 6H, H⁷, H⁹, H¹⁰), 3.31 (s, 6H, H¹⁴, H¹⁵), 2.45 – 2.40 (m, 4H, H⁸, H¹¹). ^{13}C NMR (126 MHz, DMSO- d_6) δ 181.07 (Cq, C¹³), 158.49 (Cq, C³), 153.45 (Cq, C¹), 144.30 (CH, C¹²), 137.58 (CH, C⁵), 123.34 (CH, C⁴), 118.32 (CH, C⁶), 66.67 (2CH₂, C⁹, C¹⁰), 64.38 (CH₂, C⁷), 53.78 (2CH₂, C⁸, C¹¹), 42.84 (2CH₃, C¹⁴, C¹⁵). *Z*-isomer: ^1H NMR (500 MHz, DMSO- d_6) δ 14.88 (s, 1H, H²), 8.05 (t, J = 7.8 Hz, 1H, H⁵), 7.66 (d, J = 7.7 Hz, 1H, H⁶), 7.59 (s, 1H, H¹²), 7.56 (d, J = 7.7 Hz, 1H, H⁴), 3.63 (s, 2H, H⁷), 3.62 – 3.53 (m, 4H, H⁹, H¹⁰), 3.42 (s, 6H, H¹⁴, H¹⁵), 2.40 – 2.35 (m, 4H, H¹¹, H⁸). ^{13}C NMR (126 MHz, DMSO- d_6) δ 180.61 (Cq, C¹³), 157.21 (Cq, C³), 152.00 (Cq, C¹), 139.27 (CH, C⁵), 136.59 (CH, C¹²), 125.02 (CH, C⁶), 124.41 (CH, C⁴), 66.58 (2CH₂, C⁹, C¹⁰), 64.01 (CH₂, C⁷), 53.78 (2CH₂, C⁸, C¹¹), 40.63 (2CH₃, C¹⁴, C¹⁵, overlapped with residual DMSO signal). For atom numbering and structures of *E* and *Z* isomers see SI, Scheme S1. Solubility in water $\geq$ 2.6 mg/mL. ESI-MS (methanol), positive: m/z 308 ($[\text{M} + \text{H}]^+$). IR (ATR, selected bands, $\tilde{\nu}_{\text{max}}$): 2923,

2821, 1593, 1532, 1313, 1145, 1107, 901, 821, 707, 622 cm^{-1} . UV-vis in water (48 μM), λ , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 216 (19628), 273 (13430), 315 (21488).

mPyr-FTSC (HL⁵). A suspension of methyl 1-((6-formylpyridin-2-yl)methyl)-1H-pyrrole-2-carboxylate (0.06 g, 0.24 mmol) and thiosemicarbazide (0.02 g; 0.24 mmol) in a 1 : 1 mixture of methanol/ethanol (2 mL) in a 10 mL Schlenk tube was stirred at 78 °C overnight. The next day the solvent was removed under reduced pressure and the crude product was recrystallized from water/methanol (5:1). The resulting white powder was filtered off, washed with a water/methanol 1 : 1 mixture and dried in vacuo. Yield: 0.06 g, 76%. Anal. Calcd. for $\text{C}_{14}\text{H}_{15}\text{N}_5\text{O}_2\text{S}$ (M 317.37 g/mol): C, 52.98; H, 4.76; N, 22.07; S, 10.10. Found: C, 52.98; H, 4.65; N, 21.97; S, 10.00. *E*-isomer: ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 11.66 (s, 1H, H^2), 8.35 (s, 1H, H^3), 8.20 – 8.11 (m, 2H, H^3 , H^6), 8.04 (s, 1H, H^{13}), 7.75 (t, $J = 7.9$ Hz, 1H, H^{13}), 7.33 – 7.29 (m, 1H, H^9), 7.00 – 6.92 (m, 1H, H^{11}), 6.60 (d, $J = 7.7$ Hz, 1H, H^4), 6.28 – 6.21 (m, 1H, H^{10}), 5.64 (s, 2H, H^7), 3.66 (s, 3H, H^{15}). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 178.84 (Cq, C^{15}), 161.08 (Cq, C^{12}), 158.54 (Cq, C^3), 153.30 (Cq, C^1), 142.66 (CH, C^{13}), 137.99 (CH, C^5), 131.10 (CH, C^9), 121.65 (Cq, C^8), 120.41 (CH, C^4), 119.08 (CH, C^6), 118.61 (CH, C^{11}), 109.05 (CH, C^{10}), 53.37 (CH_2 , C^7), 51.39 (CH_3 , C^{15}). *Z*-isomer: ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 14.05 (s, 1H, H^2), 8.60 (s, 1H, H^3), 8.24 (s, 1H, H^3), 8.00 (t, $J = 7.8$ Hz, 1H, H^5), 7.66 (d, $J = 7.6$ Hz, 1H, H^6), 7.64 – 7.59 (m, 1H, H^9), 7.40 (s, 1H, H^{13}), 6.98 – 6.91 (m, 2H, H^4 , H^{11}), 6.28 – 6.21 (m, 1H, H^{10}), 5.69 (s, 2H, H^7), 3.68 (s, 3H, H^{15}). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 179.40 (Cq, C^{14}), 161.08 (Cq, C^{12}), 157.34 (Cq, C^3), 151.83 (Cq, C^1), 139.84 (CH, C^5), 133.66 (CH, C^{13}), 131.63 (CH, C^9), 125.65 (CH, C^6), 122.07 (CH, C^4), 121.30 (Cq, C^8), 118.95 (CH, C^{11}), 109.37 (CH, C^{10}), 53.37 (CH_2 , C^7), 51.47 (CH_3 , C^{15}). ESI-MS (methanol), positive: m/z 340 ($[\text{M} + \text{Na}]^+$), 317 ($[\text{M} + \text{H}]^+$). IR (ATR, selected bands, $\tilde{\nu}_{\text{max}}$): 3561, 3353, 3243, 2972, 1706, 1612, 1530, 1443, 1245, 723, 653, 608 cm^{-1} . UV-vis in methanol (22 μM), λ , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 204 (13636), 237 (12182), 266 (17227), 324 (25545), 388 (1227).

mPyr-dm-FTSC (HL⁶). A solution of methyl 1-((6-formylpyridin-2-yl)methyl)-1H-pyrrole-2-carboxylate (0.18 g, 0.72 mmol) and 4,4-dimethyl-3-thiosemicarbazide (0.09 g; 0.72 mmol) in a 1 : 1 mixture of methanol/ethanol (4 mL) in a 25 mL Schlenk tube was stirred at room temperature for 6 h. The white precipitate was filtered off, washed with a water/methanol 1 : 1 mixture and dried in vacuo. Yield: 0.12 g, 47%. Anal. Calcd for $\text{C}_{16}\text{H}_{19}\text{N}_5\text{O}_2\text{S}$ (M 345.42 g/mol): C, 55.63; H, 5.54; N, 20.27; S, 9.28. Found: C, 55.43; H, 5.50; N, 20.06; S, 9.21. *E*-isomer: ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 11.18 (s, 1H, H^2), 8.17 (s, 1H, H^{13}), 7.79 – 7.73 (m, 2H, H^6 , H^5), 7.33 – 7.27 (m, 1H, H^9), 6.96

(dd, $J = 3.9, 1.8$ Hz, 1H, H^{11}), 6.69 – 6.62 (m, 1H, H^4), 6.23 (dd, $J = 3.9, 2.6$ Hz, 1H, H^{10}), 5.65 (s, 2H, H^7), 3.66 (s, 3H, H^{17}), 3.31 (s, 6H, H^{15}, H^{16}). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 180.99 (Cq, C^{14}), 161.06 (Cq, C^{12}), 158.57 (Cq, C^3), 153.55 (Cq, C^1), 144.09 (CH, C^{13}), 138.14 (CH, C^5), 131.12 (CH, C^9), 121.66 (Cq, C^8), 120.34 (CH, C^4), 118.56 (CH, C^{11}), 118.35 (CH, C^6), 108.96 (CH, C^{10}), 53.40 (CH_2 , C^7), 51.38 (CH_3 , C^{17}), 42.76 (2CH_3 , C^{15}, C^{16}). *Z*-isomer: ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 14.71 (s, 1H, H^2), 7.99 (t, $J = 7.9$ Hz, 1H, H^5), 7.65 (d, $J = 7.7$ Hz, 1H, H^6), 7.62 (s, 1H, H^{13}), 7.40 – 7.35 (m, 1H, H^9), 7.02 (dd, $J = 4.0, 1.8$ Hz, 1H, H^{11}), 6.49 (d, $J = 7.8$ Hz, 1H, H^4), 6.30 (dd, $J = 3.9, 2.6$ Hz, 1H, H^{10}), 5.73 (s, 2H, H^7), 3.64 (s, 3H, H^{17}), 3.44 (s, 6H, H^{15}, H^{16}). ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 180.60 (Cq, C^{14}), 161.01 (Cq, C^{12}), 158.01 (Cq, C^3), 151.49 (Cq, C^1), 140.11 (CH, C^5), 136.28 (CH, C^{13}), 131.12 (CH, C^9), 124.83 (CH, C^6), 121.66 (Cq, C^8), 120.10 (CH, C^4), 118.94 (CH, C^{11}), 109.49 (CH, C^{10}), 53.40 (CH_2 , C^7), 51.51 (CH_3 , C^{17}), 40.60 (C^{15}, C^{16} , overlapped with residual DMSO signal). For atom numbering and structures of *E* and *Z* isomers see SI, Scheme S1. ESI-MS (methanol), positive: m/z 368 ($[\text{M} + \text{Na}]^+$), 346 ($[\text{M} + \text{H}]^+$). IR (ATR, selected bands, $\tilde{\nu}_{\text{max}}$): 2837, 1693, 1541, 1316, 1252, 1106, 899, 757, 622 cm^{-1} . UV-vis in methanol (28 μM), λ , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 235 (23929), 266 (27321), 321 (21786), 405 (2757).

Synthesis of the copper(II) complexes

[CuCl(mPipH-FTSC-H)]Cl·0.1H₂O ((1+H)Cl·0.1H₂O). To a solution of mPip-FTSC (**HL**¹) (0.05 g, 0.17 mmol) in methanol (20 mL) was added a solution of copper(II) chloride dihydrate (0.03 g, 0.17 mmol) in methanol (5 mL). The reaction mixture was stirred at room temperature overnight. The next day a green precipitate was filtered off, washed with methanol and dried in vacuo. Yield: 0.05 g, 74%. Anal. Calcd for $\text{C}_{13}\text{H}_{20}\text{Cl}_2\text{CuN}_6\text{S}\cdot 0.1\text{H}_2\text{O}$ (M 428.66 g/mol): C, 36.43; H, 4.75; N, 19.61; S, 7.48. Found: C, 36.49; H, 4.74; N, 19.24; S, 7.48. Solubility in water ≥ 13.2 mg/mL. ESI-MS (methanol), positive: m/z 354 ($[\text{M} - \text{Cl}]^+$). IR (ATR, selected bands, $\tilde{\nu}_{\text{max}}$): 3266, 3094, 1613, 1459, 1418, 1158, 1025, 978, 825, 787, 653 cm^{-1} . UV-vis in water, λ , nm (ϵ , $\text{M}^{-1}\text{cm}^{-1}$): 286 (19545), 392 (10063) (measured at 44 μM); 607 (265) (measured at 1.88 mM). X-ray diffraction quality crystals of the composition **1**·0.15CH₃OH were obtained after slow diffusion of diethyl ether into a methanolic solution of **1** ($c \approx 5$ mg/mL) in the presence of a small amount of triethylamine.

[CuCl(mPipH-dm-FTSC-H)]Cl·0.9H₂O·0.5CH₃OH ((2+H)Cl·0.9H₂O·0.5CH₃OH). To a solution of mPip-dm-FTSC (**HL**²) (0.17 g, 0.53 mmol) in methanol (20 mL) was added a solution of copper(II) chloride dihydrate (0.10 g, 0.58 mmol) in methanol (5 mL) and triethylamine (80.8 μL ; 0.64 mmol). The reaction mixture was stirred at room temperature overnight. The next day the

solvent was concentrated under reduced pressure to about 10 mL. After slow diffusion of diethyl ether green crystals appeared which were filtered off, washed with methanol and dried in vacuo. The obtained crystals were of X-ray diffraction quality. Yield: 0.14 g, 53%. Anal. Calcd for $C_{15}H_{24}Cl_2CuN_6S \cdot 0.9H_2O \cdot 0.5CH_3OH$ (M 487.14 g/mol): C, 38.22; H, 5.75; N, 17.25; S, 6.58. Found: C, 38.23; H, 5.40; N, 17.37; S, 6.43. Solubility in water ≥ 20.2 mg/mL. ESI-MS (methanol), positive: m/z 382 ($[M - Cl]^+$). IR (ATR, selected bands, $\tilde{\nu}_{max}$): 3458, 3394, 3037, 2690, 1492, 1369, 1311, 1249, 1130, 908, 612 cm^{-1} . UV-vis in water, λ , nm (ϵ , $M^{-1}cm^{-1}$): 254 (11463), 299 (17561), 405 (15366) (measured at 41 μM); 574 (145) (measured at 1.79 mM).

[CuCl(Morph-FTSC-H)] $\cdot$ 2H₂O $\cdot$ 0.2C₂H₅OH (3 $\cdot$ 2H₂O $\cdot$ 0.2C₂H₅OH). To a solution of Morph-FTSC (**HL**³) (0.15 g, 0.54 mmol) in ethanol (35 mL) were added a solution of copper(II) chloride dihydrate (0.09 g, 0.54 mmol) in ethanol (5 mL) and triethylamine (75 μL , 0.54 mmol). The reaction mixture was stirred at room temperature overnight. The next day a green precipitate was filtered off, washed with ethanol and dried in vacuo. X-ray diffraction quality crystals were obtained after slow diffusion of diethyl ether into a methanolic solution of **3** ($c \approx 5$ mg/mL). Yield: 0.20 g, 88%. Anal. Calcd for $C_{12}H_{16}ClCuN_5OS \cdot 2H_2O \cdot 0.2C_2H_5OH$ (M 422.60 g/mol): C, 35.24; H, 5.06; N, 16.57; S, 7.59. Found: C, 35.35; H, 4.81; N, 16.42; S, 7.61. Solubility in water ≥ 1.1 mg/mL. ESI-MS (methanol), positive: m/z 341 ($[M - Cl]^+$). IR (ATR, selected bands, $\tilde{\nu}_{max}$): 3431, 3367, 3109, 1677, 1640, 1462, 1419, 1166, 1114, 783, 630 cm^{-1} . UV-vis in water, λ , nm (ϵ , $M^{-1}cm^{-1}$): 284 (20179), 389 (10893) (measured at 56 μM); 598 (252) (measured at 2.83 mM).

[CuCl(Morph-dm-FTSC-H)] $\cdot$ 0.2H₂O $\cdot$ 0.6CH₃OH (4 $\cdot$ 0.2H₂O $\cdot$ 0.6CH₃OH). To a solution of Morph-dm-FTSC (**HL**⁴) (0.15 g, 0.49 mmol) in methanol (20 mL) were added a solution of copper(II) chloride dihydrate (0.08 g, 0.49 mmol) in methanol (5 mL) and triethylamine (68 μL , 0.49 mmol). The reaction mixture was stirred at room temperature overnight. The next day the solvent was removed under reduced pressure to about 10 mL. After slow diffusion of diethyl ether green crystals appeared which were filtered off, washed with methanol and dried in vacuo. The obtained crystals were of X-ray diffraction quality. Yield: 0.21 g, 98%. Anal. Calcd for $C_{14}H_{20}ClCuN_5OS \cdot 0.2H_2O \cdot 0.6CH_3OH$ (M 428.23 g/mol): C, 40.95; H, 5.37; N, 16.35; S, 7.49. Found: C, 40.84; H, 5.29; N, 16.23; S, 7.57. Solubility in water ≥ 12.9 mg/mL. ESI-MS (methanol), positive: m/z 369 ($[M - Cl]^+$). IR (ATR, selected bands, $\tilde{\nu}_{max}$): 3499, 2859, 1593, 1359, 1242, 1123, 909, 869, 789 cm^{-1} . UV-vis in water, λ , nm (ϵ , $M^{-1}cm^{-1}$): 255 (10474), 299 (17207), 405 (15212) (measured at 40 μM); 575 (293) (measured at 1.78 mM).

[CuCl(mPyrr-FTSC-H)(H₂O)]·0.2H₂O (5·0.2H₂O). To a solution of mPyrr-FTSC (**HL**⁵) (0.05 g, 0.16 mmol) in methanol (15 mL) was added a solution of copper(II) chloride dihydrate (0.03 g, 0.17 mmol) in methanol (7 mL). The reaction mixture was stirred at 60 °C for 1 h and then allowed to stand at 4 °C for 2 h. A green microcrystalline precipitate was filtered off, washed with methanol and dried in vacuo. X-ray diffraction quality crystals were obtained after slow diffusion of diethyl ether into a DMF solution of **5** (*c* ≈ 5 mg/mL). Yield: 0.05 g, 71%. Anal. Calcd for C₁₄H₁₆ClCuN₅O₃S·0.2H₂O (M 436.98 g/mol): C, 38.48; H, 3.78; N, 16.03; S, 7.34. Found: C, 38.76; H, 3.39; N, 15.75; S, 7.05. ESI-MS (methanol), positive: *m/z* 379 ([M – Cl – H₂O]⁺). IR (ATR, selected bands, $\tilde{\nu}_{\max}$): 3346, 3105, 1704, 1622, 1463, 1406, 1332, 1253, 1111, 734, 635 cm⁻¹. UV-vis in DMF, λ , nm (ϵ , M⁻¹cm⁻¹): 298 (16447), 326 (13026, sh), 422 (11474) (measured at 76 μ M); 514 (274, sh), 684 (225) (measured at 2.29 mM).

[CuCl(mPyrr-dm-FTSC-H)(H₂O)] (6). To a warm solution of mPyrr-dm-FTSC (**HL**⁶) (0.07 g, 0.20 mmol) in methanol a solution of copper(II) chloride dihydrate (0.04 g; 0.20 mmol) was added. The formation of a green precipitate started immediately and the reaction mixture was allowed to stir at room temperature overnight. The next day the green precipitate was filtered off, washed extensively with methanol and dried in vacuo. Yield: 0.05 g, 55%. Anal. Calcd for C₁₆H₂₀ClCuN₅O₃S (M 461.43 g/mol): C, 41.65; H, 4.37; N, 15.18; S, 6.94. Found: C, 41.80; H, 4.35; N, 15.04; S, 6.81. ESI-MS (methanol), positive: *m/z* 407 ([M – Cl – H₂O]⁺). IR (ATR, selected bands, $\tilde{\nu}_{\max}$): 3420, 3006, 1705, 1508, 1377, 1246, 1113, 913, 735, 587 cm⁻¹. UV-vis in DMF, λ , nm (ϵ , M⁻¹cm⁻¹): 306 (13851), 426 (15946) (measured at 74 μ M); 519 (435), 648 (256) (measured at 2.21 mM).

pH-potentiometric measurements. The purity and aqueous phase stability of the ligands mPip-dm-FTSC (**HL**²) and Morph-dm-FTSC (**HL**⁴) were verified and the exact concentrations of the stock solutions prepared were determined by the Gran method.⁶³ The pH-metric measurements for determination of the protonation constants of the ligands and the overall stability constants of the copper(II) complexes were carried out at 298.0 ± 0.1 K in water and at an ionic strength of 0.10 M (KCl) in order to keep the activity coefficients constant. The titrations were performed with carbonate-free KOH solution of known concentration (0.10 M). The concentrations of the KOH and the HCl 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 were used for

the pH-metric measurements. The electrode system was calibrated to the $\text{pH} = -\log[\text{H}^+]$ scale in water according to the method suggested by Irving *et al.*⁶⁴ The average water ionization constant $\text{p}K_{\text{w}}$, is 13.76 ± 0.01 , which corresponds well to the literature data.⁶⁵ The reproducibility of the titration points included in the calculations was within 0.005 pH. The pH-metric titrations were performed in the pH range 2.0 – 11.5. The initial volume of the samples was 5.0 mL. The concentration of the ligands was 2 mM and metal ion-to-ligand ratios of 1:1, 1:1.5, 1:2 and 1:3 were used. The accepted fitting of the titration curves was always less than 0.01 mL. Samples were deoxygenated by bubbling purified argon through them for *ca.* 10 min prior to the measurements and argon was also passed over the solutions during the titrations.

The protonation constants of the ligands were determined with the computer program HYPERQUAD.⁶⁶ PSEQUAD⁶⁷ was utilized to establish the stoichiometry of the complexes and to calculate the stability constants ($\log \beta(\text{M}_p\text{L}_q\text{H}_r)$). $\beta(\text{M}_p\text{L}_q\text{H}_r)$ is defined for the general equilibrium $p\text{M} + q\text{L} + r\text{H} \rightleftharpoons \text{M}_p\text{L}_q\text{H}_r$ as $\beta(\text{M}_p\text{L}_q\text{H}_r) = [\text{M}_p\text{L}_q\text{H}_r]/[\text{M}]^p[\text{L}]^q[\text{H}]^r$, where M denotes the metal ion (copper(II)) and L the completely deprotonated ligand. In all calculations exclusively titration data were used from experiments, in which no precipitate was visible in the reaction mixture.

UV–vis spectrophotometric, spectrofluorimetric and ^1H NMR measurements. A Thermo Scientific Evolution 220 spectrophotometer was used to record the UV–vis spectra in the 200 to 1050 nm window. The path length was 1 or 0.5 cm. Stability constants and the individual spectra of the complexes were calculated by the computer program PSEQUAD.⁶⁷ The spectrophotometric titrations were performed on samples of the copper(II) complexes **2** and **4** of ligands mPip-dm-FTSC (**HL**²) and Morph-dm-FTSC (**HL**⁴) over the pH range between 2 and 11.5 at an ionic strength of 0.10 M (KCl) in water at 298.0 ± 0.1 K. The concentration of the metal complexes was 2.5 mM. Measurements on the copper(II) complexes **2** and **4** were also carried out by preparing individual samples, in which the 0.1 M KCl was partially or completely replaced by HCl and pH values, varying in the range of approximately 1.0 – 3.0, were calculated from the HCl content. The conditional stability constants of $[\text{CuL}]$ at pH 7.4 (10 mM HEPES) for **2** and at pH 5.6 (10 mM MES) for **4** were determined from competition titrations of the copper(II) complex of EDTA with the ligands **HL**² and **HL**⁴. Samples contained 50 μM copper(II) ion and 50 μM EDTA, and the concentration of the ligands **HL**² and **HL**⁴ was varied in the range of 0 – 170 μM . Absorbance data recorded after 1.5 h incubation time in the wavelength interval from 415 to 450 nm were used for the calculations.

Three-dimensional fluorescence spectra of the ligands mPip-dm-FTSC (**HL**²) and Morph-dm-FTSC (**HL**⁴) and their copper(II) complexes (**2** and **4**) were recorded at 240 – 500 nm excitation and at 300 – 700 nm emission wavelengths for the 10 μ M ligand containing samples in 1 cm quartz cell at pH 7.4 (10 mM HEPES) using 5 nm/5 nm slit widths at 0.1 M (KCl) ionic strength and 298.0 ± 0.1 K.

The pH-dependent ¹H NMR studies were carried out on a Bruker Ultrashield 500 Plus instrument. 4,4-Dimethyl-4-silapentane-1-sulfonic acid was used as an internal NMR standard. Ligands mPip-dm-FTSC (**HL**²) and Morph-dm-FTSC (**HL**⁴) were dissolved in a 10% (v/v) D₂O /H₂O mixture in a concentration of 3.0 and 1.5 mM, respectively. The direct pH-meter readings were corrected according to the method of Irving *et al.*⁶⁴ Spectra of the ligands were recorded using individual samples, in which the 0.1 M KCl was partially or completely replaced by HCl and pH values, varying in the range of approximately 1.0 – 2.0, were calculated from the HCl content.

Determination of the distribution coefficients (D). $D_{7.4}$ values of ligands **HL**¹–**HL**⁵ and their copper(II) complexes (**1**–**5**) were determined by the traditional shake-flask method in *n*-octanol/buffered aqueous solution at pH 7.4 at 298.0 ± 0.2 K as described previously.^{68, 69} Two parallel experiments were performed for each sample. The ligands and the complexes were dissolved at 100 μ M (~30 μ M in the case of **HL**⁵ and its complex **5**) in the *n*-octanol pre-saturated aqueous solution of the buffer (10 mM HEPES) at constant ionic strength (0.10 M KCl). The aqueous solutions and *n*-octanol with 1:1 phase ratio were gently mixed with 360° vertical rotation for 3 h to avoid the emulsion formation, and the mixtures were centrifuged at 5000 rpm for 5 min by a temperature controlled centrifuge (Sanyo) at 298 K. After separation UV–vis spectra of the ligands or complexes in the aqueous phase were compared to those of the original aqueous solutions. Since measurable amounts of the ligand **HL**⁵ and its copper(II) complex **5** were not found in the aqueous phase after partitioning, their log $D_{7.4}$ values were merely estimated.

EPR Measurements and Deconvolution of the Spectra. All continuous wave (CW)-EPR spectra were recorded with a BRUKER EleXsys E500 spectrometer (microwave frequency 9.85 GHz, microwave power 10 mW, modulation amplitude 5 G, modulation frequency 100 kHz). The pH-dependent series of isotropic EPR spectra were recorded in a circulating system, at room temperature. A Heidolph Pumpdrive 5101 peristaltic pump was used to transport the solution from the titration pot through a capillary tube into a Bruker flat cell placed in the cavity of the instrument.

The EPR titrations were performed over the pH range between 1.5 and 12.0 at an ionic strength of 0.10 M (KCl) under nitrogen atmosphere. Samples contained 1 mM mPip-dm-FTSC (**HL**²) or Morph-dm-FTSC (**HL**⁴) and 1 mM or 0.5 mM copper(II) ions. A 0.1 M KOH solution was added to the samples to adjust the pH, which was measured with an Orion 710A pH-meter equipped with a Metrohm 6.0234.100 glass electrode. For selected pH values (where predominantly complexes formed) 0.1 mL samples were introduced into quartz EPR tubes and measured individually at 77 K.

Before the simulation, the measured spectra were corrected by subtracting the spectra of water measured in the same circulating system. A phase correction of -7 degree for the series of Morph-dm-FTSC (**HL**⁴) and -8 degree for the mPip-dm-FTSC (**HL**²) containing samples was used to correct the phase of the spectra which were probably shifted due to the not perfectly perpendicular position of the flat cell to the magnetic field. Both series of the pH-dependent isotropic CW-EPR spectra were simulated by the „two-dimensional” method using the 2D_EPR program.⁷⁰ The parameters g_0 , A_0^{Cu} copper hyperfine ($I_{\text{Cu}} = 3/2$) and A_0^{N} nitrogen ($I_{\text{N}} = 1$) superhyperfine couplings have been taken into account to describe each component curve. The relaxation parameters, α , β , and γ defined the linewidths through the equation $\sigma_{\text{MI}} = \alpha + \beta M_I + \gamma M_I^2$, where M_I denotes the magnetic quantum number of the paramagnetic metal ions. The equilibrium concentrations of the copper(II) complexes were varied by fitting their overall stability constants $\beta(\text{M}_p\text{L}_q\text{H}_r)$ defined in the section of pH-potentiometric measurements. For each spectrum, the noise-corrected regression parameter (R_j for the j^{th} spectrum) is derived from the average square deviation (SQD) between the experimental and the calculated intensities. For the series of spectra, the fit is characterized by the overall regression coefficient R , calculated from the overall average SQD. The overall regression coefficient was 0.9933 for the series of Morph-dm-FTSC and 0.9928 for the series of mPip-dm-FTSC. The details of the statistical analysis were published previously.⁷⁰ The anisotropic EPR spectra, recorded at 77 K, were analyzed individually with the aid of the EPR program.⁷¹ In case of copper(II) complexes, the anisotropic EPR parameters: rhombic g -tensor (g_x , g_y , g_z), rhombic copper(II) hyperfine tensor (A_x^{Cu} , A_y^{Cu} , A_z^{Cu}) and rhombic nitrogen hyperfine tensor (a_x^{N} , a_y^{N} , a_z^{N} , for which x , y and z denotes the directions of the g -tensor) were fitted. For the description of the linewidth the orientation dependent α , β and γ parameters were used to set up each component spectra. Since a natural CuCl_2 salt was used for the measurements, both the isotropic and anisotropic spectra were calculated as the sum of the spectra of ^{63}Cu and ^{65}Cu weighted by their natural abundances. The hyperfine and superhyperfine coupling constants and the relaxation parameters were obtained in field units (Gauss = 10^{-4} T).

Crystallographic Structure Determination. X-ray diffraction measurements were performed on Bruker X8 APEXII CCD and Bruker D8 VENTURE diffractometers. Single crystals were positioned at 35, 35, 40, 35 and 40 mm from the detector, and 1645, 1386, 890, 3033 and 2542 frames were measured, each for 48, 8, 48, 65 and 24 s over 0.4, 0.4, 0.4, 0.5 and 0.4° scan width for **1**·0.15CH₃OH, **2**·2H₂O, **3**·0.5(C₂H₅)₂O, **4**·0.93CH₃OH and **5**, respectively. The data were processed using SAINT software.⁷² Crystal data, data collection parameters, and structure refinement details are given in Table 1. The structures were solved by direct methods and refined by full-matrix least-squares techniques. Non-H atoms were refined with anisotropic displacement parameters. H atoms were inserted in calculated positions and refined with a riding model. In the crystal structure of **1**·0.15CH₃OH a partly occupied (15%) co-crystallized methanol molecule position was found, while in the crystal structure of **3**·0.5(C₂H₅)₂O half molecule of diethyl ether per asymmetric unit was found to be disordered over two positions with site occupation factor (s.o.f.) 0.5:0.5. In the crystal of **4**·0.93CH₃OH one molecule of co-crystallized methanol is disordered over 3 positions with s.o.f. 0.4:0.35:0.25, while the second molecule position is populated to 85%. The disorder was solved by using SADI instructions implemented in SHELXL-97, while the atoms involved were refined with isotropic displacement parameters and the positions of H atoms were calculated. The following computer programs were used: structure solution, *SHELXS-97* and refinement, *SHELXL-97*;⁷³ molecular diagrams, *ORTEP*.⁷⁴ CCDC1052906-1052910.

Table 1. Crystal data and details of data collection for 1·0.15CH₃OH, 2·2H₂O, 3·0.5(C₂H₅)₂O, 4·0.93CH₃OH and 5.

	1·0.15CH ₃ OH	2·2H ₂ O	3·0.5(C ₂ H ₅) ₂ O	4·0.93CH ₃ OH	5
empirical formula	C _{13.15} H _{19.6} ClCuN ₆ O _{0.15} S	C ₁₃ H ₂₈ Cl ₂ CuN ₆ O ₂ S	C ₁₄ H ₂₁ ClCuN ₅ O _{1.5} S	C _{14.93} H _{23.7} ClCuN ₅ O _{1.92} S	C ₁₇ H ₂₁ ClCuN ₆ O ₃ S
Fw	395.20	490.93	414.42	435.04	488.45
space group	<i>P</i> 2 ₁ / <i>n</i>	<i>P</i> -1	<i>P</i> 2 ₁ / <i>n</i>	<i>P</i> -1	<i>P</i> -1
<i>a</i> [Å]	7.6944(5)	7.6629(3)	7.7969(6)	9.8676(7)	8.0848(4)
<i>b</i> [Å]	17.4966(10)	11.7864(3)	17.4139(14)	12.0982(8)	12.1277(6)
<i>c</i> [Å]	13.1409(8)	13.0949(4)	13.145(1)	16.606(1)	12.3563(5)
α [°]		72.114(1)		86.450(3)	63.8664(17)
β [°]	102.615(2)	73.613(1)	103.700(2)	74.015(3)	83.4075(18)
γ [°]		74.311(1)		77.176(3)	73.0771(17)
<i>V</i> [Å ³]	1726.40(18)	1057.72(6)	1734.0(2)	1858.2(2)	1040.45(8)
<i>Z</i>	4	2	4	4	2
λ [Å]	0.71073	0.71073	0.71073	0.71073	0.71073
ρ_{calcd} [g cm ⁻³]	1.520	1.541	1.587	1.555	1.559
crystal size, mm	0.26 × 0.02 × 0.02	0.17 × 0.10 × 0.04	0.09 × 0.04 × 0.03	0.50 × 0.40 × 0.40	0.10 × 0.08 × 0.06
<i>T</i> [K]	100(2)	100(2)	100(2)	100(2)	100(2)
μ [mm ⁻¹]	1.547	1.407	1.548	1.450	1.310
<i>R</i> ₁ ^[a]	0.0334	0.0303	0.0424	0.0279	0.0326
<i>wR</i> ₂ ^[b]	0.0753	0.0832	0.0950	0.0683	0.0745
GOF ^[c]	1.079	1.033	1.011	1.095	1.040

^a $R_1 = \Sigma \|F_o\| - |F_c| / \Sigma \|F_o\|$, ^b $wR_2 = \{\Sigma [w(F_o^2 - F_c^2)^2] / \Sigma [w(F_o^2)]\}^{1/2}$, ^c GOF = $\{\Sigma [w(F_o^2 - F_c^2)^2] / (n - p)\}^{1/2}$, where *n* is the number of reflections and *p* is the total number of parameters refined.

Cell lines and culture conditions. Human cervical carcinoma (HeLa), human alveolar basal adenocarcinoma (A549), human colon carcinoma (LS174) cell lines and normal human foetal lung fibroblast cell line (MRC-5) were maintained as a monolayer culture in the Roswell Park Memorial Institute (RPMI) 1640 nutrient medium (Sigma Chemicals Co, USA). RPMI 1640 nutrient medium was prepared in sterile deionized water, supplemented with penicillin (192 U/mL), streptomycin (200 µg/mL), HEPES (25 mM), L-glutamine (3 mM) and 10% of heat-inactivated foetal calf serum (FCS) (pH 7.2). The cells were grown at 310 K in a humidified 5% CO₂ air atmosphere.

MTT assay. Antiproliferative activity of the investigated ligands and complexes was determined using 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay.⁷⁵ Cells were seeded into 96-well cell culture plates (Thermo Scientific Nunc™), at a cell density of 4000 cells/well (HeLa), 6000 cells/well (A549), 5000 cells/well (MRC-5) and 7000 cells/well (LS174) in 100 µL of culture medium. After 24 h of growth, cells were exposed to the serial dilutions of the tested compounds. The investigated compounds were dissolved in sterile water at a concentration of 10 mM as stock solution (complexes **2** and **4**), 5 mM (complex **1**), or 2 mM (complex **3**), and prior the use diluted with nutrient medium to the desired final concentrations (in range up to 300 µM). Ligands were dissolved in sterile water at a concentration of 10 mM **HL**¹ and **HL**² and 5 mM **HL**⁴, while ligand **HL**³ was dissolved in 1% DMSO at a concentration of 3 mM. Each concentration was tested in triplicates. After incubation periods of 48 h, 20 µL of MTT solution (5 mg/mL in phosphate buffer solution, pH 7.2) were added to each well. Samples were incubated for 4 h at 310 K, with 5% CO₂ in a humidified atmosphere. Formazan crystals were dissolved in 100 µL of 10% sodium dodecyl sulfate (SDS). Absorbances were recorded after 24 h, on an enzyme-linked immunosorbent assay (ELISA) reader (Thermo Labsystems Multiskan EX 200–240 V), at the wavelength of 570 nm. The IC₅₀ value, defined as the concentration of the compound causing 50% cell growth inhibition, was estimated from the dose-response curve.

Results and Discussion

These studies examined the effects of attachment of methylpiperazine, morpholine and methylpyrrole-2-carboxylate to the pyridine ring of the parent thiosemicarbazone on the aqueous solubility, lipophilicity, ability to form copper(II) complexes, their thermodynamic

stability in aqueous solution, and antiproliferative activity in human cancer cell lines HeLa, A549 and LS174, as well as in nontumorigenic cell line MRC5.

Synthesis and Characterization of HL^1 – HL^6 . The organic hybrids were synthesized in six steps, as shown in Scheme S2. The first four steps were described in detail previously.^{61, 76} The key aldehydes were prepared in two steps. First, 2-(chloromethyl)-6-(dimethoxymethyl)pyridine (**D**) was allowed to react with methylpiperazine or morpholine in THF in the presence of triethylamine or with methylpyrrole-2-carboxylate in DMF in the presence of NaH, following a literature procedure,⁷⁷ affording compounds **E–G** in 79, 93 and 58% yields, respectively. The aldehydes **H–J** were obtained by hydrolysis of species **E–G** in acidic aqueous solution or in acetone/water 1:5 mixture. Finally, condensation reactions of the aldehydes with thiosemicarbazide and/or 4,4-dimethyl-3-thiosemicarbazide afforded the hybrids HL^1 – HL^6 in 47–91% yields. One- and two-dimensional ^1H and ^{13}C NMR spectra confirmed the expected structures for HL^1 – HL^6 and the presence of *E* and *Z* isomers in DMSO. The *E/Z* ratio is 1:0.12, 1:0.66, 1:0.34, 1:0.62, 1:0.27 and 1:0.36 for HL^1 – HL^6 respectively, (measured at a concentration of approximately 10 mM). The presence of *E* and *Z* isomers is typical for thiosemicarbazones and our data are in good agreement with those reported for other α -pyridyl-TSCs.⁷⁸ The purity of HL^1 – HL^6 was further evidenced by elemental analysis. The positive-ion ESI mass spectra of HL^1 – HL^6 showed strong peaks at *m/z* 293, 321, 280, 308, 317 and 346, respectively, which were assigned to the $[\text{M}+\text{H}]^+$ ion. The lipo-hydrophilic character of the ligands (HL^1 – HL^5) is discussed in the section Solution Chemistry.

Synthesis and Characterization of Copper(II) Complexes. By reaction of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ with HL^1 and HL^2 in methanol two complexes $[\text{CuCl}(\text{mPipH-FTSC-H})]\text{Cl} \cdot 0.1\text{H}_2\text{O}$ ((**1**+H) $\text{Cl} \cdot 0.1\text{H}_2\text{O}$) and $[\text{CuCl}(\text{mPipH-dm-FTSC-H})]\text{Cl} \cdot 0.9\text{H}_2\text{O} \cdot 0.5\text{CH}_3\text{OH}$ ((**2**+H) $\text{Cl} \cdot 0.9\text{H}_2\text{O} \cdot 0.5\text{CH}_3\text{OH}$) were obtained in 74 and 53% yields, respectively. The formulation of both complexes was in accord with X-ray diffraction measurements (vide infra) and elemental analyses. Re-crystallization of the first complex from methanol in the presence of a small amount of triethylamine led to crystallization of the complex with deprotonated piperazine moiety, namely $[\text{CuCl}(\text{mPip-FTSC-H})] \cdot 0.15\text{CH}_3\text{OH}$ (**1**·0.15 CH_3OH), the structure of which was established by single crystal X-ray crystallography. ESI mass spectra of copper(II) complexes with HL^1 and HL^2 showed peaks

with m/z 354 and 382, attributed to $[M-Cl]^+$ ion. The copper(II) complexes $[CuCl(Morph-FTSC-H)] \cdot 2H_2O \cdot 0.2C_2H_5OH$ (**3**·2H₂O·0.2C₂H₅OH) and $[CuCl(Morph-dm-FTSC-H)] \cdot 0.2H_2O \cdot 0.6CH_3OH$ (**4**·0.2H₂O·0.6CH₃OH) were prepared in 88 and 98% yields, by reaction of copper(II) chloride with **HL**³ and **HL**⁴ in ethanol, and methanol, respectively. The composition of both complexes was confirmed by elemental analysis, X-ray diffraction data and ESI mass spectra. The latter showed the presence of peaks at m/z 341 and 369, assigned to $[M-Cl]^+$ ion. Starting from copper(II) chloride and **HL**⁵ and **HL**⁶ in methanol the complexes $[CuCl(mPyrr-FTSC-H)(H_2O)] \cdot 0.2H_2O$ (**5**·0.2H₂O) and $[CuCl(mPyrr-dm-FTSC-H)(H_2O)]$ (**6**) were obtained in 71 and 55% yield, respectively. This was confirmed by elemental analysis and ESI mass spectra. The latter showed the presence of peaks with m/z 379 and 407, assigned to $[M-Cl-H_2O]^+$ ion. Re-crystallization of $[CuCl(mPyrr-FTSC-H)(H_2O)] \cdot 0.2H_2O$ from DMF afforded the complex $[CuCl(mPyrr-FTSC-H)(DMF)]$ (**5**) via replacement of coordinated water molecule by DMF, as was confirmed by single crystal X-ray diffraction analysis (see section X-ray Crystallography). Lipo-hydrophilicity data of the copper(II) complexes **1–5** are discussed in the section Solution Chemistry.

X-ray Crystallography. The results of single crystal X-ray diffraction studies of **1**·0.15CH₃OH, **[2+H]Cl**·2H₂O, **3**·0.5(C₂H₅)₂O, **4**·0.93CH₃OH and **5** are shown in Figures 1–5. The complexes **1**·0.15CH₃OH and **4**·0.93CH₃OH crystallized in the centrosymmetric monoclinic space group $P2_1/n$, while **2**·2H₂O, **3**·0.5(C₂H₅)₂O and **5** crystallized in the centrosymmetric triclinic space group $P-1$. The piperazine-thiosemicarbazone and morpholine-thiosemicarbazone hybrid ligands **HL**¹, **HL**³ and **HL**⁴ in **1**, **3** and **4** act as tetradentate monodeprotonated ligands coordinating to copper(II) via the pyridine nitrogen atom, the azomethine nitrogen, the thiolato S atom and the piperazine or morpholine nitrogen atom, while in **[2+H]Cl**·2H₂O the organic ligand **HL**² is overall neutral being deprotonated at N3 and protonated at N6. The coordination number of copper(II) is five in complexes **1–4** and the coordination polyhedron can be described as a square-pyramid⁷⁹ ($\tau = 0.13, 0.11, 0.12$ and 0.10 (0.07 for another crystallographically independent complex), respectively). The apical position is occupied by a chlorido ligand. Three five-membered metallocycles are formed upon binding of the monodeprotonated ligands (**L**¹)[–] – (**L**⁴)[–] to copper(II). Two of them are essentially planar, while the N1–C1–C8–N5–Cu in **1** and **3**, or N1–C1–C10–N5–Cu in **2** and **4** is markedly distorted. The dihedral angle N1–C1–C8–N5/N1–C1–C10–N5 used here

as a measure of the deviation of the chelate ring from planarity is at $-28.1(3)$ and $-28.3(3)^\circ$ in **1** and **3**, and at $-29.62(19)$ and $-22.5(3)^\circ$ in **2** and **4** (for one of the two crystallographically independent molecules), respectively. This is not surprising if one takes into account the presence of an aliphatic carbon atom (C8/C10) in this chelate ring.

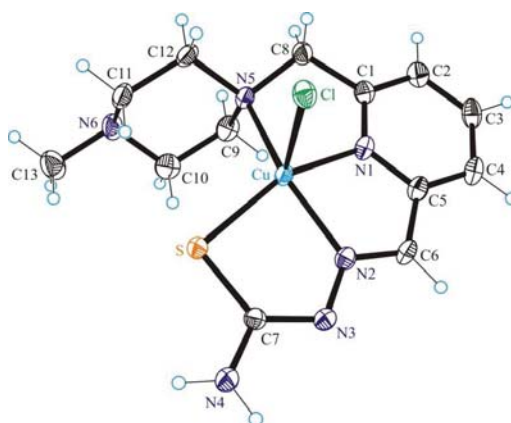


Figure 1. ORTEP view of **1** with thermal ellipsoids drawn at the 50% probability level. Selected bond distances (Å) and bond angles (deg): Cu–N1 1.959(2), Cu–N2 2.011(2), Cu–S 2.2725(7), Cu–N5 2.172(2), Cu–Cl 2.4724(7), N2–N3 1.352(3), C7–S 1.749(3), N1–Cu–N2 79.07(9), N2–Cu–S 83.43(7), N1–Cu–N5 78.60(9), N1–Cu–S 158.26(7), N5–Cu–S 112.56(6), Cl–Cu–N1 96.58(7), Cl–Cu–N2 106.20(7), Cl–Cu–S 100.62(3), Cl–Cu–N5 95.53(6), N2–Cu–N5 150.35(9).

The terminal amine nitrogen N4 of the thiosemicarbazone moiety is involved as a proton donor in hydrogen bonding to the nitrogen atom N3ⁱ of a neighboring molecule of **1** forming pairs of molecules as displayed in Figure S1 and in hydrogen bonding to Cl1ⁱⁱ, where i and ii denote the atoms generated by symmetry transformations $-x + 2$, $-y + 1$, $-z + 1$ and $x + 1$, y , z , respectively.

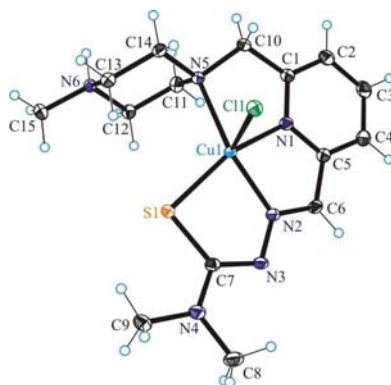


Figure 2. ORTEP view of $[2+H]^+$ with thermal ellipsoids drawn at the 50% probability level. Selected bond distances (Å) and bond angles (deg): Cu–N1 1.9508(13), Cu–N2 1.9901(13), Cu–S 2.2719(4), Cu–N5 2.2370(13), Cu–Cl 2.4295(4), N2–N3 1.3549(18), C7–S 1.7620(16), N1–Cu–N2 79.91(6), N1–Cu–N5 77.91(2), N2–Cu–S 83.31(4), N5–Cu–S 113.45(4), Cl–Cu–N1 96.25(4), Cl–Cu–N2 109.71(4), Cl–Cu–S 101.600(15), Cl–Cu–N5 89.22(4), N1–Cu–S 158.77(4), N2–Cu–N5 152.23(5).

The protonated atom N6 acts as a proton donor to the chloride counterion with $N6\cdots Cl2^i$ at 3.0837(14) Å, where i denotes atom positions generated by symmetry transformation $x - 1, y + 1, z$, and $N6-H\cdots Cl2^i$ 159.5°. Four other hydrogen bonds are formed between the co-crystallized water molecules and the chloride counterion.

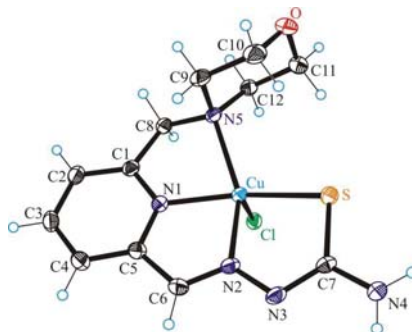


Figure 3. ORTEP view of **3** with thermal ellipsoids drawn at the 50% probability level. Selected bond distances (Å) and bond angles (deg): Cu–N1 1.951(2), Cu–N2 2.004(2), Cu–S 2.2711(7), Cu–N5 2.169(2), Cu–Cl 2.4786(7), N2–N3 1.353(3), C7–S 1.744(2), N1–Cu–N2 79.09(8), N1–Cu–N5 78.88(8), N2–Cu–S 83.37(6), N5–Cu–S 112.30(6), Cl–Cu–N1 96.08(6), Cl–Cu–N2 107.42(6), Cl–Cu–S 101.66(2), Cl–Cu–N5 93.83(6), N1–Cu–S 158.09(6), N2–Cu–N5 150.76(8).

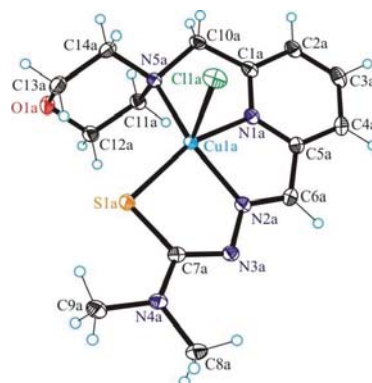


Figure 4. ORTEP view of one crystallographically independent molecule of **4** with thermal ellipsoids drawn at the 50% probability level. Selected bond distances (Å) and bond angles (deg): Cu1a–N1a 1.9388(17), Cu1a–N2a 1.9948(17), Cu1a–S1a 2.2523(6), Cu1a–N5a 2.1406(17), Cu1a–Cl1a 2.5088(6), N2a–N3a 1.358(2), C7a–S1a 1.754(2), N1a–Cu1a–N2a 79.68(7), N1a–Cu1a–N5a 79.90(7), N2a–Cu1a–S1a 83.88(5), N5a–Cu1a–S1a 111.88(5), Cl1a–Cu1a–N1a 91.49(5), Cl1a–Cu1a–N2a 103.19(5), Cl1a–Cu1a–S1a 103.65(2), Cl1a–Cu1a–N5a 93.40(5), N1a–Cu1a–S1a 159.83(5), N2a–Cu1a–N5a 153.91(7). (0.10 and 0.07)

Unlike, the hybrid ligand **HL**⁵ acts as a tridentate monodeprotonated ligand binding to copper(II) via pyridine nitrogen N1, azomethine atom N2 and thiolato atom S. Like in complexes **1–4** the coordination number of the copper(II) center in **5** is five, and the coordination geometry shows a slight tendency to square-pyramidal ($\tau = 0.43$), the remaining two places being occupied by the DMF molecule and the chlorido ligand. The pyrrol nitrogen atom N5, due to its sp^2 hybridization remains unbound to copper(II). Note that sp^3 -hybridized proline nitrogen atom in proline-thiosemicarbazone conjugates was involved in binding to first-row transition metals and became a chiral center upon coordination.⁸⁰

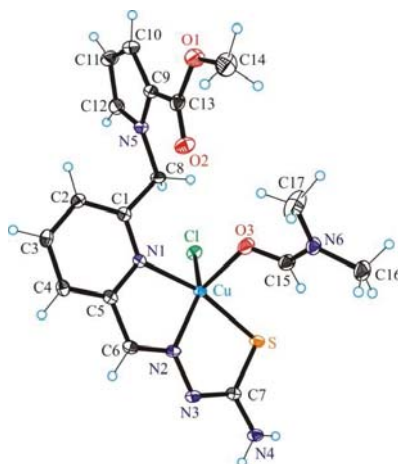


Figure 5. ORTEP view of **5** with thermal ellipsoids drawn at the 50% probability level. Selected bond distances (Å) and bond angles (deg): Cu–N1 2.0912(14), Cu–N2 1.9820(13), Cu–S 2.2785(5), Cu–O3 2.1852(13), Cu–Cl 2.3069(4), N2–N3 1.3641(19), C7–S 1.7387(17), N1–Cu–N2 80.48(6), N2–Cu–S 83.13(4), O3–Cu–S 97.27(2), Cl–Cu–N1 98.97(4), Cl–Cu–N2 137.78(4), Cl–Cu–S 94.701(16), Cl–Cu–O3 107.32(4), N1–Cu–S 163.45(4).

Solution Chemistry: Proton Dissociation Processes of Ligands HL² and HL⁴, Lipophilicity of ligands HL¹ – HL⁵. Morph-dm-FTSC (HL⁴), which forms the most biologically active copper(II) complex among the studied ligands (*vide infra*), was chosen for the detailed solution equilibrium studies together with its methylpiperazine analogue, mPip-dm-FTSC (HL²) (Chart 1) for comparison. Deprotonation processes of these ligands were followed in aqueous solution by pH-potentiometric and ¹H NMR titrations. Consecutive multiple pH-potentiometric titrations showed that no ligand decomposition occurred in the pH range studied (2.0–11.5) under an argon atmosphere. Morph-dm-FTSC (HL⁴) possesses three, while mPip-dm-FTSC (HL²) four functional groups which, presumably, dissociate. The proton dissociation constants determined by pH-potentiometry are listed in Table 2. The identical N-terminally dimethylated α-N-pyridyl thiosemicarbazone moiety of the ligands is expected to have a relatively low pK_a value for the N_{pyridyl}H⁺ and a significantly higher value for the N_{hydrazinic}H functional group based on the proton dissociation constants of structurally similar HCTs, such as 2-formylpyridine N⁴,N⁴-dimethylthiosemicarbazone (PTSC, pK₁: 3.38 and pK₂: 10.54) or 3-aminopyridine-2-carboxaldehyde N⁴,N⁴-dimethylthiosemicarbazone (APTSC, pK₁: 4.31 and pK₂: 10.29).⁸¹ Taking into account these data we attributed the pK₂ of

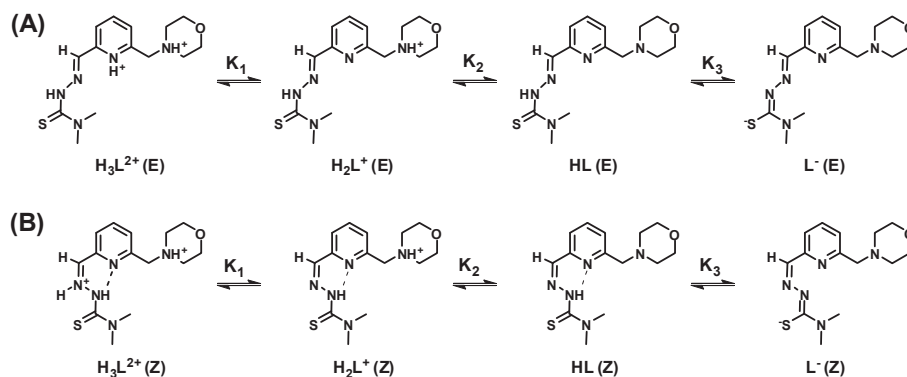
Morph-dm-FTSC to the deprotonation of the morpholinium ion. It should also be noted that the assignment of the pK_a values for the methylpiperazine-thiosemicarbazone hybrid is not so straightforward. The proton dissociation steps of the ligands studied were assigned to the different functional groups by careful analysis of the results of the ^1H NMR titrations and are shown in Schemes 1 and S3.

Table 2. Macroscopic and microscopic proton dissociation constants (pK_a) of ligands mPip-dm-FTSC (HL^2) and Morph-dm-FTSC (HL^4) determined by pH-potentiometry and ^1H NMR titrations [$T = 298\text{ K}$, $I = 0.10\text{ M}$ (KCl)]

	method		pK_1	pK_2	pK_3	pK_4
mPip-dm-FTSC	pH-metry		1.69 ± 0.02	3.29 ± 0.01	7.88 ± 0.01	10.23 ± 0.01
	^1H NMR	isomer <i>E</i>	–	3.59 ± 0.06	7.94 ± 0.01	10.05 ± 0.03
	^1H NMR	isomer <i>Z</i>	–	2.24 ± 0.05	7.82 ± 0.01	>11.5
	^1H NMR ^a		–	3.33	7.90	10.35
Morph-dm-FTSC	pH-metry		2.27 ± 0.02	5.91 ± 0.01	10.18 ± 0.01	–
	^1H NMR	isomer <i>E</i>	2.28 ± 0.01	6.08 ± 0.01	10.14 ± 0.01	–
	^1H NMR	isomer <i>Z</i>	<1	5.18 ± 0.01	>11.5	–
	^1H NMR ^a		2.21	5.90	10.30	–

^aEstimated from the summed concentration distribution curves of the *E/Z* isomers in Figures 6B ad S4B.

Scheme 1. Deprotonation steps of the H_3L^{2+} form of ligand Morph-dm-FTSC (HL^4) for its *E* (A) and *Z* (B) isomers



The pH-dependent ^1H NMR spectra of Morph-dm-FTSC (HL^4) (Figure 7) revealed that most of the proton resonances are fairly sensitive to stepwise proton dissociation processes. In addition, the presence of *Z* and *E* isomers was observed. These were found to be involved in slow interconversion processes with regard to the NMR time scale ($t_{1/2}(\text{obs}) > \sim 1$ ms) in a wide pH-range. Their proton resonances were well-separated in most of the cases. However, the lines tend to broaden at $pH < \sim 4$ due to faster isomerization around the $CH^{12}=N^1$ double bond. Integrated signals of the different ligand protons belonging to the *E* and *Z* isomers were converted to molar fractions showing the predominant formation of the *E* isomer in the whole pH range, although the ratio of the isomers is undoubtedly changing due to the deprotonation steps (Figure 6A). The *E* isomer was also found to be the major species in DMSO- d_6 and its molar fraction (0.62) corresponds well to that found for aqueous solution (0.61) between $pH \sim 7$ and ~ 9 , where the neutral *HL* form predominates. Based on the pH-dependence of the ^1H NMR signals (Figure S2) microscopic proton dissociation constants could be computed for both *Z* and *E* isomers (Table 2). Concentration distribution curves were calculated based on these data providing the macroscopic constants as well (Table 2), which are in good agreement with the results of the pH-potentiometry. The first deprotonation process was accompanied by significant changes of the chemical shifts of the C^6H pyridine ring proton and $C^{14,15}H_3$ terminal methyl protons. The morpholine ($C^{8,11}H_2$, $C^{9,10}H_2$) and C^7H_2 protons were very sensitive to the second deprotonation step, as were, also, the pyridine ring protons, while the chemical shifts of protons of the thiosemicarbazone moiety ($C^{12}H$, $C^{14,15}H_3$) remain unaltered during the process. In the pH-range where the third proton dissociation occurs the

signals of the last mentioned protons were shifted exclusively. These observed changes strongly support the subsequent deprotonation steps of the $N^4_{\text{pyridyl}}H^+$, $N^5_{\text{morpholinium}}H^+$ and $N^2_{\text{hydrazinic}}H$ functional groups of both isomers of Morph-dm-FTSC as indicated in Scheme 1. On the other hand, marked differences are found between the pK_a values of the *Z* and *E* isomers (Table 2). Most probably the hydrogen bond between the pyridyl nitrogen and the $N^2_{\text{hydrazinic}}H$ moiety in the H_2L^+ , HL forms of the *Z* isomer is responsible for these differences. Namely, it decreases pK_1 of the *Z* isomer via stabilization of the conjugate base (H_2L^+) as well as pK_2 due to the diminished π -electron density in the pyridine ring, which results in an easier deprotonation of the $N^5_{\text{morpholinium}}H^+$ group. The pK_3 of the *Z* isomer is higher than that of the *E* form, since the dissociation of the $N^2_{\text{hydrazinic}}H$ functional group participating in the hydrogen bonding is more difficult.

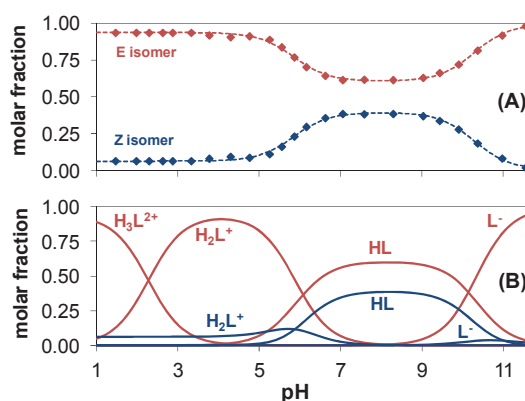
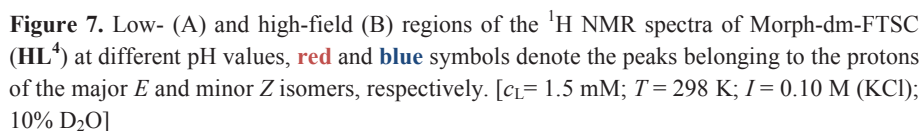


Figure 6. pH-Dependence of the molar fraction of the *E* (red symbols) and *Z* (blue symbols) isomers of the ligand Morph-dm-FTSC (HL^4) calculated on the basis of the integrated areas of the signals of the various ligand protons (A). Concentration distribution curves for the isomeric ligand species (*E*: labelled in red; *Z*: labelled in blue) calculated with the aid of the microscopic proton dissociation constants (B). [c_L = 1.5 mM; T = 298 K; I = 0.10 M (KCl); 10% D_2O]



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double bond. The *E* isomer was found to be predominant in the whole pH range studied (Figure S5).

It is worth noting that the pK_a values of the $N_{\text{pyridyl}}H^+$ functional group of the studied thiosemicarbazone-based hybrids are significantly lower compared to those of ligands PTSC, APTSC⁸¹ due to the electron withdrawing effect of the charged morpholinium and methylpiperazinium moieties.

Both ligands mPip-dm-FTSC (**HL**²) and Morph-dm-FTSC (**HL**⁴) possess intrinsic fluorescence. 3-Dimensional fluorescence spectra recorded in aqueous solution at pH 7.4 (Figure S6) reveal their fairly similar excitation (330 nm) and emission maxima (420 nm), although the emission intensity of the morpholine-thiosemicarbazone hybrid is by a factor of 3 higher in comparison to that of **HL**².

Table 3. Log $D_{7.4}$ values (*n*-octanol/water) for the ligands **HL**¹ – **HL**⁵ and for the copper(II) complexes **1** – **5** [$T = 298$ K, pH = 7.40 (10 mM HEPES) and $I = 0.10$ M (KCl)]

Ligand		log $D_{7.4}$	complex	log $D_{7.4}$
mPip-FTSC	HL ¹	−0.07±0.01	1	−1.53±0.09
mPip-dm-FTSC	HL ²	−0.03±0.03	2	−0.95±0.10
Morph-FTSC	HL ³	+0.60±0.02	3	−1.15±0.09
Morph-dm-FTSC	HL ⁴	+0.61±0.01	4	−0.90±0.09
mPyr-FTSC	HL ⁵	>1.8	5	>1.8

The lipo-hydrophilic character of the ligands **HL**¹ – **HL**⁵ was studied at pH 7.4 via the partitioning between *n*-octanol and water. The log $D_{7.4}$ values determined by the analysis of the UV–vis spectra of the aqueous phases before and after separation are listed in Table 3. The results indicate a slightly higher lipophilicity of the terminally dimethylated derivatives (**HL**² and **HL**⁴) compared to that of the corresponding non-methylated ligands (**HL**¹ and **HL**³). Compounds containing the morpholine moiety (**HL**³ and **HL**⁴) possess significantly higher log $D_{7.4}$ values compared to those of the methylpiperazine derivatives (**HL**¹ and **HL**²) most probably due to the different protonation states of the ligands at physiological pH. According to the pK_a values of the ligands studied (Table 2) mPip-dm-FTSC (**HL**²) is partly protonated (74% H_2L^+ , 26% HL), while Morph-dm-FTSC (**HL**⁴) is mainly neutral (97% HL,

3% H_2L^+ at pH 7.4. On the other hand, the methyl ester mPyrr-FTSC (HL^5) is much more lipophilic than the other ligands studied and its high $\log D_{7.4}$ value is manifested in a strongly reduced aqueous solubility compared to that of the corresponding proline-thiosemicarbazone conjugates (L- and D-Pro-FTSC: $\log D_{7.4} < -1.7$).³⁵ It should be also noted that all the ligands studied except mPyrr-FTSC (HL^5) are more hydrophilic than Triapine ($\log D_{7.4} = +0.85$)⁶⁹ at physiological pH.

Solution Chemistry: Complex Formation Equilibria of Copper(II) with Ligands HL^2 and HL^4 and lipophilicity of the complexes. The main aim of the studies on complexation reactions of ligands mPip-dm-FTSC (HL^2) and Morph-dm-FTSC (HL^4) with copper(II) was monitoring the stability of the complexes **2** and **4** in aqueous solution especially at physiological pH. The complex formation processes were investigated by the combined use of pH-potentiometric, UV-vis and EPR titrations. The stoichiometries and cumulative stability constants of the complexes furnishing the best fits to the experimental data are listed in Table 4. EPR spectra were recorded at various pH values at 1:1 and 1:2 metal-to-ligand ratios at room temperature and at 77 K; the fitted experimental and simulated isotropic spectra are depicted in Figures 8A,B and S7A,B. The simulation of the EPR spectra resulted in the individual isotropic and anisotropic EPR spectra and parameters of the various species (Figures 8C, S7C and S8; Table 5). The EPR measurements at both temperatures revealed the predominant formation of mononuclear mono-ligand complexes in different protonation states. The proton displacement by the metal ion due to complex formation is almost complete already at the starting pH value of the pH-potentiometric titrations (pH~2) and a negligible amount of free copper(II) was detected by EPR at this pH, indicating the prominently high stability of the complexes formed with both ligands. Therefore, conditional stability constants for $[\text{CuL}]^+$, which predominates in a wide pH-range, were determined by competition reactions with EDTA. The displacement of EDTA from the $[\text{Cu}(\text{EDTA})]^{2-}$ complex by the ligands were followed by UV-vis spectrophotometry at pH 7.4 and 5.6, in the case of mPip-dm-FTSC (Figure S9) and Morph-dm-FTSC, respectively. Absorbance values recorded at $\lambda > 415$ nm were used for calculations of the conditional stability constants ($\log \beta'$) of $[\text{CuL}]^+$, the only species contributing to the measured absorbance. The cumulative stability constants ($\log \beta$) of $[\text{CuL}]^+$ were computed (Table 4) taking into account the protonation of the ligands at these pH values, which were kept constant during subsequent data evaluation.

Table 4. Cumulative ($\log\beta$), derived and stepwise stability constants of the copper(II) complexes of ligands mPip-dm-FTSC (**HL**²) and Morph-dm-FTSC (**HL**⁴) determined by pH-potentiometry, UV-vis and EPR spectroscopy [$T = 298\text{ K}$, $I = 0.10\text{ M}$ (KCl)]

	pH-metry	UV-vis	EPR
mPip-dm-FTSC	$\log\beta [\text{CuLH}_2]^{3+}$	–	27.5±0.1
	$\log\beta [\text{CuLH}]^{2+}$	26.53±0.01	26.47±0.01
	$\log\beta [\text{CuL}]^+$	20.26±0.03 ^a	26.49±0.03
	$\log\beta [\text{CuLH}_{-1}]$	8.4±0.1	7.8±0.1
	$\log\beta [\text{CuL}_2\text{H}]^+$	33.43±0.02	33.84±0.06
	$\log\beta [\text{CuL}_2]$	–	23.71±0.08
	$\text{p}K_a [\text{CuLH}_2]^{3+}$	–	1.4
	$\text{p}K_a [\text{CuLH}]^{2+}$	6.27	6.23
	$\text{p}K_a [\text{CuL}]^+$	11.9	12.5
	$\log K [\text{CuL}_2]$	–	3.45
Morph-dm-FTSC	$\log\beta [\text{CuLH}]^{2+}$	20.9±0.1	20.7±0.1
	$\log\beta [\text{CuL}]^+$	18.86±0.08 ^a	20.7±0.1
	$\log\beta [\text{CuLH}_{-1}]$	7.2 ±0.1	7.0±0.1
	$\log\beta [\text{CuL}_2\text{H}_3]^{3+}$	40.2±0.1	39.90±0.08
	$\log\beta [\text{CuL}_2]$	–	21.71±0.02
	$\text{p}K_a [\text{CuLH}]^{2+}$	2.0	1.8
	$\text{p}K_a [\text{CuL}]^+$	11.7	11.9
	$\log K [\text{CuL}_2]$	–	2.85

^a Determined via the EDTA displacement reactions by the ligand **HL**² or **HL**⁴ by UV-vis spectrophotometry. Data for the $\text{p}K_a$ values of EDTA (0.9; 1.6; 2.0; 2.66; 6.16; 10.24) and $\log\beta$ of the $[\text{Cu}(\text{EDTA})]^{2-}$ complex (18.92) are taken from ref. 61 and conditional stability constants of $[\text{Cu}(\text{EDTA})]^{2-}$ calculated for pH 7.4 and 5.6 are 16.06 and 13.61, respectively. Conditional stability constants ($\log\beta$) of the $[\text{CuL}]^+$ species: 16.83 ± 0.03 (**HL**²) at pH 7.4 (10 mM HEPES) and 13.79 ± 0.03 (**HL**⁴) at pH 5.6 (10 mM MES). β values of $[\text{CuL}]^+$ are calculated as $\beta = \beta^\circ \times \alpha_{\text{H}}$; where $\alpha_{\text{H}} = 1 + \sum \beta(\text{H}_p\text{L}) \times [\text{H}^+]^p$.

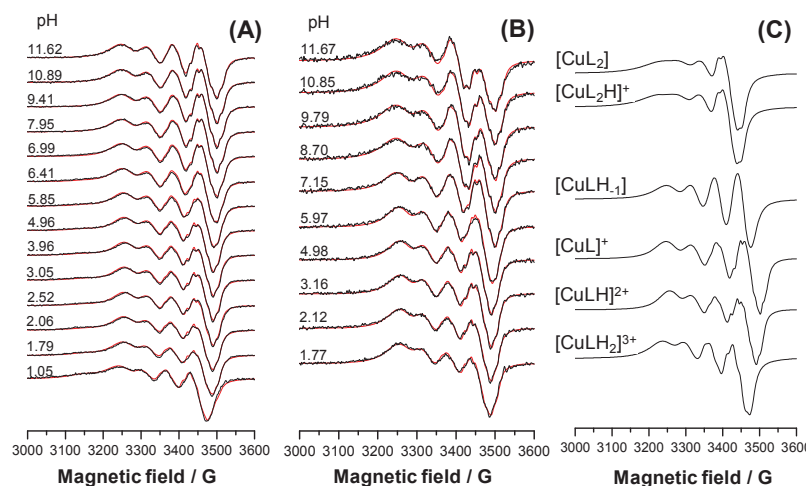


Figure 8. Experimental (black) and simulated (red) solution EPR spectra recorded for the copper(II) – mPip-dm-FTSC (HL^2) system at 1:1 (A) and 1:2 (B) metal-to-ligand ratios. Calculated component EPR spectra obtained for the different copper(II) – mPip-dm-FTSC (HL^2) species (C). [$c_{\text{ligand}} = 1.0 \text{ mM}$; $c_{\text{Cu}} = 1.0 \text{ mM}$ (A) or $c_{\text{Cu}} = 0.5 \text{ mM}$ (B); $T = 298 \text{ K}$; $I = 0.10 \text{ M}$ (KCl)]

In the case of Morph-dm-FTSC, $[\text{CuL}]^+$ predominates between pH ~ 4 and ~ 10 . This is clearly indicated by the unaltered UV–vis spectra in the wavelength range of both the d-d (Figure 9) and CT (Figure S10B) bands. EPR spectra were also intact in this particular pH range (Figure S7A). Based on the EPR parameters of $[\text{CuL}]^+$ (Table 5, the superhyperfine couplings to three nitrogen atoms is resolved in the spectra) the coordination of the ligand via the ($\text{S}^-, \text{N}^1, \text{N}^4, \text{N}^5$) donor set is the most probable in solution. The rhombic g -tensor determined from the anisotropic EPR spectra indicates a strong rhombic distortion which is probably due to the three conjugated five-membered chelate rings formed by the four donor atoms. The single-crystal X-ray crystallography revealed the same binding mode for the ligand in **4** in the solid state (Figure 4). Upon decreasing the pH complex $[\text{CuL}]^+$ becomes protonated and the significant UV–vis (Figures 9 and S10B) and EPR (Figure S7) spectral changes at pH $< \sim 3$ indicate the alteration of the coordination mode. λ_{max} values of both the d-d and CT bands are shifted to the higher wavelengths upon the formation of species $[\text{CuLH}]^{2+}$ (578 nm $\rightarrow$ 690 nm, 406 nm $\rightarrow$ 410 nm, 302 nm $\rightarrow$ 323 nm). Most likely, the morpholine nitrogen is protonated and not involved in coordination in $[\text{CuLH}]^{2+}$ as indicated by its higher g_0 value

compared to that of $[\text{CuL}]^+$ (Table 5). On the other hand the deprotonation of $[\text{CuL}]^+$ observed at $\text{pH} > \sim 10$, is accompanied by only minor changes of the UV-vis spectra (see changes at $\sim 256 \text{ nm}$ in Figure S10B). However, the decreasing ligand field (lower A_0) supports the formation of a mixed hydroxido complex, $[\text{CuL}(\text{OH})]$, in which the ligand binds through ($\text{S}^-, \text{N}^1, \text{N}^4$) donor atoms. A fairly similar deprotonation process of $[\text{CuL}]^+$ is characteristic for mPip-dm-FTSC. The formation of $[\text{CuLH}_{-1}]$ ($= [\text{CuL}(\text{OH})]$) could be also admitted at the highly basic pH values, although additional changes could be detected in the neutral and acidic pH ranges. Namely, the inflection point of the titration curve recorded at 1:1 metal-to-ligand ratio (not shown here) at $\text{pH} 6.26$ strongly suggests an additional (de)protonation process which was not observed in the case of Morph-dm-FTSC. pK_a of species $[\text{CuLH}]^{2+}$ was also calculated on the basis of the minor changes of the d-d bands of the UV-vis spectra and the pH-dependent EPR spectra (Figure 8A). The data obtained by the three different methods are in good agreement (Table 4). The similar g_0 values of $[\text{CuLH}]^{2+}$ and $[\text{CuL}]^+$ (Table 5) indicate the same coordination mode of mPip-dm-FTSC in these complexes via a ($\text{S}^-, \text{N}^1, \text{N}^4, \text{N}^5$) donor atoms both in solution and in the solid state established by X-ray diffraction (Figure 2), while the ligand field is slightly increased (somewhat higher A_0) due to the deprotonation of $[\text{CuLH}]^{2+}$. These results strongly indicate that the process is assigned to the deprotonation of the N^6 of the methylpiperazine moiety which is not involved in the binding to copper(II). The observed UV-vis spectral changes (Figure S10A) and EPR parameters (Table 5) at $\text{pH} < \sim 3$ were found to be similar to those found for the Morph-dm-FTSC system, thus the ($\text{S}^-, \text{N}^1, \text{N}^4$) coordination is suggested for $[\text{CuLH}_2]^{3+}$ in which the methylpiperazine N^5 atom is protonated.

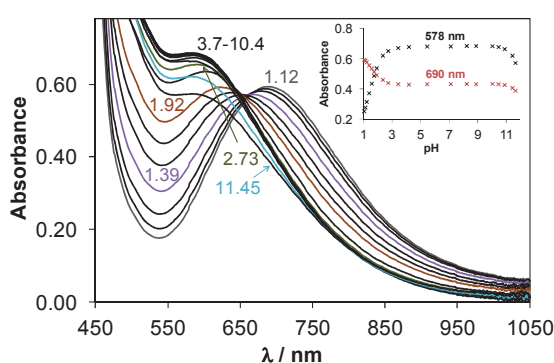


Figure 9. UV–vis absorbance spectra of **4** recorded in the pH range 1.1 – 11.7. Inset shows the absorbance values recorded at 578 and 690 nm. [$c_{\text{complex}} = 2.5 \text{ mM}$; $T = 298 \text{ K}$; $I = 0.10 \text{ M}$ (KCl); $l = 1 \text{ cm}$]

Formation of merely mono-ligand copper(II) complexes for **HL**² and **HL**⁴ was expected. However at ligand excess ($c_L/c_{\text{Cu}} > 2$) bis-ligand complexes were detected mainly in the basic pH range. Formation of the neutral bis-ligand complexes $[\text{CuL}_2]$ resulted in precipitation which hindered the accurate determination of their stability constants by pH-potentiometry and UV–vis spectrophotometry, although these were estimated by the EPR measurements (Table 4). The EPR data for this kind of complexes represent quite high g_0 and low A_0 values (Table 5) and strong rhombic distortion. The ligands in these complexes coordinate most probably via ($\text{S}^-, \text{N}^1, \text{N}^4$) and ($\text{S}_{\text{equatorial}}, \text{N}^1_{\text{axial}}$) donor sets. The stepwise stability constants $\log K$ $[\text{CuL}_2]$ are lower by many orders of magnitude than $\log K$ $[\text{CuL}]^+$ indicating the non-favored formation of the bis-ligand complexes. Constants for these minor charged bis-ligand complexes such as $[\text{CuL}_2\text{H}_3]^{3+}$ (Morph-dm-FTSC) and $[\text{CuL}_2\text{H}]^+$ (mPip-dm-FTSC) could be calculated by pH-potentiometry as well. The former complex displays a well resolved solution EPR spectra with two coordinating N atoms, and large A_0 value which indicate a symmetrical structure with (S^-, N^1) (S^-, N^1) binding mode, while the latter one has a similar coordination pattern as species $[\text{CuL}_2]$.

It is worth noting that the isotropic g and A values calculated by averaging the anisotropic values ($g_{0,\text{calc}}$ and $A_{0,\text{calc}}$ in Table 5) are in relatively good agreement with the corresponding values measured in solution, indicating that the coordination modes adopted by the ligands in solution are preserved upon freezing.

Representative concentration distribution curves were calculated by using the overall stability constants (average values obtained by the 3 methods) for the the copper(II) – mPip-dm-FTSC (**HL**²) and copper(II) – Morph-dm-FTSC (**HL**⁴) systems at 1:1 metal-to-ligand ratio to represent the complex formation processes in the pH range studied (Figure 10). It can be concluded that complexes $[\text{CuL}]^+$ predominate at physiological pH even at submicromolar concentrations, although 6% of the complex is protonated in the case of mPip-dm-FTSC.

In order to compare the copper(II) binding ability of mPip-dm-FTSC (**HL**²) and Morph-dm-FTSC (**HL**⁴) with other thiosemicarbazones pCu values ($pCu = -\log[Cu(II)]$; $c_L/c_{Cu} = 10$; $c_{Cu} = 1 \mu M$) have been computed at physiological pH. The higher pCu value indicates stronger chelating ability. For mPip-dm-FTSC and Morph-dm-FTSC pCu values of 17.6 and 17.0 were obtained, respectively, which are significantly higher than those reported for tridentate HCTs such as Triapine (11.6) at pH 7.4 in 30% (w/w) DMSO/H₂O⁸² and are comparable to that of the pentadentate L-Pro-FTSC conjugate (17.5) in pure water.³⁵

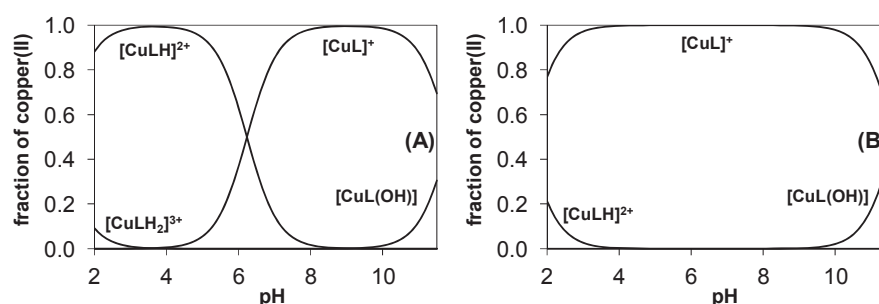


Figure 10. Concentration distribution curves for the copper(II) – mPip-dm-FTSC (**HL**²) (A) and copper(II) – Morph-dm-FTSC (**HL**⁴) (B) systems. [$c_L = 1.0 \text{ mM}$; $c_{Cu} = 1.0 \text{ mM}$; $T = 298 \text{ K}$; $I = 0.10 \text{ M (KCl)}$]

$\log D_{7.4}$ values were determined for the copper(II) complexes **1–5** and are collected in Table 3 in order to characterize the hydro-lipophilic character of these species. Comparing these values to those of the metal-free ligands it can be concluded that the same lipophilicity trend is obtained. Namely, the terminal dimethylation results in somewhat increased values and complexes of the morpholine-thiosemicarbazone derivatives possess enhanced lipophilic character. Note that the copper(II) complexes are much more hydrophilic than the corresponding ligands since the positively charged $[CuL]^+$ species predominate at physiological pH. Complex **5** is much more lipophilic than the other complexes, although its $\log D_{7.4}$ value cannot be determined exactly and compared to that of mPyr-FTSC (**HL**⁵).

Table 5. EPR parameters of the components obtained for the copper(II) complexes of mPip-dm-FTSC (HL^2) and Morph-dm-FTSC (HL^4)

	Isotropic parameters ^a			Anisotropic parameters ^b			Calculated parameters ^c		
	g_o	A_o/G	a_o^N/G	g_x, g_y, g_z	$A_x, A_y, A_z/G^d$	$a_x^N, a_y^N, a_z^N/G$	$g_{o,calc}$	$A_{o,calc}$	G
Morph-dm-FTSC	[CuLH] ²⁺	2.1037(2)	68.6(4)	13.8(5) 10.7(7)	2.035, 2.058, 2.216	18.7, 30.4, 155.9	18.5, 13.9, 11.9 12.8, 15.7, 6	2.103	70.7
	[CuL] ⁺	2.08856(1)	63.3(1)	17.8(1) 11.9(1)	2.032, 2.053, 2.176	8.9, 9.8, 160.1	18.2, 9.6, 8.4 10.4, 15.9, 9.8	2.087	61.7
	[CuLH ₂] ^e	2.0953(6)	62.1(8)	8.9(2) 9.0(8)	2.05, 2.07, 2.249	16, 19, 156	10.4, 15.9, 9.8 8, 17, 8	2.121	67.7
	[CuL ₂ H ₃] ³⁺	2.0745(5)	85.5(6)	12.3(8) 12.3(8)			15, 10, 8		
	[CuL ₂] ⁺	2.106(2)	53(2)	17(1) 14(1)					
mPip-dm-FTSC	[CuLH ₂] ³⁺	2.1026(4)	58.0(5)	17.4(4) 10.3(9)	2.035, 2.059, 2.214	-18.9, 30.4, 155.7	18.4, 14, 12 12, 16.3, 6	2.103	58.5
	[CuLH] ²⁺	2.0899(1)	58.3(1)	18.2(1) 11.5(2)	2.031, 2.055, 2.176	4.4, 8.3, 155.9	16.9, 10.9, 10.9 11.3, 16.8, 9.7	2.087	58.3
	[CuL] ⁺	2.0894(1)	64.2(1)	7.8(2) 17.9(1)	2.033, 2.053, 2.177	6.7, 10.9, 159.2	11.3, 16.8, 9.7 16.7, 9.4, 9	2.088	61.1
	[CuLH ₂] ^e	2.0959(7)	59(1)	12.1(2) 9.0(3)	2.05, 2.07, 2.249	16, 19, 156	11.7, 16.7, 10 11.7, 16.7, 10	2.121	67.7
	[CuL ₂ H] ⁺	2.1144(6)	54(1)	10(1) 15(1)			8, 17, 8 15, 10, 8		
	[CuL ₂] ^e	2.1118(6)	53(1)	12(2) 16(1)	2.03, 2.05, 2.200	25, -11, 150	17, 10, 10 10, 17, 10	2.093	57

^aUncertainties (SD) are shown in parentheses. ^bThe experimental errors were ± 0.002 for g_x and g_y , and ± 0.001 for g_z , ± 2 G for A_x and A_y , and ± 1 G for A_z . ^cIsotropic values calculated via the equation $g_o = (g_x + g_y + g_z)/3$, and $A_o[\text{MHz}] = (A_x + A_y + A_z)/3$. ^dThe signs of the couplings were derived from a comparison of $A_{o,calc}$ with the experimental A_o values. ^eHigher uncertainties of anisotropic parameters were obtained for *minor* species.

Cytotoxicity in Cancer Cell Lines. The antiproliferative activity of the ligands **HL**¹–**HL**⁴ and the copper(II) complexes **1**–**4** was evaluated for 48 h of continuous drug action, using colorimetric MTT assay. The study was performed in three human neoplastic cell lines, namely HeLa (cervical carcinoma), A549 (alveolar basal adenocarcinoma) and LS174 (colon carcinoma), and one human foetal lung fibroblast cell line (MRC-5), which was used as a noncancerous model for the *in vitro* cytotoxicity evaluation. The results for the ligands and their copper(II) complexes are summarized in Table 6 in terms of IC₅₀ values with their standard deviations. The results revealed that compounds **1**–**4** exhibited significant antiproliferative activity (IC₅₀ < 100 μM) against all cell lines used, with complex **4** showing the highest cytotoxic potential. The most sensitive to the investigated compounds was proved to be the cell line LS174, as indicated by the calculated IC₅₀ values varying from 13.1 to 17.5 μM. In contrast, the ligands showed significantly lower activity than the parent 2-formyl- and/or 2-acetylpyridine thiosemicarbazones, except ligand **HL**⁴, which exhibited significant cytotoxic activity against both the MRC5 and LS174 cell lines (63.2±4.2 and 15.9±0.6 μM, respectively).

Table 6. Results of the MTT assay presented as IC₅₀ values obtained after 48 h treatment.

Complex	IC ₅₀ * [μM] (mean ± SD)			
	HeLa	A549	LS174	MRC5
1	38.3±1.7	62.7±4.7	16.4±4.2	50.6±3.5
2	65.1±5.7	131.3±3.9	17.4±0.2	38.6±5.9
3	63.3±2.7	208.0±0.1	17.5±1.6	132.1±9.2
4	25.5±5.3	42.8±3.7	13.1±2.1	28.3±3.8
HL ¹	>300	>300	>300	>300
HL ²	>300	>300	>300	>300
HL ³	>300	>300	>300	>300
HL ⁴	>300	>300	15.9±0.6	63.2±4.2

*The sign > (in front of the maximum value of the concentration) indicates that IC₅₀ value is not reached in the examined range of concentrations.

The metal-free 2-formylpyridine thiosemicarbazone (FTSC) showed high cytotoxicity against human cancer cell lines 41M (ovarian carcinoma), SK-BR-3 (mammary carcinoma), SW480 (colon carcinoma) and HL60 (leukemia) after 96 or 72 h treatment with IC_{50} values of 2.9 ± 0.6 , 3.2 ± 0.6 , 10.6 ± 0.1 and 3.3 ± 0.5 μM , respectively.^{83, 84} The effect of substitution of azomethine hydrogen atom by a methyl group is cell line dependent. While there was no change in antiproliferative activity for 2-acetylpyridine thiosemicarbazone (APTSC) in the first two cancer cell lines, a considerable increase was observed against the other two cell lines ($IC_{50} = 0.4 \pm 0.01$ and 0.2 ± 0.02 μM). Terminal N^4 -dimethylation of FTSC resulted in a very strong enhancement of antiproliferative activity reaching IC_{50} values of 0.0040 ± 0.0009 and 0.0098 ± 0.0011 μM in 41M and SK-BR-3 cells after exposure for 96 h.⁸³ The favorable effect of N^4 -dimethylation is also well-documented for other related α -heterocyclic thiosemicarbazones.³¹ The coordination of FTSC to copper(II) was reported to increase or decrease the activity depending on the cell type.⁸⁵⁻⁸⁸ In particular, $[\text{Cu}(\text{FTSC})\text{Cl}_2]$ revealed an increase of cytotoxicity by a factor of 3 in SW480 cells when compared to that of FTSC, while against HL60 cells the activity of FTSC and the copper(II) complex was very similar.⁸⁴ The proline-FTSC hybrids, we synthesized previously,⁸⁰ showed a different activity compared to the compounds reported herein. Hybrids that were not methylated at N^4 (L- and D-Pro-FTSC) and their nickel(II), copper(II) and zinc(II) complexes lacked activity ($IC_{50} > 300$ μM) in both the studied human cancer cell lines HeLa (cervical carcinoma) and A549 (adenocarcinoma), as well as in the non-carcinogenic cell line MRC5 (foetal human fibroblast). The terminally dimethylated hybrid dm-L-Pro-FTSC showed moderate to low anticancer activity with IC_{50} values of 224.6 ± 6.4 , 204.3 ± 4.8 and 178.4 ± 1.5 μM in the HeLa, A549 and MRC5 cell lines respectively. Complex formation with copper(II) led to an increased cytotoxicity with IC_{50} values of 93.3 ± 5.5 , 176 ± 1.7 and 69.4 ± 4.7 in the same cell lines, respectively. Complex formation with zinc(II) or nickel(II) had no favorable effect on the activity. It should be also noted that the copper(II) complex of dm-L-Pro-FTSC showed significant RNR-inhibition activity under reductive conditions at a concentration of 20 μM .⁸⁰ Comparison of IC_{50} values for **3** and **4** indicates that terminal N^4 -dimethylation enhances the cytotoxicity in accord with the general trend observed in the literature.^{31, 83} In contrast, the effect is opposite, although also cell type dependent, if the activity of compounds **1** and **2** is compared. The observed divergent effects of terminal N^4 -dimethylation suggest that structural

modifications at the pyridine ring (coupling to piperazine and morpholine moieties which increases the denticity of the ligands) play an important role in structure-activity relationships.

Conclusions

The synthesis of new hybrid species as potential ligands for transition metals permitted the study of the effects of the methylpiperazine, morpholine and methylpyrrole-2-carboxylate attachment to the parent 2-formylpyridine thiosemicarbazone on the aqueous solubility, lipophilicity, ability to form copper(II) complexes, their thermodynamic stability and antiproliferative activity in human cancer cell lines HeLa, A549 and LS174, as well as in nontumorigenic cell line MRC5. The hybrid species **HL**⁵ and **HL**⁶ proved to be almost insoluble in water precluding any biological investigations. Attempts to hydrolyze the ester group to –COOH in these two compounds in order to improve the aqueous solubility and ascertain the biological potency of new compounds are undergoing in our laboratory. In contrast, the hybrids **HL**¹–**HL**⁴ possess excellent water solubility. The solution speciation of copper(II) complexes of **HL**² and **HL**⁴ has been characterized in pure aqueous solution via a combined approach using pH-potentiometry, EPR spectroscopy and UV–vis spectrophotometry. The two hybrid compounds were found to act as tetradentate ligands in solution coordinating to copper(II) via the (N_{py}, N, S[–], N_{hetero}) donor atoms. This binding mode was confirmed by X-ray crystallography in the case of complexes **1**–**4**. Predominant formation of highly stable [CuL]⁺ complexes was found at pH 7.4 in aqueous solution and based on the stability constants their decomposition cannot occur even at biologically more relevant micromolar concentrations. The morpholine derivatives **HL**³ and **HL**⁴ possess markedly higher log *D*_{7.4} values compared to those of the piperazine counterparts **HL**¹ and **HL**² most probably due to the different protonation states of the hybrid ligands at physiological pH. At the same time they are more hydrophilic than Triapine. Compounds prepared in this work were tested for antiproliferative activity in different human cancer cell lines. Coordination of hybrid ligands **HL**¹–**HL**⁴ to copper(II) significantly increased the cytotoxicity *in vitro*. While **HL**¹–**HL**⁴ possess low cytotoxicity with IC₅₀ > 300 μM, their copper(II) complexes revealed high antiproliferative activity. The most active compound **4** exhibited IC₅₀ values in the range 13.1–42.8 μM in all three human cancer cell lines. Nevertheless the toxicity of the most active complex remains considerably lower when compared to parent 2-formylpyridine and 2-acetylpyridine thiosemicarbazones and their copper(II) complexes which showed IC₅₀ values in the nanomolar concentration range and are

characterized by very high general toxicity, and, as a consequence have a low therapeutic index. Further experimental work to get insight into the mechanism of action of the prepared copper(II) complexes with hybrid ligands is required to ascertain whether they are really good candidates for further development as potential anticancer drugs.

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† Electronic Supplementary Information (ESI) available: NMR numbering scheme for **HL**¹–**HL**⁶ (Scheme S1), synthesis scheme for **HL**¹–**HL**⁶ (Scheme S2), deprotonation steps of **HL**² (Scheme S3), part of the crystal structure of **1** showing complex pairing via intermolecular hydrogen bonding interactions (Figure S1), pH-dependence of the chemical shifts of various protons of **HL**⁴ (Figure S2), low- (A) and high-field (B) regions of the ¹H NMR spectra **HL**² at different pH values (Figure S3), pH-dependence of the chemical shifts of various protons of **HL**² in the low- (A) and in the high-field (B) regions (Figure S4), pH-dependence of the molar fraction of the *E* and *Z* isomers of **HL**² (Figure S6), 3-dimensional fluorescence spectra of **HL**² and **HL**⁴ (Figure S6), experimental and simulated solution EPR spectra recorded for the copper(II) – **HL**⁴ system at 1:1 (A) and 1:2 (B) metal-to-ligand ratio (Figure S7), calculated component EPR spectra obtained for copper(II) complexes of **HL**² and **HL**⁴ in frozen solution (Figure S8), UV–vis spectra of [Cu(EDTA)]^{2–} in the presence of **HL**²

(Figure S9), UV-vis spectra of the copper(II) – **HL**² and **HL**⁴ systems at 1:1 metal-to-ligand ratio (Figure S10).

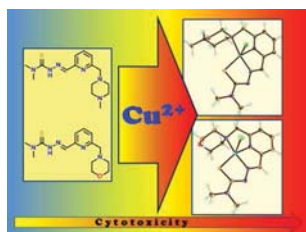
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Table of Contents



Strong enhancement of antiproliferative activity in human cancer cell lines was found upon coordination of new hybrid ligands to copper(II).

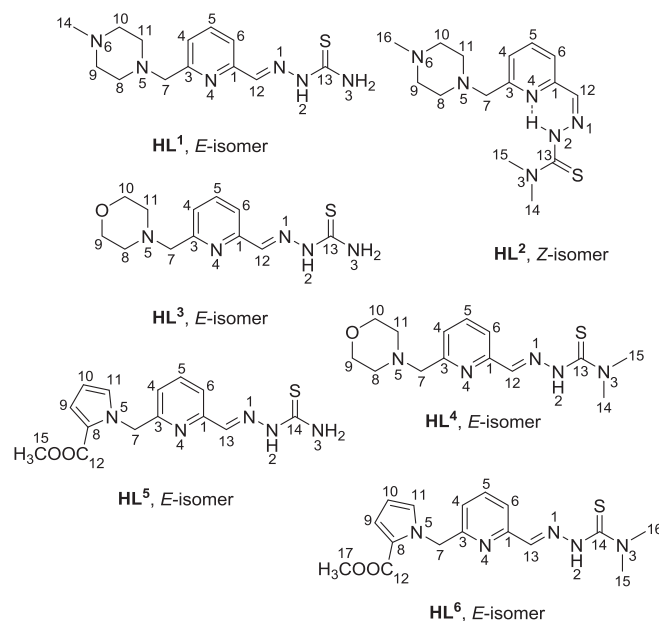
Supporting Information
for

**Strong Effect of Copper(II) Coordination on Antiproliferative Activity of
Thiosemicarbazone-Piperazine and Thiosemicarbazone-Morpholine Hybrids**

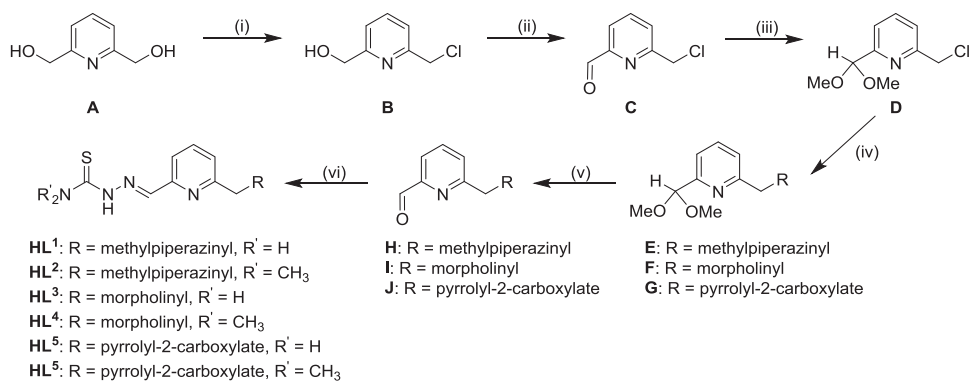
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Keywords: Thiosemicarbazone-piperazine, Thiosemicarbazone-morpholine,
Thiosemicarbazone-methylpyrrole-2-carboxylate hybrids, Solution equilibrium, Stability
constants, Antitumor activity

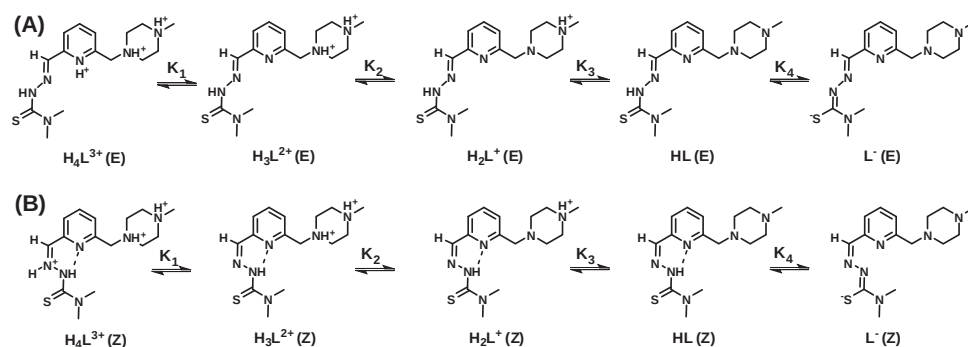


Scheme S1. Atom numbering scheme for ^1H and ^{13}C NMR data for **HL¹ – HL⁶**. **HL²** is depicted as Z-isomer for clarity reasons.



Scheme S2. Synthesis of **HL¹–HL⁶**.^a

^aReagents and conditions: (i) and (ii) see ref.1; (iii) trimethyl orthoformate, methanesulfonic acid, methanol, 78 °C, 3 h; (iv) **E**: methylpiperazine, triethylamine, THF/CH₂Cl₂ 1:1, 40 °C, 12 h, purification by column chromatography; **F**: morpholine, triethylamine, THF/CH₂Cl₂ 1:1, 40 °C, 12 h, purification by column chromatography; **G**: pyrrole-2-carboxylate, sodium hydride, DMF, 0° C, 1 h, then room temperature, 12 h, purification by column chromatography; (v) **H** and **I**: HCl, water, 60 °C, 12 h, sat. aqueous NaHCO₃, extraction with CH₂Cl₂; **J**: water/acetone 5:1, reflux, 12 h; (vi) **HL¹**: thiosemicarbazide, EtOH, 78 °C, 12 h, purification on prep. HPLC; **HL²**: 4,4-dimethyl-3-thiosemicarbazide, EtOH, room temperature, 12 h, purification on prep. HPLC; **HL³**: thiosemicarbazide, EtOH, 78 °C, 12 h, recrystallization from water/methanol 5:1; **HL⁴**: 4,4-dimethyl-3-thiosemicarbazide, EtOH, room temperature, 12 h. **HL⁵**: thiosemicarbazide, EtOH/MeOH 1:1, 78 °C, 12 h, recrystallization from water/methanol 5:1. **HL⁶**: 4,4-dimethyl-3-thiosemicarbazide, EtOH/MeOH 1:1, room temperature, 6 h.



Scheme S3. Deprotonation steps of the H_4L^{3+} form of ligand mPip-dm-FTSC (HL^2) for its *E* (A) and *Z* (B) isomers

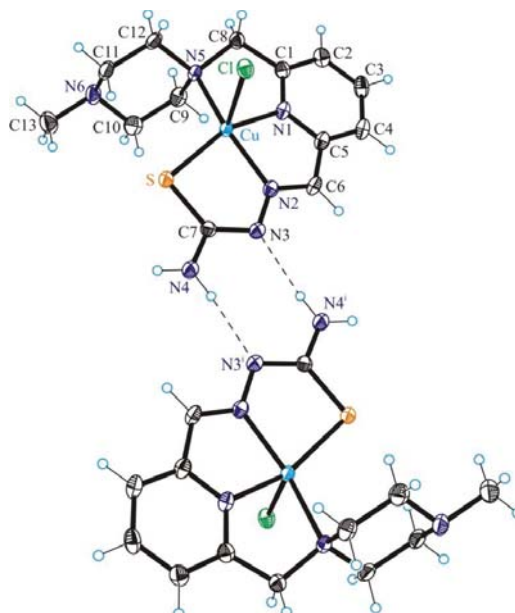


Figure S1. Part of the crystal structure of **1** showing complex pairing via intermolecular hydrogen bonding interactions. Donor...acceptor ($D\cdots A$) distances ($\text{\AA}$) and donor-hydrogen...acceptor ($D-H\cdots A$) angles (deg) of the intermolecular hydrogen bonds: $N4\cdots N3^i$ 2.946(3) $\text{\AA}$, $N4-H\cdots N3^i$ 173.7°; atoms marked with *i* are at symmetry position $-x + 2, -y + 1, -z + 1$.

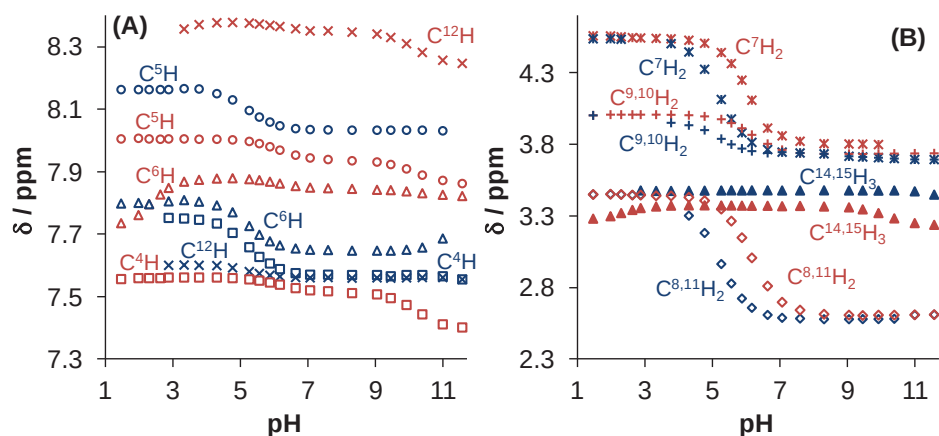


Figure S2. pH-Dependence of the chemical shifts of the various protons of the ligand Morphdm-FTSC (HL^4) in the low- (A) and in the high-field (B) regions. **Red** and **blue** symbols denote the protons belonging to the major *E* and minor *Z* isomers, respectively. [$c_L = 1.5 \text{ mM}$; $T = 298 \text{ K}$; $I = 0.10 \text{ M}$ (KCl); 10% D_2O]

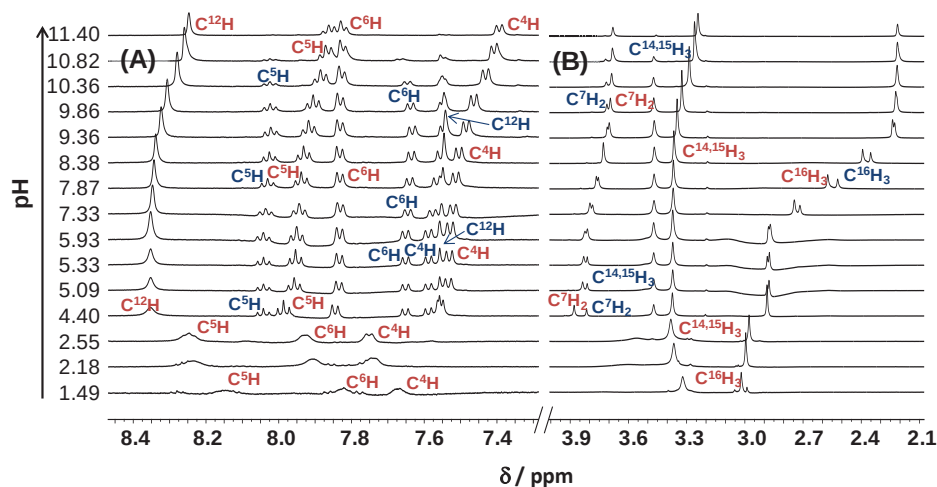


Figure S3. Low- (A) and high-field (B) regions of the ^1H NMR spectra of ligand mPip-dm-FTSC (HL^2) at different pH values, **red** and **blue** symbols denote the peaks belonging to the protons of the major *E* and minor *Z* isomers, respectively. [$c_L = 3 \text{ mM}$; $T = 298 \text{ K}$; $I = 0.10 \text{ M}$ (KCl); 10% D_2O]

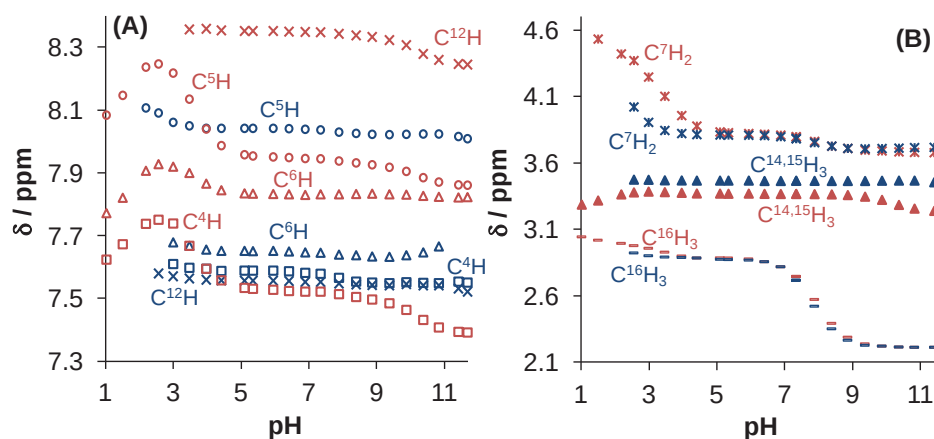


Figure S4. pH-Dependence of the chemical shifts of the various protons of the ligand mPip-dm-FTSC (HL^2) in the low- (A) and in the high-field (B) regions. Red and blue symbols denote the protons belonging to the major *E* and minor *Z* isomers, respectively. [$c_L = 3 \text{ mM}$; $T = 298 \text{ K}$; $I = 0.10 \text{ M (KCl)}$; 10% D_2O]

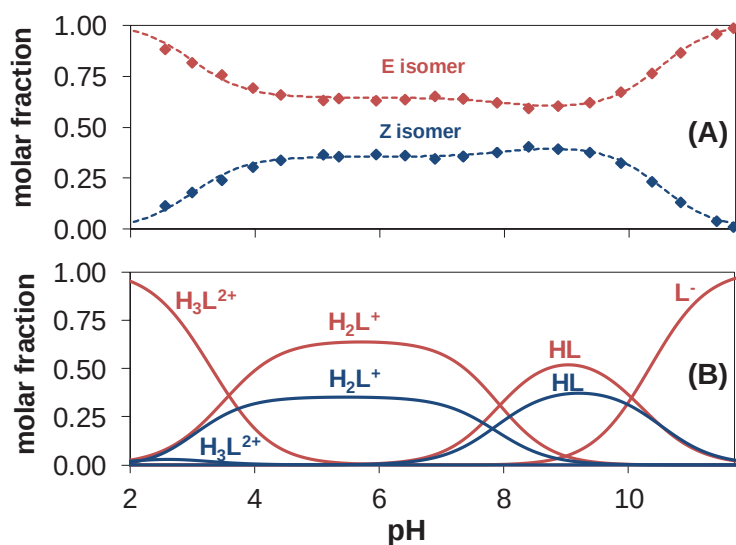


Figure S5. pH-Dependence of the molar fraction of the *E* (red symbols) and *Z* (blue symbols) isomers of the ligand mPip-dm-FTSC (HL^2) calculated on the basis of the integrated areas of the signals of the various ligand protons (A). Concentration distribution curves for the isomeric ligand species (*E*: red letters; *Z*: blue letters) calculated with the aid of the microscopic proton dissociation constants (B). [$c_L = 3 \text{ mM}$; $T = 298 \text{ K}$; $I = 0.10 \text{ M (KCl)}$; 10% D_2O]

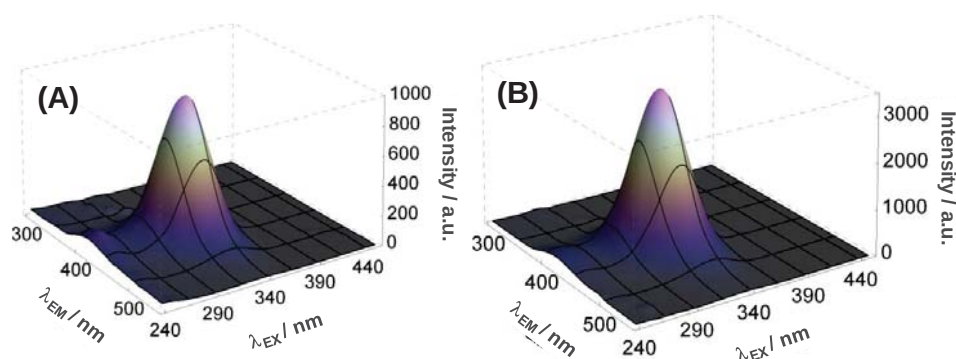


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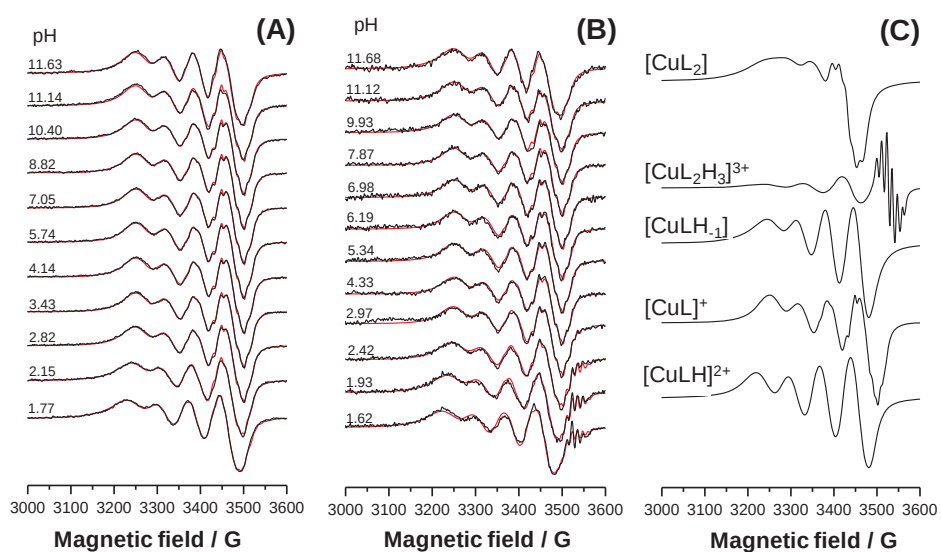


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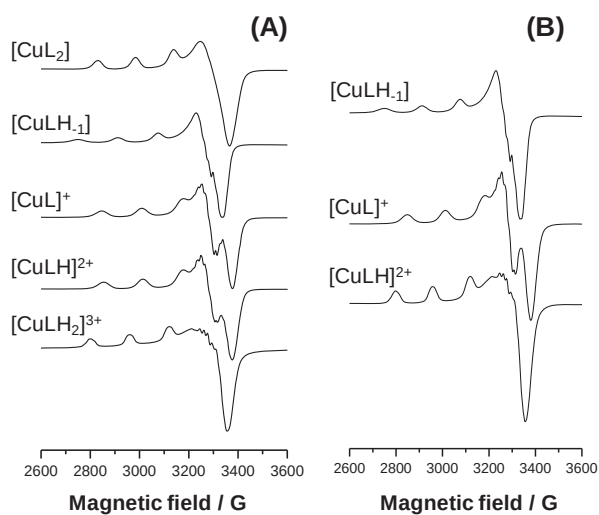


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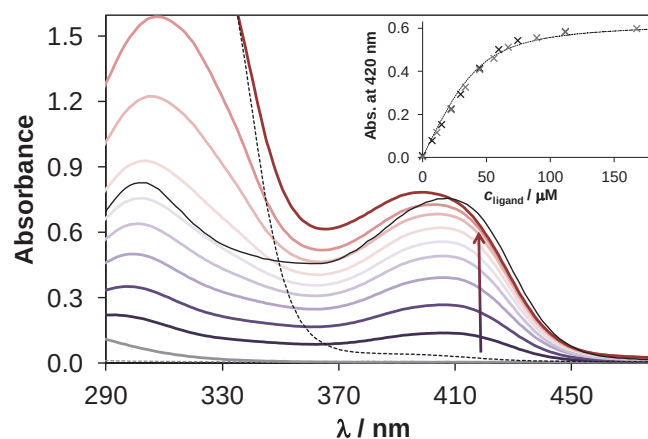


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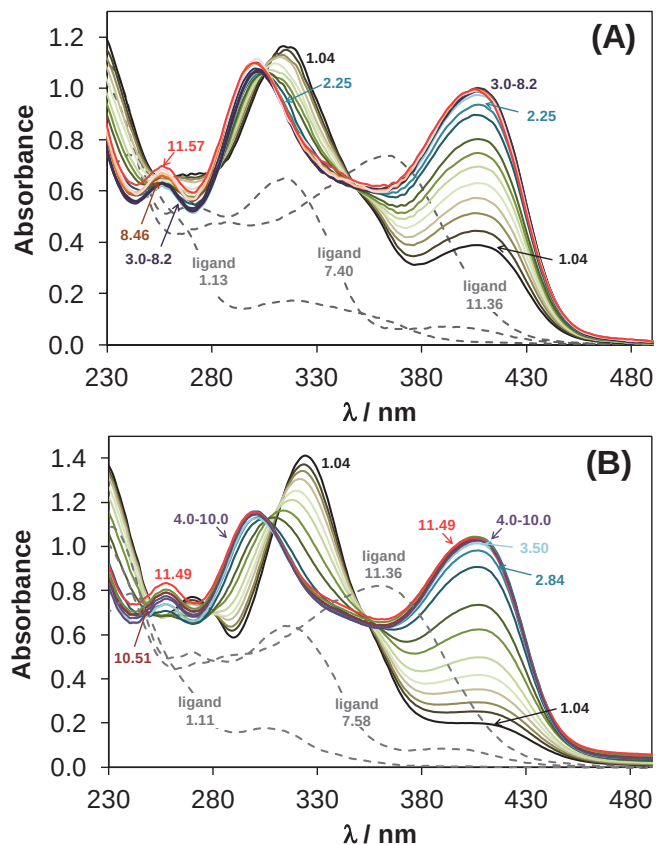


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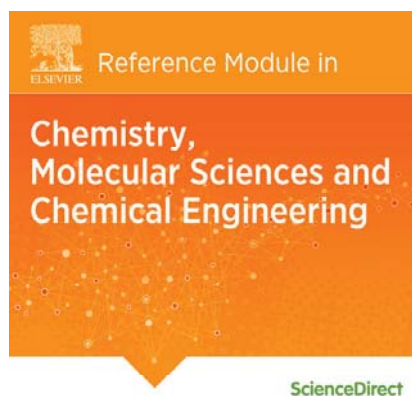
2.3 Ruthenium Compounds as Antitumor Agents: New Developments

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Ruthenium Compounds as Antitumor Agents: New Developments

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Introduction

Coordination and organometallic chemistry offers additional options for the design and synthesis of chemotherapeutics when compared to organic chemistry.^{1–3} These advantages arise mainly due to metal coordination and realization of stereochemical preferences of metal ions leading ultimately to structural diversity distinct from that of organic compounds, as well as the possibility to form covalent metal-pharmacological target bonds. Coordination numbers from 2 to 8 and the formation of varied coordination geometries for the same coordination number and metal oxidation state are characteristic for transition metal complexes, while the sp, sp², and sp³ hybridization of valence electrons of the carbon atom leads to a limited number of possible geometries in organic compounds, namely, linear, trigonal-planar, and tetrahedral. One asymmetrical sp³ hybridized carbon atom generates one pair of enantiomers, while an asymmetrical sp³d² or d²sp³ hybridized metal ion (in terms of valence bond theory) coordinated by six different monodentate ligands generates 15 pairs of enantiomers. In addition, the event of ligand exchange at the metal ion (occurring with rates ranging from 10^{−9} to 10⁹ s^{−1} for water exchange)⁴ with controls of both the thermodynamics and kinetics of these substitution reactions,⁵ redox activity,⁶ and the ability of metal-containing species to bind reversibly to biomolecular targets, for example, enzymes,⁷ can be explored to optimize the reactivity required for biological efficacy. Favorable effects resulting from coordination of biologically active organic compounds include improved aqueous solubility, enhancement of lipophilicity facilitating the cellular uptake, stabilization of less flexible ligand conformations via binding to metal ions, changed reactivity, and biologically accessible redox activity (metal or ligand centered), as well as control of acid–base properties of coordinated ligands. Fine-tuning of diverse parameters by metal coordination may help in the development of more effective chemotherapeutics by optimization of pharmacokinetic and pharmacodynamic properties of lead drug candidates. However, these opportunities have been little explored so far, although the worldwide use of cisplatin, oxaliplatin, and carboplatin, approved by FDA for medicinal use in 1978, 1989, and 2002, respectively, and the regional use of nedaplatin, lobaplatin, and heptaplatin have significantly raised the reputation of coordination chemistry in the fight against cancer.^{8–10} The majority of drugs approved for medicinal use and introduced to the market since 2002 are organic compounds, with those containing metal ions constituting less than 1% mainly as diagnostic agents or earlier approved platinum anticancer drugs used in combination with new organic drugs.^{11–20} Table 1 summarizes the anticancer products launched worldwide from 2011 to 2013 with exception of biological drugs.^{20–22} Figure 1 displays the new chemical entities of the active ingredients of the marketed anticancer drugs. Examples of current clinical interest involving metal-based compounds and chelators as theranostics have also been listed in the literature.²³

Although platinum complexes dominate the field of metal-based cancer chemotherapy,⁷ they are far from being optimal drugs. The limitations imposed by general toxicity as well as the intrinsic and acquired tumor resistance to clinically used drugs have motivated the exploration of other metal complexes as chemotherapeutic agents. Complexes of ruthenium, osmium, gold, gallium, titanium, and some first row transition metals have been investigated in order to create drugs with other than DNA-binding

2 Ruthenium Compounds as Antitumor Agents: New Developments

Table 1 New anticancer products launched in 2011–2013

Trade name/ country/year	Company (developing/ marketing)	Active ingredient ^a	Indication	Underlying mechanism of action
Zytiga [®] / USA/2011	Cougar Biotechnology/ Johnson & Johnson ^b	Abiraterone acetate	Treatment of castration-resistant prostate cancer	Inhibition of CYP17A1 enzyme expressed in testicular, adrenal, and prostatic tumor tissues
Giotrif [™] / USA/2013	Boehringer Ingelheim	Afatinib	Treatment of metastatic non-small cell lung cancer (NSCLC) with common epidermal growth factor receptor (EGFR) mutations	Irreversible binding and inhibition of EGFR (c-ErbB-1), HER2 (c-ErbB-2) and c-ErbB-4 receptors
Inlyta [®] /USA/ 2012	Pfizer	Axitinib	Treatment of advanced renal cell carcinoma	Inhibition of tyrosine kinase and of vascular endothelial growth factor receptor (VEGFR-1, -2, and -3)
Cometriq [™] / USA/2013	Exelixis	Cabozantinib S-malate	Treatment of progressive, metastatic medullary thyroid cancer	Inhibition of multiple receptor tyrosine kinases, including Ret, Met, VEGFR-1, VEGFR-2 and VEGFR-3, Trk-B, FLT-3, AXL, and TIE-2 involved in oncogenesis, metastasis, angiogenesis, and maintenance of the tumor microenvironment
Kyprolis [™] / USA/2012	Onyx Pharmaceuticals	Carfilzomib	Treatment of multiple myeloma	Inhibition of proteasome
Xalkori [®] / USA/2011	Pfizer	Crizotinib	Treatment of metastatic NSCLC that is caused by the echinoderm microtubule associated protein-like 4 (EML4) mutation of anaplastic lymphoma kinase (ALK)	Inhibition of mesenchymal epithelial transition factor/anaplastic lymphoma kinase (cMET/ALK)
Tafinlar [®] / USA/2013	GlaxoSmithKline	Dabrafenib mesylate	Treatment of adult patients with unresectable or metastatic melanoma with BRAF V600E mutation	Inhibition of Raf kinase B
Dacogen [®] / EU/2012	Astex/Janssen- Cilag	Decitabine	Treatment of adult patients with newly diagnosed <i>de novo</i> or secondary acute myeloid leukemia (AML)	Inhibition of DNA methyltransferase
Xtandi [®] / USA/2012	Medivation/ Astellas	Enzalutamide	Treatment of metastatic castration-resistant prostate cancer (mCRPC)	Inhibition of androgen binding to androgen receptors
Imbruvica [™] / USA/2013	Pharmacyclics/ Janssen	Ibrutinib	Treatment of mantle cell lymphoma (MCL)	Inhibition of Bruton tyrosine kinase (BTK)
Conmana [®] / China/ 2011	Zhejiang Bata Pharma Inc.	Icotinib hydrochloride	Treatment of NSCLC	Inhibition of epidermal growth factor receptor (EGFR) tyrosine kinase
Pixuvri [®] /EU/ 2012	Cell Therapeutics	Pixantrone maleate	Treatment of multiple relapsed or refractory aggressive non-Hodgkin's B-cell lymphomas	DNA intercalation
Pomalyst [®] / USA/2013	Celgene	Pomalidomide	Treatment of multiple myeloma	Immunomodulation
Iclusig [™] / USA/2013	Ariad Pharmaceuticals	Ponatinib	Treatment of chronic, accelerated, or blast phase Philadelphia chromosome-positive (Ph+) chronic myelogenous leukemia (CML)	Inhibition of tyrosine kinases with antiangiogenesis effects
Xofigo [®] / USA/2013	Algeta/Bayer Schering Pharma	Radium Ra223 dichloride	Treatment of CRPC, with symptomatic bone metastases and without known visceral metastases	Alpha particle-emitting radioactive therapy
Stivarga [®] / USA/2012	Bayer/Onyx Pharmaceuticals	Regorafenib	Treatment of metastatic colorectal cancer (mCRC)	Multikinase inhibition
Jakafi [®] / USA/2011	Incyte	Ruxolitinib phosphate	Treatment of myelofibrosis (MF), post- polycythemia vera MF, and post-essential thrombocythemia MF	ATP competitive inhibition of tyrosine- protein kinases JAK1 and JAK2
Marqibo [®] / USA/2013	Talon Therapeutics/ Spectrum Pharmaceuticals GlaxoSmithKline	Sphingosomal vincristine	Treatment of Philadelphia chromosome- negative acute lymphoblastic leukemia (ALL)	Inhibition of mitosis

(Continued)

Table 1 (Continued)

Trade name/ country/year	Company (developing/ marketing)	Active ingredient ^a	Indication	Underlying mechanism of action
Mekinist™/ USA/2013	AstraZeneca	Trametinib dimethyl sulfoxide	Treatment of unresectable or metastatic melanoma with BRAF V600E and V600K mutations	Inhibition of the mitogen-activated protein (MAP) kinase (MEK1/MEK2)
Caprelsa®, Zactima®, Zictifa®/ USA/2011		Vandetanib	Treatment of symptomatic or aggressive medullary thyroid cancer (MTC)	Inhibition of VEGF, EGF, and RET receptor tyrosine kinases
Zelboraf®/ USA/2011	Plexxikon/Roche	Vemurafenib	Treatment of metastatic melanoma with BRAF V600E mutation	Inhibition of BRAF gene

^aProducts are listed in alphabetical order by active ingredient. The structure of the chemical entity is shown in Figure 1.

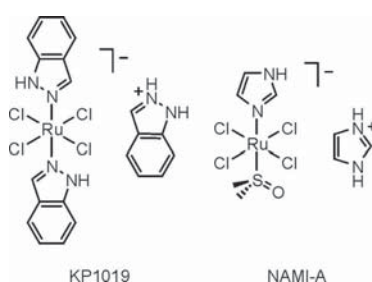
^bThis drug was originally discovered by researchers at the Cancer Research UK Centre for Cancer Therapeutics.

mechanisms of action at the molecular level. In particular, synthesis of compounds binding to specific proteins expressed exclusively in cancer cells can provide high selectivity and significantly reduce or completely eliminate the side effects. At the same time, many compounds are still prepared as mimics of cisplatin and focus on DNA binding.⁷ However, their efficacy can be confirmed only by *in vivo* studies.⁷

Interest in and the enormous importance of the topic motivate more and more researchers for the synthesis of new metal-based drugs^{8,24–26} and investigation of different issues, such as their behavior in biological media, interaction with biological ligands, antiproliferative activity both *in vitro* and *in vivo*, uptake into the cell and intracellular distribution, cell response to metal-based drug-induced stress, and, finally, the underlying mechanism of their action, which determine the progress of the field. This article describes the most recent developments in ruthenium complexes as potential antitumor drugs comprehensively covering the literature published in the last 3 years.

Ruthenium Complexes in Clinical Trials and Preclinical Development

Advances in the development of potential ruthenium-based anticancer drugs are made evident by a number of review articles published recently.^{2,27–36} Among the most notable achievements over the past 30 years are surely the clinical phase I trials of two ruthenium(III) complexes, namely, NAMI-A and KP1019. The results of early-stage clinical and pharmacokinetic studies have been reported for both NAMI-A³⁷ and KP1019^{38–41} and discussed.⁴² One of 24 and five of six evaluable patients all of which had progressive disease at trials entry achieved stable disease status after treatment with NAMI-A and KP1019, respectively. Dose-limiting toxicity (DLT), manifested as the formation of blisters, and maximum tolerated dose (MTD) of 300 mg m⁻² day⁻¹ were determined for NAMI-A and recommended for phase II studies. The lack of marked renal toxicity in the phase I study with NAMI-A is probably due to a 3-week drug-free interval used between consecutive cycles of treatment, as concluded from the full reversibility of the renal toxicity of this drug studied in mice.⁴³



In the case of KP1019, the MTD could not be reached, and no DLTs were established because of the limitations imposed by drug solubility requiring increasingly large infusion volumes. As a result, current studies are shifted on the sodium salt of the [RuCl₄(Hind)₂]⁻ complex, also named KP1339, whose aqueous solubility is 35 times higher than that of indazolium salt.⁴⁴ Further, disease-specific studies are under way to elucidate the safety and efficacy of this compound in patients.⁴² It has been claimed recently that sodium *trans*-[tetrachloridobis(1*H*-indazole)ruthenate(III)] is particularly effective in inhibiting the growth of glioma cells,⁴⁵ prostate cancer cells,⁴⁶ and pancreatic cancer,⁴⁷ both sensitive and resistant to accepted chemotherapeutics for

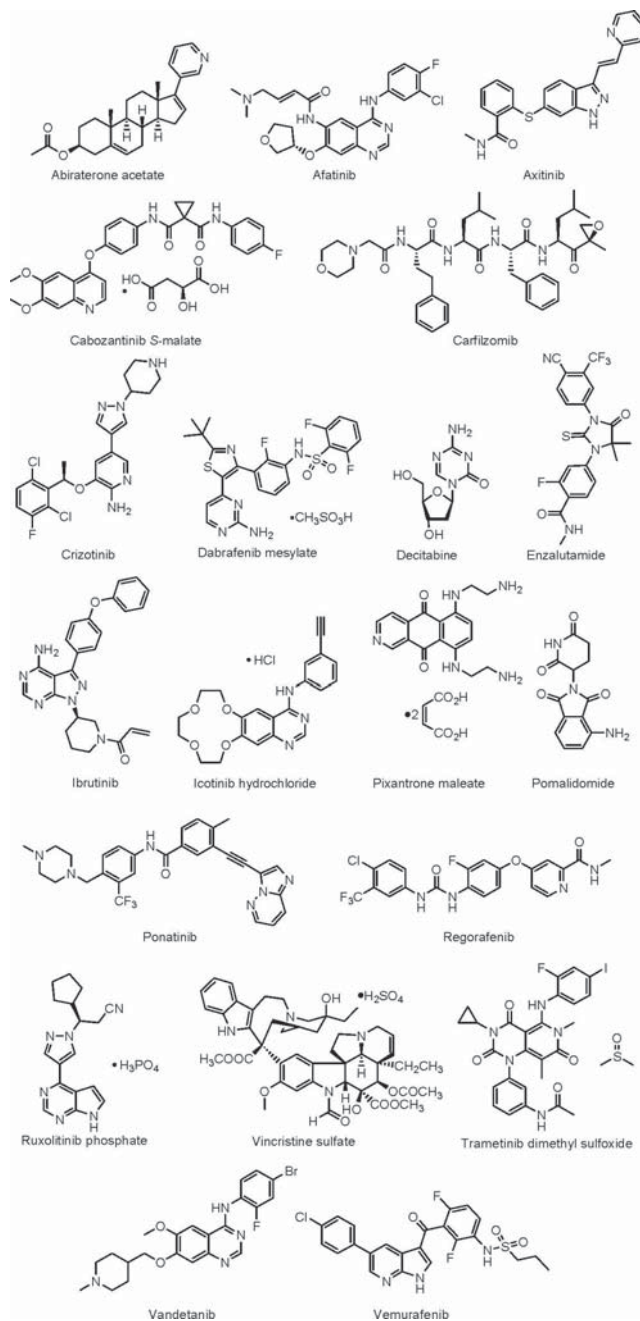


Figure 1 Structures of the active ingredients of anticancer drugs launched worldwide in 2011–2013.

treatment of these types of cancer. Based on MTT assay on gastric cancer cell line NCI-N87 (differentiated carcinoma), it has also been reported that KP1339 is effective in treating, preventing, or delaying the onset of gastric cancer and in treating refractory (tumor fails to respond favorably to or is resistant to the treatment involving chemotherapeutics other than KP1019 or, alternatively, relapses after responding first positively to the treatment) gastric cancer.⁴⁸ Similar efficacy by application of KP1339 against refractory colorectal cancer, melanoma, and lung cancers has also been claimed.⁴⁹ Favorable synergistic effects have been found upon using this drug in combination with platinum-based drugs, taxanes, anthracyclines, 5-FU, nitrosourea compounds, etc. for treatment of different cancers,⁵⁰ as well as with sorafenib in the Hep3B hepatoma xenograft with a 2.4-fold increase in the animal life span compared to 1.9-fold on applying sorafenib as a single agent.⁵¹

Further investigations into the still poorly understood modes of action of NAMI-A and KP1339 have been performed. Interaction with the cell cycle and accumulation of cells in G₂/M phase,⁵² inhibition of matrix metalloproteinases, increase of extracellular matrix around tumor vasculature preventing invasion of tumor cells into adjacent tissues, and direct binding to DNA are considered the major events in the mechanism of action of NAMI-A.⁴² Although DNA as a possible cellular target is still under debate, because little is known about ruthenium–DNA interactions *in vivo*, investigations into the elucidation of other cellular targets relevant for ruthenium drugs have been reported. In particular, binding of hydrolysis products of NAMI-A to RNA and ruthenium accumulation in cellular RNA (two to five ruthenium ions per ribosome) were identified by ICP-MS on RNA extracted from the single-celled organism *Saccharomyces cerevisiae* treated with NAMI-A.⁵³ The ruthenium–RNA adduct formation is favored in acidic medium and reducing environments typical for tumor tissues.

The ‘activation by reduction’ hypothesis was usually used to explain why ruthenium is less toxic than platinum drugs. However, recently, both the hypothesis and the opinion about the low host toxicity of ruthenium drugs have become a subject of criticism,²⁹ mainly because of the lack of experimental evidence for the reduction of potential ruthenium drugs *in vivo*. In this respect, the results reported recently for a NAMI-A analog⁵⁴ have revived the literature debate. In particular, by reaction of [(DMSO)₂][*trans*-RuCl₄(DMSO)₂] with purine derivatives, a number of NAMI-A analogs have been prepared. The complexes have shown low cytotoxicity with IC₅₀ > 100 μM against human cancer K562 (leukemia), G361 (melanoma), HOS (osteosarcoma), and MCF-7 (breast) cell lines. The complex with L = 6-(2-methoxybenzylamino)purine was found to undergo a metal-centered quasireversible one-electron reduction ($E_{1/2} = -6$ mV vs. NHE) with the formation of a ruthenium(II) species in tumor tissue isolated from the treated animal. This was shown by intracellular visualization of the arising ruthenium(II)–bipyridine complex by fluorescence microscopy. To prove the validity of the method proposed, ruthenium(III) complexes with large negative reduction potentials, which cannot be reduced under physiological conditions (the reduction potential is about –240 mV vs. NHE in proliferating cells) or in a tumor environment, would be required. Note that the immediate reduction of NAMI-A already after intravenous administration by the reductants present in blood accompanied by slowing down the formation of apo–Tf–ruthenium adducts has also been suggested.⁵⁵ Exposure of KP1019 or KP1339 adducts with holo-transferrin to glutathione at simulated cancer cytosol concentration was claimed to result in ruthenium(II) species, [RuCl₄(Hind)(GSH)]^{2–} and [RuCl₄(Hind)(GSSG)]^{2–}, based on tandem electrospray ionization mass spectrometry data.⁵⁶ In addition, this adduct was found to release both ruthenium and iron when treated with glutathione and ascorbic acid.⁵⁷

In the case of KP1019, the induction of apoptosis via the mitochondrial pathway⁵⁸ and oxidative stress⁵⁹ is considered to play a major role in the mechanism contributing to cytotoxicity, while interaction with DNA⁶⁰ and topoisomerase II⁶¹ is most likely of secondary relevance. Nevertheless, quite recently, the induction of DNA damage by KP1019, leading to cell cycle delay and cell death in yeast, has been reported.⁶² *In vivo* analysis of KP1339 in mouse plasma by LC- and CZE-ICP-MS has confirmed the high affinity of ruthenium(III) compounds to proteins and the major role of the adducts formed in the transport of the drug through the bloodstream.⁶³ In addition, the effect of the applied dosage on the protein content and adducts formed has been established. Plasma samples treated with 40 mg kg^{–1} and tested after 24 h showed mainly binding to the albumin/transferrin (60–80 kDa) fraction, while a broader distribution of the adducts formed was observed upon a dosage increase to 50 mg kg^{–1}. The formation of ruthenium–protein adducts is considered responsible, at least in part, for the low side effects exerted by the compound in clinical trials. The effect of axial indazole ligand substitution in KP1019 by pyridine-substituted ligands on hydrophobic interactions with human serum albumin (HSA) and antiproliferative activity in a SW480 cell line has been reported.⁶⁴ Dibenzyloxy-functionalized complexes preincubated with albumin showed increased cytotoxicity when compared to ruthenium complex-exposed cells, consistent with hydrophobic HSA interactions, which may enhance the bioavailability of the drug by inhibiting protein coordination. Given the extensive protein binding of KP1339, as well as KP1019,⁶⁵ a somewhat subordinate role for ruthenium–DNA interactions *in vivo* was recently suggested. However, coinubation of KP1019 with ascorbic acid, the main reducing agent in blood,⁶⁶ was demonstrated to enhance the interaction of the ruthenium species with DNA in SW480 cells and with plasmid DNA under cell-free conditions⁶⁷ and to increase the cytotoxicity *in vitro* when concentrations of ascorbic acid varied from 50 to 700 μM. A strong chemoprotective effect was observed in SW480 cells in the presence of low concentrations of ascorbic acid (2.7–50 μM).

By performing studies on kinetics and mechanism of reduction of NAMI-A by 2-mercaptoethane sulfonate, a chemoprotectant against kidney and bladder toxicity, a combination chemotherapy regimen involving NAMI-A and an anticancer drug inducing bladder and kidney cytotoxicity was proposed.⁶⁸

Modern physical methods have been applied extensively to gain more insight into the mechanism of action of both NAMI-A and KP1019. In particular, we note the recent use of X-ray absorption and synchrotron-based micro-X-ray fluorescence (XRF) spectroscopy for the investigation of biotransformations of anticancer ruthenium(III) complexes.^{69–71} NAMI-A and KP1019 undergo transformations in aqueous buffers, protein solutions or gels, undiluted blood serum, cell culture medium, and human liver (HepG2) cancer cells with the formation of Ru^{IV/III} clusters and binding of ruthenium(III) to S-donors, amine/imine, and

carboxylate groups of proteins.⁷¹ XANES spectra measured on tumor samples taken from mice treated with KP1019 or KP1339 indicate the presence of both ruthenium(II) and ruthenium(III) bound to Cl, N, and possibly O donors.⁷⁰ At the same time, EPR spectroscopy used for probing the interactions of NAMI-A and KP1019 with cells of *S. cerevisiae* has provided evidence for the presence of paramagnetic low-spin ruthenium(III) species, which prevalently remain unaffected by endogenous reducing agents.⁷² The formation μ -oxido-bridged ruthenium(III) oligomers from NAMI-A was suggested in physiological medium at pH 7.4 24 h after dissolution.⁷³ The addition of ascorbic acid to NAMI-A in physiological buffer results in quantitative reduction of paramagnetic ruthenium(III) (d^5 , $S = 1/2$) to diamagnetic ruthenium(II) (d^6 , $S = 0$). The reduction is prevented when the complex binds covalently to HSA.⁷³ The distribution of ruthenium in human neuroblastoma cells SHSY-5Y treated with KP1019 and NAMI-A has for the first time been studied by XRF imaging.⁷⁴ The results showed that ruthenium is accumulated both in cytosol and in the nucleus when cells were treated with KP1019, in accord with previously reported results obtained by utilizing techniques involving cell lysis,⁷⁵ while ruthenium was not detected in the cells treated with NAMI-A, indicating membrane-binding underlying mechanism for its biological activity, in accord with previous findings.^{76–78} Moreover, colocalization of ruthenium and iron upon treatment of the cells with KP1019 provides evidence for the drug involvement in the iron uptake via transferrin receptor pathway and metabolism. Difference in gel electrophoresis (DIGE) proteomics combined with mass spectrometry measurements was also applied for investigation of the A2780/S cell response to the treatment with NAMI-A.⁷⁹ The proteomic alterations were limited in number, moderate in intensity, and different from those induced by cytotoxic platinum compounds. Of note are two upregulated proteins, namely, PRDX, a redox-active protein playing a role in destroying peroxides, and CATD, a lysosomal aspartic protease involved in proteolysis, cell invasion, and apoptosis. Their upregulation may indicate that NAMI-A induces the generation of damaging ROS and activates the mitochondrial death pathway in the cells.

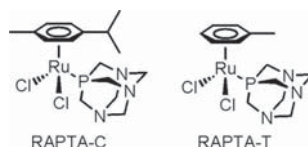
AziRu, a closely related NAMI-A derivative (1), in which the imidazole ligand was replaced by pyridine, was recently prepared and characterized.^{80,81} Like NAMI-A, this compound is rather reactive and undergoes hydrolysis under physiological conditions. The lability of this complex was confirmed by combined Raman spectroscopy and protein crystallography studies of the products resulted from the interaction of AziRu with two small proteins consisting of 129 and 124 amino acid residues, respectively, namely, hen egg white lysozyme (HEWL) and bovine pancreatic ribonuclease (RNase A).^{82,83} The complex forms one adduct per macromolecule with both proteins by covalent bonding to His15 and Asp87 in the case of HEWL and to His105 or His119 (of two crystallographically independent adducts) with RNase A and losing all ligands initially bound to ruthenium. The exchange of chloride ligands by water is faster for AziRu than for the HEWL–AziRu adduct. The binding to proteins does not alter their structure significantly but inactivates the enzymatic activity of RNase A. The formation of Ru–O–Ru oligomers adsorbed on the protein surface was suggested upon interaction with HEWL. To increase the circulating lifetime of AziRu, the complex was coupled to amphiphilic nucleosides⁸⁴ resulting in the formation of conjugates 2–5, and others,⁸⁵ and further stabilized by incorporation into the liposome bilayer of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or into the cationic lipid 1,2-dioleoyl-3-trimethylammoniumpropane chloride (DOTAP).⁸⁶ These ruthenium-containing liposomes proved to be stable for months and showed markedly higher antiproliferative activity in human cancer cell lines MCF-7 (breast) and WiDr (colorectal) and rat C6 glioma cells.

In particular, the 3/POPC liposome after 48 h incubation with the two human cancer cell lines showed IC_{50} values of 7 ± 4 and 40 ± 5 μ M in MCF-7 and WiDr cells, respectively, compared to 305 ± 16 and 441 ± 20 μ M for 1.⁸⁵

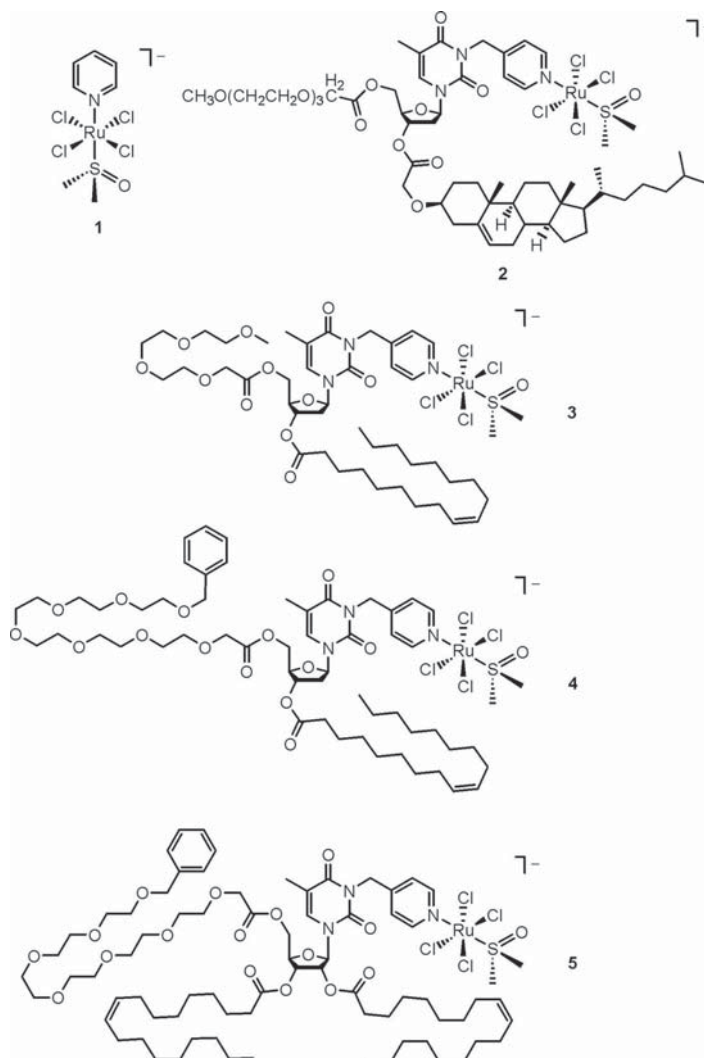
Iodinated indazole analogs of KP1019 and NAMI-A, namely, $Me_4N[trans-RuCl_4(5\text{-iodoindazole})_2]$ and $Me_4N[trans-RuCl_4(dmso)(5\text{-iodoindazole})]$, were prepared and tested for cytotoxicity in A549 cells.⁸⁷ The IC_{50} data indicated similar antiproliferative activity with those of the parent compounds KP1019 and NAMI-A. XRF imaging of single cells provided strong evidence that the indazole ligand(s) remains bound to ruthenium after cellular uptake. By reaction of $RuCl_3$ with 5,7-di-*tert*-butyl-1,2,4-triazolo[1,5-*a*]pyrimidine (dbtp) in 1:2 and 1:3 molar ratios, in boiling ethanol, two different complexes, namely, $trans-[RuCl_3(H_2O)(dbtp)_2]$, an analog of KP1019, and $mer-[RuCl_3(dbtp)_3]$, have been isolated and tested for cytotoxicity *in vitro*.⁸⁸ The first complex showed IC_{50} values of 0.3 ± 0.1 and 2.4 ± 1.4 μ M compared to 10.2 ± 3.2 and 11.2 ± 1.8 μ M for cisplatin in T47D and A549 cell lines, respectively.

RAPTA-C and Model Compounds

Anticancer properties of ruthenium–arene PTA (RAPTA)-type complexes and structure–activity relationships have been surveyed recently.²⁹ Therefore, we will briefly discuss only the works reported after publication of that review.



Selectivity toward specific proteins is believed to play a major role in the mechanism of action of RAPTA-C.⁸⁹ In particular, RAPTA compounds were shown to form adducts with histone proteins.^{90,91} Intriguingly, about 5% of the intracellular ruthenium content was associated with chromatin, which was considered as a possible target for this type of compound, although other targets



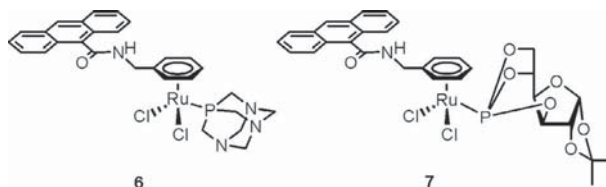
can also be envisaged. RAPTA-C and a number of other related compounds were found to inhibit cathepsin B,⁹² while one other RAPTA-C-related arene-modified compound was shown to inhibit the glutathione transferase enzyme.⁹³ The reactivity of RAPTA-C in the presence of proteins differs from that of cisplatin.⁹⁴ The antimetastasis effect of RAPTA-C is presumably realized by inhibition of angiogenesis and induction of apoptosis in endothelial cells via DNA fragmentation.⁹⁵ RAPTA complexes were shown to form covalent adducts with plasmid DNA pCMV-GLuc, and at high ruthenation level, these adducts are able to inhibit RNA transcription in live HCT-116 cells.⁹⁶

Downregulation of the cytoskeletal proteins such as vimentin has been identified as the strongest cellular response upon treatment with RAPTA-T by an advanced metallomics/proteomics approach based on application of multidimensional protein identification technology (MudPIT),⁹⁷ providing evidence for the significance of histones to the RAPTA-T-mediated mechanism of antimetastasis activity.⁹⁸

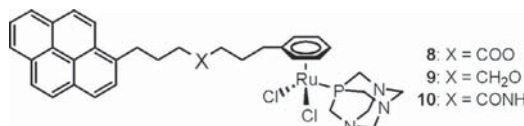
Two anthracene-tethered ruthenium(II)-arene complexes 6 and 7 have been prepared as models of the parent RAPTA-C compound for visualization of their intracellular accumulation by fluorescence microscopy.⁹⁹ Both complexes are generally

8 Ruthenium Compounds as Antitumor Agents: New Developments

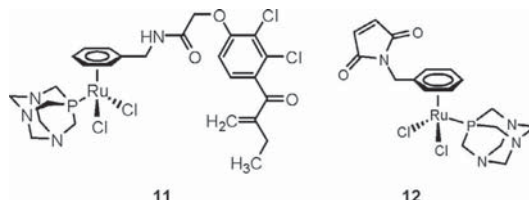
nontoxic in human cancer cell lines after 72 h incubation and do not localize in the nucleus of A549 lung carcinoma cells after being exposed to drugs for 24 h.



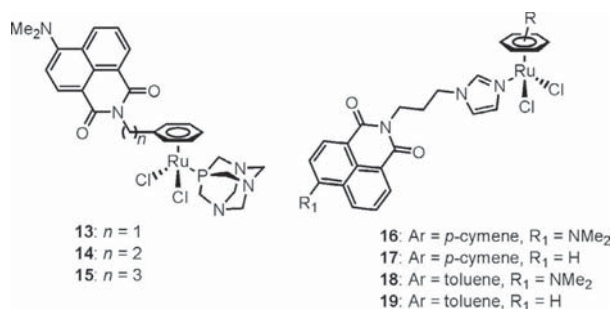
Three fluorescent pyrenyl-arene ruthenium complexes 8–10 have been also reported and evaluated for cytotoxicity in a panel of human cancer cell lines A549 (lung), A2780 (ovarian), A2780cisR, Me300 (melanoma), and HeLa (cervix), which was substantially superior to that of parent compound RAPTA-C.¹⁰⁰ Fluorescence microscopy studies of HeLa cervix cells exposed to 2.5 μM complex 9 have shown fast uptake reaching saturation after 6 h incubation time.



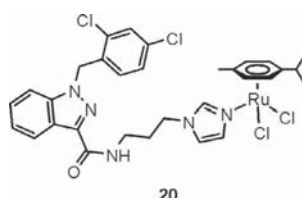
Complex 11, also termed ethaRAPTA, is more effective than RAPTA-C in inhibiting amplification of an important tumor suppressor gene in humans, namely, BRCA1.¹⁰¹ The compound induces apoptosis in the cisplatin-resistant MCF-7 (breast) cancer cell line via multiple pathways.¹⁰² Maleimide-functionalized RAPTA complex 12 and some other related compounds with carbohydrate-derived P-containing or triphenylphosphine ligands were shown to selectively react with Cys34 of HSA, a possible carrier for this type of compounds.¹⁰³ The compounds generally showed higher antiproliferative activity than the parent RAPTA-C compound against CH1 (ovarian), SW480 (colon), and A549 (lung) cancer cells.



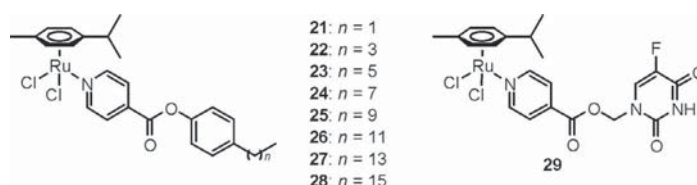
Successful attempts to prepare RAPTA-polymer hybrid-type complexes via PTA alkylation and coupling to a suitably designed polymer as potential anticancer drugs have been described.¹⁰⁴ Substitution of PTA in RAPTA-type complexes by fluorescent BODIPY-phosphane,¹⁰⁵ phosphinoferrocene amino acid conjugates,¹⁰⁶ ω -diphenylphosphino-functionalized alkyl phenyl sulfides,¹⁰⁷ or oligocationic triarylphosphine ligands¹⁰⁸ resulted in complexes with broadly varied cytotoxic potency. A pendant DNA intercalating naphthalimide group has been attached to RAPTA-type complexes via conjugation at the arene moiety or via an imidazole linker substituting the PTA ligand and bound covalently to ruthenium.¹⁰⁹ The two series of compounds 13–15 and 16–19 showed respectable antiproliferative activity in ovarian cancer cell lines A2780 and A2780cisR.



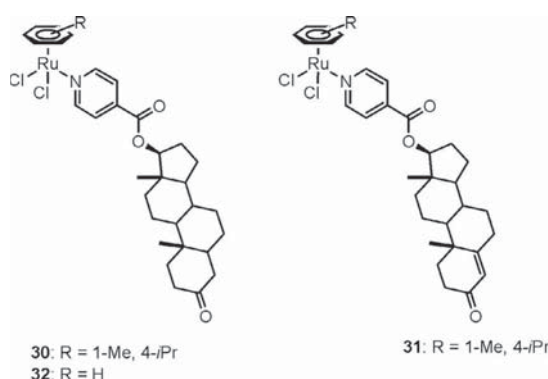
Another RAPTA-C model compound 20, in which the PTA ligand was substituted by a lonidamine–imidazole hybrid ligand, has shown remarkable cytotoxicity in glioblastoma cell lines LN18, LN229, and LN2308 with IC_{50} values of 6.4 ± 2.1 , 8.3 ± 2.4 , and 5.7 ± 0.9 μ M, respectively, which is superior to that found in primary neuronal cultures of the cerebral cortex ($IC_{50} = 20.2 \pm 5.1$ μ M) after 72 h drug exposure.¹¹⁰

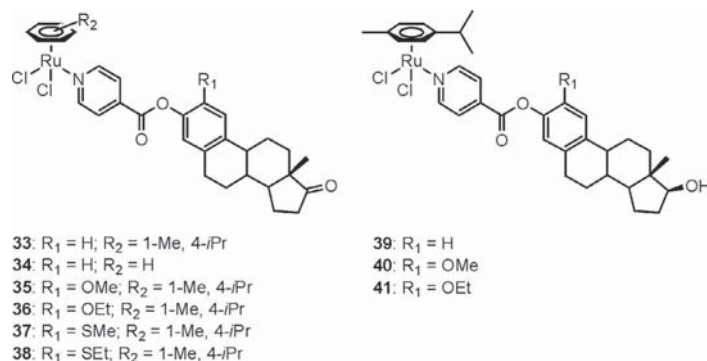


A series of *p*-cymene ruthenium(II) complexes with amino derivatives of biologically active ligands such as flavone, chromone, benzofuran, and *ortho*-, *meta*-, and *para*-methylbenzenes, modified azole heterocycles, as well as with substituted pyridines, including that of 5-fluorouracil-1-methyl isonicotinamide, 21–29, have been prepared by exploring the μ -chlorido-bridge splitting reaction of $[(p\text{-cymene})RuCl(\mu\text{-Cl})_2]_2$ with amine or pyridine-derived ligands.^{111–115} Complex 24 has shown the highest antiproliferative activity with IC_{50} values in the low micromolar concentration range in both human ovarian cancer cell lines A2780 and A2780cisR. Substitution of the terminal methyl group in this complex by a hydroxyl group led to further increase of antiproliferative activity with IC_{50} values in the submicromolar concentration range in an A2780 cell line.

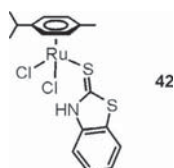


Another series of arene dichlorido ruthenium(II) complexes 30–32 with 4-pyridine-carboxylic acid coupled to steroids was prepared and tested for their affinity to the estrogen receptor ($ER\alpha$), sex hormone-binding globulin (SHBG), and antiproliferative activity in hormone-dependent breast and cervix carcinoma cells MCF-7 and KB-VI, respectively, as well as in hormone-independent melanoma cells 518A2, human leukemia cells HL60, and nontumorigenic HF foreskin fibroblasts.¹¹⁶ The complexes did not show any affinity for the estrogen receptor $ER\alpha$, while some affinity for SHBG was found for androgen derivatives 30 and 32, estrone complexes 33 and 34 and 2-substituted estrone complexes 35–38, and estradiol complexes 39–41. The affinity for SHBG, however, was lower than that of metal-free steroids. Binding of steroids to a ruthenium–arene fragment does not diminish and in some cases even slightly enhances the cytotoxicity. Selectivity toward cancer cell lines for complexes 36, 38, and 41 is also of note.

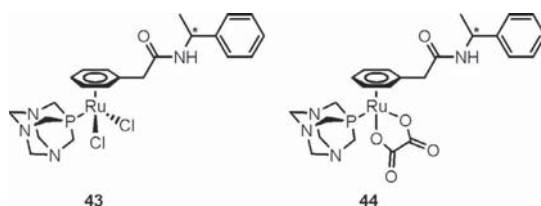




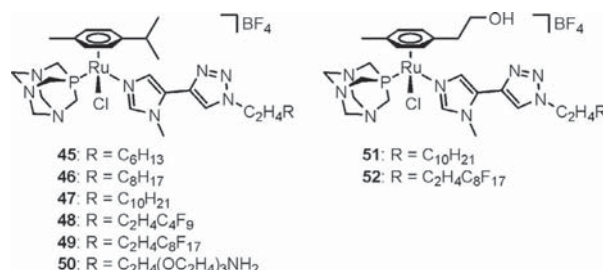
p-Cymene ruthenium(II) complexes of the general formula $[(p\text{-cymene})\text{RuCl}_2(\text{kN,S-heterocycle})]$, where *N,S*-heterocycle is 2-mercaptobenzothiazole, 5-chloro-2-mercaptobenzothiazole, 3-methyl-2-benzothiazolinthion, thiouracil, mercaptosuccinic acid, 2-mercaptobenzoxazole, 2-mercaptobenzimidazole, 2-mercaptobenzimidazole-5-carboxylic acid, methoxy-2-mercaptobenzimidazole-5-carboxylate, and 2-mercapto-5-nitroimidazole, were reported, in which the heterocyclic ligand is bound to ruthenium via the thion sulfur atom.¹¹⁷ The most active complex 42 in the human lung carcinoma cell line H460 ($\text{IC}_{50} = 2.97 \mu\text{M}$) was also highly cytotoxic in HCT116 (colon) and HaCat (keratinocyte) cells showing selectivity for cancer cells when compared to its activity against the normal cell line HEL299. Although the complex-induced cell arrest in the G1 phase, apoptosis was not observed even upon cell treatment with higher concentrations of 42.



Two pairs of enantiomerically pure RAPTA complexes 43 and 44, containing the chiral arene (*R*)- or (*S*)-2-phenyl-*N*-(1-phenylethylene)acetamide and either two chlorido ligands or one bidentate oxalato ligand, were prepared and tested for antiproliferative activity in human ovarian cancer cell lines A2780 and A2780cisR as well as in human embryonic kidney cells as a model of nontumorigenic cancer cells.¹¹⁸ These complexes were more cytotoxic than the parent RAPTA-C and oxalato-RAPTA-C. In addition, *S*-enantiomers were generally more potent in inhibiting cell proliferation than *R*-enantiomers.

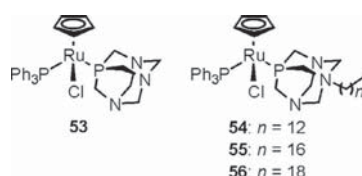


A series of ruthenium-arene complexes 45–52 with hybrid imidazole-triazole ligands containing hydrophobic tails have been prepared by exploring click chemistry and tested for antiproliferative activity in ovarian cancer cell lines A2780 and A2780cisR, as well as in nontumorigenic human endothelial cells HCEC (the last two compounds).¹¹⁹ Overall, moderate cytotoxicity was correlated with the lengths of alkyl chains, which determine the lipophilicity of the compounds. Of note is also the selectivity of the compounds toward cancer cell lines.

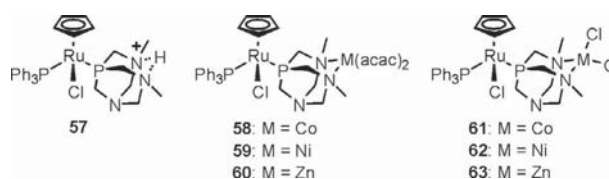


A related complex $[(\eta^6\text{-benzene})\text{RuCl}(\text{letrozole})(\text{PPh}_3)]\text{BF}_4$ ·letrozole has shown antiproliferative activity in MCF-7 cells superior to that of metal-free letrozole with an IC_{50} value of $36 \pm 6 \mu\text{M}$.¹²⁰ Letrozole is an FDA-approved drug that coordinates to the iron center of the aromatase enzyme and deactivates it by blocking the access of testosterone molecule to iron. The enzyme catalyzes the conversion of testosterone into estradiol, an estrogen responsible for the growth of breast cancer tumors.¹²¹

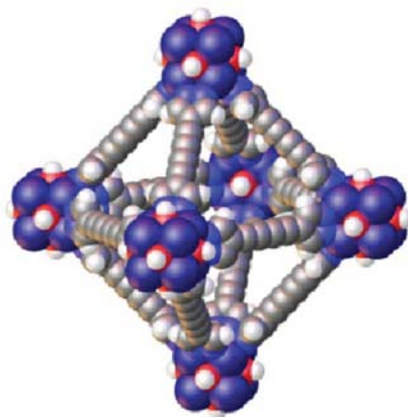
By alkylation of PTA in 53, complexes 54–56 have been prepared and tested for cytotoxicity in two human cancer cell lines SKOV3 (ovarian) and SW480 (colon).¹²² Alkylated complexes with a long tail show IC_{50} values at 9–10 μM compared to >100 μM for the parent complex.



Reaction of $[\text{CpRuCl}(\text{PPh}_3)_2]$ with trifluoromethanesulfonate salt of *N,N'*-dimethylPTA (dmPTA) in refluxing acetone afforded 57, closely related to 53, in 87% yield, containing a new ligand, 3,7-*H*-3,7-dimethyl-1,3,7-triaza-5-phosphabicyclo[3.3.1]nonane (HdmoPTA).¹²³ Note that the same ligand can be obtained by saponification of trifluoromethanesulfonate salt of *N,N'*-dimethylPTA in methanol in the presence of KOH in 65% yield.¹²⁴ Complex 57 was then used for the preparation of heterodinuclear complexes 58–63.^{43,125} The antiproliferative activity of 57–60 was studied in human cancer cell lines HeLa (cervix), Ishikawa (endometrial), SW1573 (lung), T-47D (breast), and WiDr (colon). All complexes showed GI_{50} values in the range 0.8–6.5 μM . The first row transition metal had no or only marginal impact on cytotoxicity.



Attempts toward preparation of metal-organic frameworks (MOFs) with the ultimate aim of controlled delivery to and release of metallodrugs in cancer cells have been reported. In particular, incorporation and release of four molecules of RAPTA-C on a highly porous and robust MOF with remarkable thermal and chemical resistance also in biological medium has been recently described.¹²⁶ The compound $[\text{Ni}_8(\mu_4\text{-OH})(\mu_4\text{-OH}_2)(\mu_4\text{-4,4'-(buta-1,3-diene-1,4-diyl)bispyrazolato})_6]_n$ (64) contains octahedral and tetrahedral internal voids of 1.6 and 1.0 nm size, respectively, with the estimated diameter of a RAPTA-C molecule of 0.9 nm.

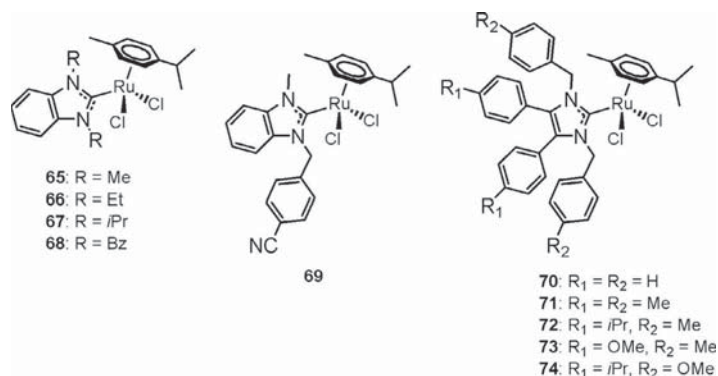


Conjugation of RAPTA-C to degradable polymeric micelles resulted in an enhanced delivery of the metallodrug into the lysosome of the cells, confirmed by confocal microscopy images, indicating an endocytic pathway for drug uptake into the cell.¹²⁷ A tenfold increase of cytotoxicity for the macromolecular drugs compared to RAPTA-C in ovarian cancer cell lines A2780, A2780cisR, and Ovar3 has been claimed.

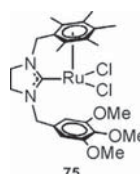
Thermodynamic and spectroscopic properties of RAPTA-C and other closely related complexes have been studied using DFT methods,¹²⁸ and the effect of bidentate carboxylate ligands on the molecular properties of these systems has been described.¹²⁹ Theoretical basis for activation of RAPTA-type anticancer complexes has been provided,¹³⁰ and attempts to find a correlation between hydrolysis transformations and the anticancer activity were reported.¹³¹ Molecular docking simulations for a series of RAPTA complexes with cathepsin B and kinases have also been performed,^{132,133} but the absolute energies were in separate cases too large and the results very sensitive to calculation details.¹³⁴ By using B3LYP hybrid functional and IEF-PCM solvation models, the binding of the hydrolyzed drug $[(\eta^6\text{-C}_6\text{H}_5(\text{CH}_2)_2\text{OH})\text{RuCl}_2(\text{DAPTA})]$ (DAPTA = 3,7-diacetyl-1,3,7-triaza-5-phosphabicyclo-[3.3.1]nonane) to guanine (G), adenine (A), cytosine (C), cysteine (Cys), and histidine (His) was studied.¹³⁵ The diaqua complex proved to be slightly more reactive than the chloridoaqua species. The order of the activation free energy was $\text{G} > \text{His} > \text{A} > \text{Cys} > \text{C}$ for the chloridoaqua complex and $\text{G} > \text{C} > \text{A} > \text{His} > \text{Cys}$ for the diaqua product, indicating that amino acids may be preferential targets for the fully hydrolyzed compound *in vivo*. Surprisingly, the N7 atom of guanine was found to be an unfavored binding site for both hydrolysis products.

Ruthenium–Arene NHC Complexes

Ruthenium–arene complexes with *N*-heterocyclic carbene (NHC) ligands are also currently being considered as potential metal-based anticancer drugs. Ruthenium *p*-cymene complexes with a series of benzimidazolyldiene, as well as imidazolyldiene carbenes, have been synthesized quite recently^{136,137} and evaluated for antiproliferative activity and inhibition of cathepsin B and thioredoxin reductase. Antiproliferative activity of 65–68 strongly correlating with lipophilicity and potent enzyme inhibitory effects should be noted. The IC_{50} values in two human cancer cell lines MCF-7 (breast) and HT29 (colon) progressively decreased on going from compound 65 to 68, from $>100\ \mu\text{M}$ in both cell lines (65) to 2.07 ± 0.93 and $2.40 \pm 0.97\ \mu\text{M}$ (68), respectively.

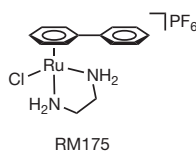


Likewise, the antiproliferative activity of complexes 69–74 in Caki1 (renal) and MCF-7 cells varied broadly in terms of IC_{50} values from $>500 \mu M$ for 69 to 13 ± 2 and $2.4 \pm 0.7 \mu M$ for 74 (Caki1) and 73 (MCF-7), respectively. Treatment of rats with ruthenium(II)–arene NHC complex 75 was found to cause oxidative damage of cells in heart tissue via peroxidation of fatty acids by ROS as a result of suppressing the antioxidant defense capacity of the cells (decreasing the levels of glutathione, superoxide dismutase, and catalase),¹³⁸ as well as damaging the male reproductive system.¹³⁹



$[(\eta^6\text{-bip})\text{Ru}(\text{en})\text{Cl}]\text{PF}_6$ (RM175/ONC04417) and Related Compounds

The mode of action of RM175 is believed to have similarities to that of cisplatin. Upon reaching the cell, the complex undergoes hydrolysis with the formation of a monoaqua species.^{140,141}



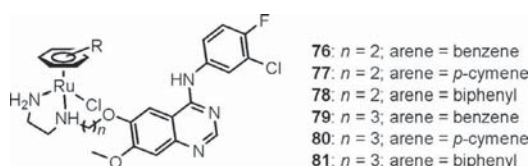
Then, the aqua complex reacts with nuclear DNA via ligand exchange resulting in the formation of a covalent bond to the N7 atom of guanine.¹⁴² Mechanistic details of these transformations have been studied by DFT calculations.^{143–146} Thymine bases were shown to compete kinetically with guanine for binding to ruthenium upon interaction with single-stranded oligonucleotides and may be involved in ruthenation of DNA via the formation of thermodynamically more stable adducts with the guanine.^{147,148} At the same time, interstrand migration of ruthenium G-adducts formed initially upon interaction of the complex with the 15-mer duplex nucleotide via an S_N1 reaction mechanism has been demonstrated in a recent study,¹⁴⁹ indicating that ruthenium–arene complexes can be removed from G-bound DNA. The biological consequences of this observed lability have to be investigated further. It has also been suggested that marked affinity of $[(\eta^6\text{-bip})\text{Ru}(\text{en})\text{Cl}]^+$ to the sulfur atoms of cysteine, glutathione (GSH), and HSA and binding to these biological thiols may also play a role in the underlying mechanism of action of this drug candidate.¹⁵⁰ In this context, the ability of $[(\eta^6\text{-bip})\text{Ru}(\text{en})\text{Cl}]^+$ to inhibit human glutathione-S-transferase π (GST π), an enzyme overexpressed in many types of solid tumors, is of particular note, because S donor atoms in this protein can serve as a potential target site for this ruthenium drug.¹⁵¹ Interestingly, a MALDI-TOF-MS study has shown that this ruthenium complex is significantly less reactive than cisplatin to metallothioneins (MTs), a nonenzymatic thiol-rich polypeptide involved in important biological functions and overexpressed in cancer tissues with increased cisplatin resistance.¹⁵² The competition between GSH and the single-stranded 14-mer oligonucleotide for $[(\eta^6\text{-bip})\text{Ru}(\text{en})\text{Cl}]^+$ has been studied under physiologically relevant conditions by ESI-MS and high-resolution ESI-FTICR-MS coupled to HPLC.¹⁵³ The data indicated that the only resulting product is the monoruthenated at G-site oligonucleotide, in agreement with previous findings that the complex can be involved in cellular redox chemistry with the formation of a sulfenate adduct followed by its displacement by guanine in native DNA.¹⁵⁰ The same authors suggested that the observed lability of the sulfenate adduct may indicate a different role of GSH in the mechanism of action of ruthenium drugs when compared to that of cisplatin, which may contribute to the lack of cross-resistance with platinum drugs. By performing a complex study¹⁵⁴ involving tryptic digestion, ICP-MS, LC-ESI-MS, molecular modeling, receptor assay, and MTT tests, it was found that this ruthenium complex and its *p*-cymene analog, as well as cisplatin, bind to certain surface exposed amino acid residues in both apo- and holo-hTf and could be internalized via TfR-mediated endocytosis. The ruthenium complexes are released into the cell, while cisplatin bioavailability was considerably reduced, resulting in a dramatic drop of drug cytotoxicity.

Complexes of the RM175 type contain three different building blocks, which can be varied in order to improve the biological activity: an arene, a bidentate chelating ligand, and a monodentate ligand initially explored as an activation site.

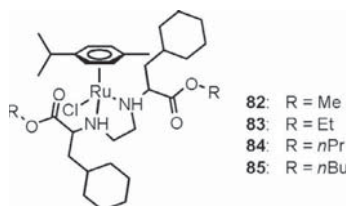
It is well documented¹⁵⁵ that the antiproliferative activity of $[(\eta^6\text{-arene})\text{Ru}(\text{en})\text{Cl}]^+$ complexes in the human ovarian cancer cell line A2780 in terms of IC_{50} values increases with the size of the coordinated arene. Moreover, it was shown¹⁵⁶ that of the three complexes of *ortho*-, *meta*-, and *para*-terphenyl, the *para*-isomer showed much higher cytotoxicity than the other two structural isomers. The increased potential of this latter isomer is probably due to its dual binding mode to DNA via intercalation and monofunctional covalent binding.¹⁴² Induction of apoptosis and G₀/G₁ cell cycle arrest mediated by tumor suppressor protein p53 were the main cellular responses observed on treatment of A2780 cells with the *para*-isomer.¹⁵⁷ The complex did not show mutagenicity, which increases its suitability for further development as anticancer drug.

It should be noted that replacement of the chelating ethylenediamine ligand by two ammine ligands led to loss of cytotoxicity in the human cancer cell lines CH1 (ovarian), A549 (non-small cell lung), and SW480 (colon), due to instability of the complex in biological medium.^{158,159} Rapid hydrolysis and loss of cytotoxicity were also found for related cis-dichlorido ruthenium ammine complexes, namely, $[(\eta^6\text{-}p\text{-cymene})\text{Ru}(\text{NH}_3)\text{Cl}_2]$ and $[(\eta^6\text{-biphenyl})\text{Ru}(\text{NH}_3)\text{Cl}_2]$.¹⁶⁰

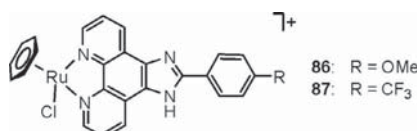
Attempts to develop multifunctional anticancer agents by coordination of ethylenediamine-4-anilinoquinazoline hybrid ligand(s) to a ruthenium(II)-arene moiety have been reported.¹⁶¹ 4-Anilinoquinazolines are derivatives of the epidermal growth factor receptor (EGFR) inhibitor gefitinib. The prepared complexes 76–81 preserved the high inhibitory potential for EGFR, which is also characteristic for gefitinib, and the ability of the $(\eta^6\text{-arene})\text{Ru}(\text{en})^+$ moiety to react with 9-ethylguanine as a DNA model.



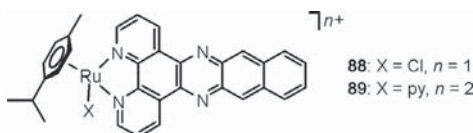
Combination of biologically active methyl, ethyl, *n*-propyl, or *n*-butyl esters of (*S,S*)-ethylenediamine-*N,N'*-di-2-(3-cyclohexyl)propanoic acid with the $(\eta^6\text{-}p\text{-cymene})\text{Ru}^{2+}$ fragment resulted in products 82–85, which showed better antiproliferative activity than the corresponding ethylenediamine-modified ligands or the parent ruthenium–arene compound, as well as selectivity toward leukemia cells.¹⁶² Induction of apoptosis via the intrinsic mitochondrial pathway, distinct from that of cisplatin, has been suggested as a possible mode of action for these metallodrugs.



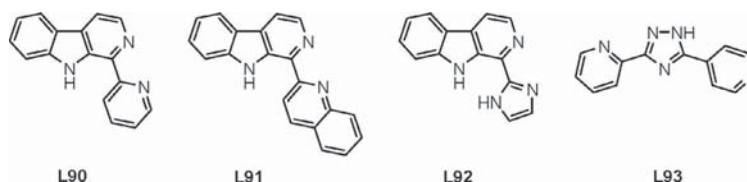
The use of a series of rigid phenanthroimidazole derivatives as bidentate ligands instead of ethylenediamine resulted in rather low cytotoxicity *in vitro*, with some exceptions for particular cell lines.^{163,164} Lack of biological activity was noted for the ruthenium-*p*-cymene complex with 2,4-diamino-6-(2-pyridyl)1,3,5-triazine isolated as tetrafluoroborate salt, despite its propensity to interact with DNA.¹⁶⁵ The complexes 86 and 87 were shown by a series of biophysical methods to react with DNA oligonucleotides 5'-G3 (T2AG3)3-3' (HTG21) and stabilize G-quadruplex DNA. A change in G-quadruplex conformation led to inhibition of telomerase activity and *in vitro* cytotoxicity.¹⁶⁶ Telomerase enzyme activity is reported in 80–90% of tumors, and therefore, G-quadruplex DNA is considered an important target for anticancer chemotherapy.¹⁶⁷



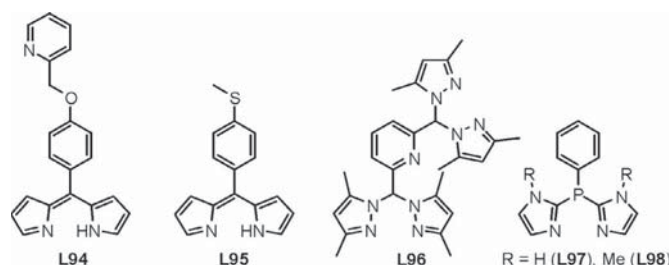
Binding a fluorescent ligand derived from phenanthroline, namely, 4,5,9,16-tetraazadibenzo[*a,c*]naphthacene, to a (*p*-cymene) Ru^{2+} moiety resulted in two complexes, 88 and 89, which retained fluorescence and showed antiproliferative activity in MCF-7 cells with IC₅₀ values of 5.5 ± 2 and 60 ± 5 μM and accumulation in the nucleus.¹⁶⁸



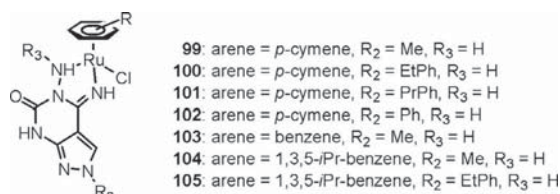
A combination of β -carboline derivatives L90–L92, which are potential cdk inhibitors, with a ruthenium–benzene or ruthenium-*p*-cymene moiety afforded highly active compounds, which may directly target cdk1.¹⁶⁹ Interestingly, another ruthenium–benzene complex with L93, isolated as the hexafluorophosphate salt, $[(\eta^6\text{-benzene})\text{Ru}(\text{L93})\text{Cl}]\text{PF}_6$, showed excellent antitumor activity in Dalton's lymphoma ascites (DLA)-bearing albino mice with a T/C value of 211%.¹⁷⁰



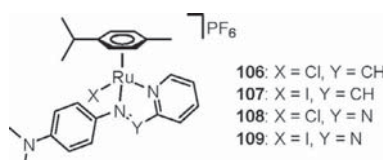
Ruthenium(II)–arene complexes with dipyrromethenes **L94** and **L95** with a six-membered chelate ring have been reported to exhibit antiproliferative activity in Dalton's lymphoma cell lines,^{171,172} while those with $\alpha,\alpha,\alpha',\alpha'$ -tetra(pyrazol-1-yl)-2,6-lutidine (**L96**), both mono- and dinuclear, were active in the human breast (MCF-7), lung (A549), and colon (HCT116) cancer cells.¹⁷³ On the other hand, [(*p*-cymene)Ru(**L97**)Cl]Cl and [(*p*-cymene)Ru(**L98**)Cl]Cl, in which the ligands adopt $\kappa N, \kappa N$ coordination mode, were inactive in human HCT116 (colon) and A2780 (ovarian) and rat H411E (hepatoma) cells.¹⁷⁴ Mono- and dinuclear ruthenium(II)–arene complexes were also prepared with other nitrogen-containing ligands, such as 2,2'-bipyrimidine,¹⁷⁵ but the mononuclear ones have been found to be inactive toward A2780 human ovarian cancer cells.¹⁷⁶

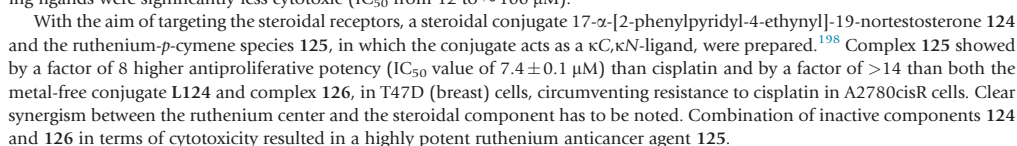
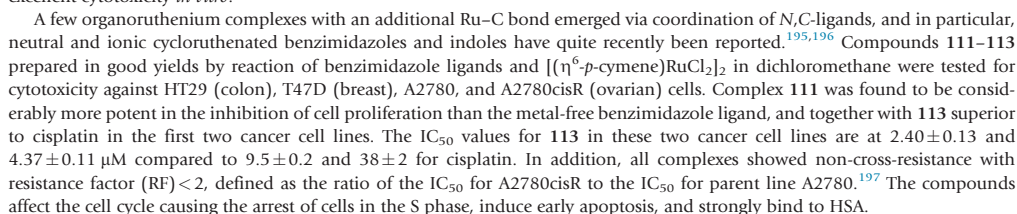


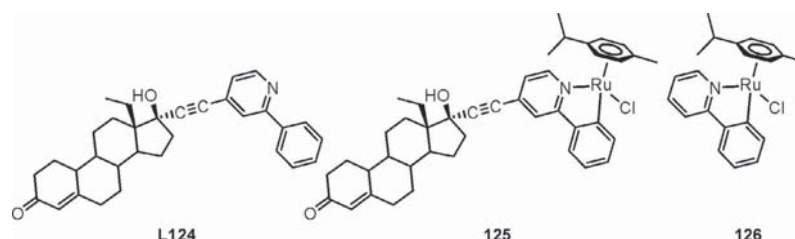
Attempts to prepare effective antagonists of human A_3 adenosine receptors, which are overexpressed in tumor cell lines as well as in solid tumors, have been described by synthesis of organoruthenium complexes and variation of the arene ligand or substitution pattern at pyrazolo-[3,4-*d*]pyrimidine ligand chelated to ruthenium in **99**–**105**.¹⁷⁷



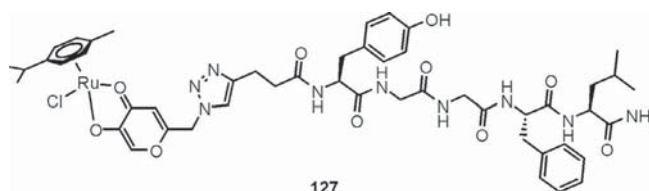
The results indicate that subtle changes in the structures of the complexes have marked effects on their biological properties. In this context, a very illustrative and thorough study on the alteration of cellular properties of complexes, such as cell recognition, transport across cell membrane, accumulation, cytotoxicity, etc., by replacement of coordinated chlorido ligand with an iodo ligand has been reported.^{178,179} The synthesized complexes **106**–**109** show antiproliferative activity in human cancer cell lines. However, the IC_{50} assays showed that iodo complexes **107** and **109** are significantly more cytotoxic compared to chlorido species **106** and **108**. For comparison, the IC_{50} values for **109** in A2780, A549, HCT116, and MCF-7 are at 0.69 ± 0.04 , 1.27 ± 0.01 , 1.37 ± 0.04 , and 0.8 ± 0.1 μM , while those for **108**, at 13.1 ± 0.5 , 15 ± 1 , 16.7 ± 0.8 , and 11.9 ± 0.9 μM , respectively. In addition, the iodo complexes are more selective to cancer cell lines, do not show cross-resistance with cisplatin or oxaliplatin, and preserve their potency in p53 tumor suppressor mutant cells indicating a clear advantage of these complexes for clinical development.



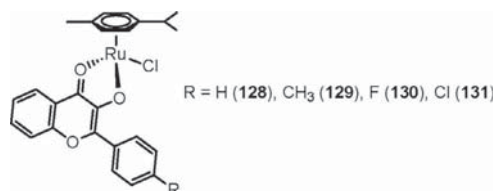




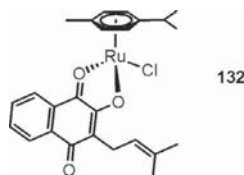
In contrast, similar complexes with bidentate *O,O*-ligands, derived from the biomolecule maltol, such as hydroxypyrones¹⁹⁹ and hydroxypyridinones,²⁰⁰ are generally noncytotoxic, as also were the compounds with clinically used quinolone antibacterials ofloxacin, nalidixic acid, and cinoxacin,²⁰¹ models of the quinolone elvitegravir,²⁰² or hydroxamic acids.²⁰³ Interestingly, the complexes with closely related *S,O*-chelating ligands, such as thiopyrones and thiopyridinones, were cytotoxic.²⁰⁴ Thionation of nalidixic acid also had a favorable but much weaker effect on the antiproliferative properties of the resulting ruthenium–arene complex.²⁰⁵ Solution equilibrium studies on ruthenium(II)–arene complexes with hydroxypyrene and hydroxypyridone ligands by pH potentiometry, ¹H NMR spectroscopy, and UV–vis spectrophotometry have shown the formation of 1:1 Ru(arene)–ligand complexes of moderate stability predominating at physiological pH.^{206,207} At more basic pH, the formation of the dinuclear species [$\text{Ru}^{\text{II}}(p\text{-cymene})_2(\text{OH})_3$]⁺ was observed accompanied by release of metal-free ligand (HL = hydroxypyrene or hydroxypyridone), along with generation of a hydroxido complex [$\text{Ru}^{\text{II}}(p\text{-cymene})(\text{L})(\text{OH})$]. The hydroxythiopyrone and hydroxythiopyridone ligands form significantly more stable complexes with the $\text{Ru}^{\text{II}}(p\text{-cymene})$ moiety of 1:1, 2:1, or even 3:1 stoichiometry in the presence of excess ligand. The three available binding sites are shared between two or three organic ligands binding as mono- and/or bidentate in different protonation states depending on pH. ESI-ion trap mass spectrometry studies have shown that complexes with pyronato and pyridonato ligands react rapidly with ubiquitin (Ub) with the release of these ligands and formation of [$\text{Ub} + \text{Ru}^{\text{II}}(p\text{-cymene})$]⁺ adducts, while complexes with thiopyronato ligand are significantly more inert and form only minor amounts of adducts upon prolonged incubation time (3 days).²⁰⁸ In addition, attachment of the neuropeptide [Leu⁵]-enkephalin to a hydroxypyrene and further complexation to a $\text{Ru}^{\text{II}}(p\text{-cymene})$ moiety have recently been reported.²⁰⁹ The metal-based conjugate **127** showed high cytotoxicity in the chemosensitive human ovarian cancer cell line CH1 with an IC_{50} value of $13 \pm 5 \mu\text{M}$ compared to $>640 \mu\text{M}$ for the metal-free pyrone–peptide conjugate. Note, however, that **127** is only moderately active in SW480 (colon) and A549 (lung) carcinoma cell lines with IC_{50} values at 170 ± 32 and $159 \pm 52 \mu\text{M}$.



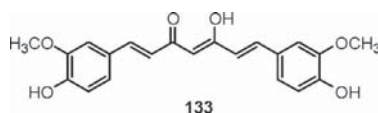
Of the other biologically active ligands bound to the ruthenium–arene moiety with the aim of creating more potent anticancer agents, of note are the 3-hydroxyflavones, which belong to the flavonoids, a class of natural compounds with antioxidant, anti-inflammatory, antiviral, and anticancer properties; their quinolinone analogs and lapachol are endowed with antibiotic and anticancer properties. The complexes **128**–**131** exhibit very high antiproliferative activity *in vitro* with IC_{50} values in the low μM concentration range, which correlated with their potency to inhibit topoisomerase II α enzyme activity.²¹⁰ In addition, both the flavonoids and their ruthenium–arene complexes are fluorescent enabling the study of their intracellular distribution by fluorescence microscopy. Complex **130** was found to accumulate in the endoplasmic reticulum (ER) as the primarily targeted organelle, acting as a reservoir for this cytotoxic agent. The complexes with related quinolinone derivatives were found equipotent in inhibition of cancer cell proliferation, while variation of arene and halido ligands had minor effects on the cytotoxic activity.²¹¹



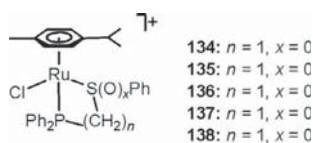
The ruthenium complex with lapachol, 132, showed an enhanced activity compared to the free ligand in SW480 (colon), A549 (lung), and HCT-116 (colon) cancer cells, based mainly on ROS-induced apoptosis and cell cycle arrest in the G₂/M phase.²¹²



The chelating ability of another natural molecule, curcumin, 133, a major constituent of the *Curcuma longa* plant, with a broad spectrum of biological activity, has also been exploited and the ruthenium–arene complexes prepared,^{213,214} which showed very good antiproliferative activity in HCT116 (colon), MCF-7 (breast), and A2780 (ovarian) cell lines. The complexes $[(\eta^6\text{-arene})\text{-Ru}(\text{curcuminato})\text{Cl}]$, where arene = *p*-cymene, benzene, or hexamethylbenzene, were found to inhibit the proteasome inducing apoptosis in HCT116 (colon) cells, the most potent being the benzene derivative.

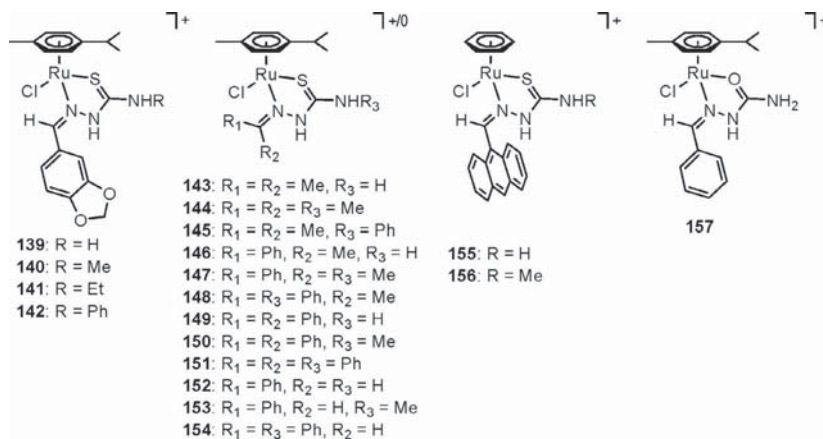


Highly cytotoxic ruthenium(II) *p*-cymene complexes with $\kappa P, \kappa S$ -chelating ligands 134–138 were prepared from $[(\eta^6\text{-}p\text{-cymene})\text{RuCl}_2]_2$ and ω -diphenylphosphino-functionalized alkyl phenyl sulfide and sulfoxide ligands $\text{Ph}_2\text{P}(\text{CH}_2)_n\text{S}(\text{O})_x\text{Ph}$ ($n = 1\text{--}3$, $x = 0, 1$).²¹⁵ The complexes are especially active in cisplatin-resistant tumor cell lines 8505C (anaplastic thyroid tumor), MCF-7 (breast), and SW480 (colon).



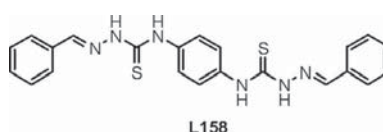
Ruthenium–Arene Complexes with Thiosemicarbazones, Isothiosemicarbazones, and Semicarbazones and Hydrazones

The ethylenediamine in RM175 can also be substituted by other biologically active ligands, and, in particular, thio- or semicarbazones. By exploring the μ -chlorido-bridge splitting reaction of the $[\text{Ru}(\eta^6\text{-}p\text{-arene})\text{Cl}_2]_2$ with different thiosemicarbazones or semicarbazones, a series of half-sandwich complexes 139–157, in which the thio- or semicarbazones act as bidentate *N,S* or *N,O*-ligands, have been prepared and evaluated for biological activity. Complexes 141 and 142 with piperonal thiosemicarbazones²¹⁶ and 155 and 156 with anthraldehyde thiosemicarbazones²¹⁷ were tested for antiproliferative activity in two human cancer cell lines, HCT116 (colon) and Caco2 (epithelial colorectal), as well as in a nontumorigenic (CCD-18Co) cell line. The IC₅₀ values varied from 23.3 to 153 μM after 72 h drug exposure time in HCT116 and Caco2 cells and were of 59.6 ± 1.9 , 168 ± 9 , 205 ± 4 , and 48.7 ± 5.6 μM for 141, 142, 155, and 156, respectively, against CCD-18Co cells. Complexes 141 and 142 were found to act as catalytic inhibitors of the human topoisomerase II enzyme at concentrations as low as 20 μM .

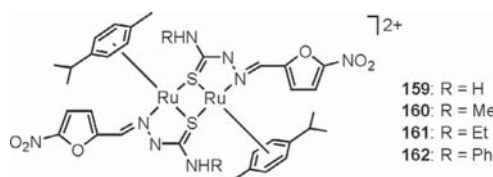


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Complexes 143–151 and the corresponding ruthenium-free thiosemicarbazones were assayed for cytotoxicity in two human cancer cell lines SGC-7901 (gastric) and BEL-7404 (liver) and also in noncancerous cell line HEK-293 T.²¹⁸ There was no selectivity for cancer cells observed. The ruthenium complexes were generally more cytotoxic than the metal-free thiosemicarbazones with IC₅₀ values varying from 15.9 ± 2.2 to 47.7 ± 1.3 μM in the two cancer cell lines. Compound 154 was markedly more cytotoxic than 152 and 153 in a panel of four human cancer cell lines CNE-1 (nasopharyngeal), H292 (lung), SK-BR-3 (breast), and Hey1-B (ovarian) with IC₅₀ values of 19.6 ± 3.5, 31.2 ± 4.2, 10.4 ± 2.3, and 34.8 ± 3.8 μM, respectively.²¹⁹ Lower cytotoxicity was found for semicarbazone complex 157 in SGC-7901 (gastric), BEL-7404 (liver), and CNE-1 (nasopharyngeal) carcinoma cells, with the IC₅₀ values of 20.7 ± 2.3, 71.1 ± 6.2, and 42.6 ± 3.5 μM, respectively.²²⁰ A potential dinucleating ligand L158 was allowed to react with [(η⁶-arene)RuCl₂]₂, where arene = *p*-cymene or C₆H₅C₃H₆COOH, to give two dinuclear η⁶-arene ruthenium thiosemicarbazone complexes, [{Ru(*p*-cymene)Cl₂]₂(L158)]Cl₂ and [{Ru(C₆H₅C₃H₆COOH)Cl₂]₂(L158)]Cl₂.²²¹ The dinucleating ligand L158 was considerably more active against esophageal cancer cells WHCO1 than benzaldehyde thiosemicarbazone (IC₅₀ 0.21 vs. >200 μM). Coordination to the ruthenium–arene moiety affects the cytotoxicity of the ligand, reducing it markedly, while the arene also plays a role. The IC₅₀ values for the two complexes are >200 and 8.96 μM for [{Ru(*p*-cymene)Cl₂]₂(L158)]Cl₂ and [{Ru(C₆H₅C₃H₆COOH)Cl₂]₂(L158)]Cl₂, respectively.

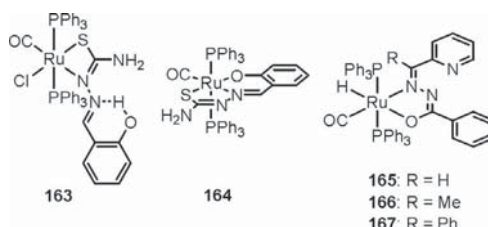


A series of another type of diruthenium arene complexes with thiosemicarbazones (159–162) was prepared starting from [Ru(η⁶-*p*-arene)Cl₂]₂ and 5-nitrofurfuraldehyde thiosemicarbazones.²²²



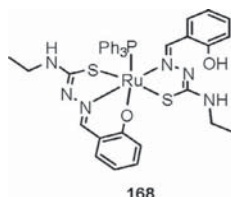
Complexation of 5-nitrofurfuraldehyde thiosemicarbazones to ruthenium diminished their antiproliferative activity in HL-60 (leukemia), A2780 (ovarian), MCF-7 (breast), and PC3 (prostate) cell lines. Complex 162 was the most active showing IC₅₀ values of 4.11 ± 0.76, 2.4 ± 0.5, 8.7 ± 1.9, and 4.6 ± 0.8 μM, respectively. In addition, 159 and 162 were found to interact with tubulin-inducing apoptotic processes.

As starting materials for the synthesis of other organoruthenium(II) complexes with thiosemicarbazones, *S*-methylisothiosemicarbazones and hydrazones were used [RuHCl(CO)(EPh₃)₃] (E = P or As), as well as [RuH₂(CO)(PPh₃)₃] or [RuCl₃(EPh₃)₃] (E = P or As).^{223–229} For example, 2-hydroxybenzaldehyde thiosemicarbazone reacts with [RuHCl(CO)(PPh₃)₃] in benzene under reflux to form two different products, 163 and 164, which were separated by column chromatography.²²⁶ In 163, the ligand acts as *N,S*-bidentate monodeprotonated and coordinates to ruthenium with the formation of a four-membered chelate ring, while in 164, the thiosemicarbazone is doubly deprotonated and acts as *O,N,S*-tridentate ligand binding to ruthenium in a meridional fashion. Despite their different composition and structure, both compounds show similar antiproliferative activities in terms of IC₅₀ values in A549 (lung) and HepG2 (liver) human cancer cell lines, namely, 20 ± 1 and 26 ± 1 μM, induced through ROS generation and oxidative stress. Complexes 165–167 prepared by reaction of [RuH₂(CO)(PPh₃)₃] with the respective hydrazine in boiling alcohol show moderate to good cytotoxicities in A431 (skin) and HeLa (cervical) cells with IC₅₀ values varying from 18 to 122 μM without causing marked damage to the mouse embryonic NIH 3T3 cells (IC₅₀ 347–449 μM).²²⁷



Classical ruthenium thiosemicarbazones with or without ancillary organic coligands, which do not contain a Ru–C bond, have also been reported as potential anticancer agents.^{230–236} Ruthenium(III) complex 168 was prepared by reaction of

[RuCl₃(PPh₃)₃] with 2-hydroxybenzaldehyde 4*N*-ethylthiosemicarbazone in a 1:1 or 1:2 molar ratio in boiling benzene.²³⁵ The two ligands adopt different coordination modes and protonation states. One acts as a tridentate doubly deprotonated ligand binding to ruthenium via the phenolate oxygen, thiolato sulfur atom, and nitrogen atom of the hydrazine moiety, while the second as a monodeprotonated bidentate ligand, coordinating to ruthenium via the azomethine nitrogen atom and thiolato sulfur atom. The sixth coordination place is occupied by PPh₃. The complex showed potent antiproliferative activity on human lymphocytes and an IC₅₀ value of ~12 μM against lung carcinoma cell line A549. In addition, the ability of the complex to scavenge the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical was reported.



Ruthenium Polypyridyl Complexes

Ruthenium polypyridyl complexes have been known for their biological activity for more than six decades, when some tris-homoleptic ruthenium(II) complexes were found to possess toxicity in mice, showed inhibition of acetylcholinesterase, and possessed antibacterial properties. This type of compound exists in two enantiomeric forms, displayed in Figure 2, differing in their biological activity.²³⁷ *In vivo* anticancer properties were studied some years later in a Landschütz ascites tumor mice model, showing promising results and prompting further investigations in this field of bioinorganic chemistry.²³⁸ Today, polypyridyl ruthenium complexes are promising anticancer drug candidates.

The classical mechanism of anticancer action of ruthenium polypyridyl complexes is DNA intercalation,^{239–246} the first X-ray structures with atomic resolution were published recently, showing intercalative/semi-intercalative interaction of Λ -[Ru(TAP)₂(dppz)]²⁺ (TAP=L178 in Figure 3) with d(TCGGCGCCGA) DNA strands.²⁴⁷ Along with that, other mechanisms are assumed to be operative, namely, stabilization of G-quadruplex DNA and^{248–250} reactive oxygen species (ROS) formation,²⁵¹ followed by cell death via the mitochondrial pathway,^{252,253} inhibition of ROS formation,²⁵⁴ mitochondria targeting without involvement of ROS,²⁵⁵ and inhibition of enzymes such as histone deacetylase,²⁵⁶ pancreatic phospholipase A₂,²⁵⁷ topoisomerases I and II,^{258–260} and telomerase (due to G-quadruplex DNA stabilization).^{261–263} A broad variety of photoactive ruthenium polypyridyl complexes have been prepared, which in the ideal case exhibit low cytotoxicity in the dark and high cytotoxicity when irradiated with visible or NIR light, providing a platform for tumor targeting and a relatively safe therapy (photodynamic therapy, PDT). The mechanism of action for most photoactive compounds implies the production of ROS, especially ¹O₂, when falling back from a photon-induced excited state to a state with lower energy.^{264,265} In particular cases, photoinduced covalent ligand–peptide bond formation may also play a role in the mechanism of action.²⁶⁶ It is worth noting that the vast majority of ruthenium polypyridyl complexes exhibit their biological activity as intact complexes, while most standard anticancer metal drugs in the clinic and in clinical development involve ligand dissociation in their underlying mechanism of action. This could be an advantage in gaining nonstandard mechanisms of action, a promising way to fight resistances, which cause a major problem in chemotherapy.

However, there exist several examples of ruthenium polypyridyl complexes with labile ligands allowing a covalent metal–target (i.e., DNA and protein) interaction.²⁶⁷ The ligand loss is often photoinduced, referring to the previously mentioned PDT principle.^{268–271} The unique photophysical properties of ruthenium polypyridyl complexes also renders them interesting for analytical purposes.^{272–277}

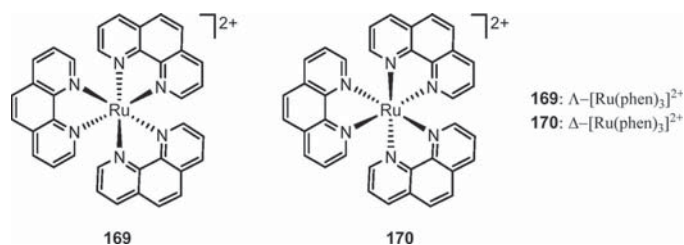


Figure 2 Stereoisomers of tris(1,10-phenanthroline)ruthenium(II).

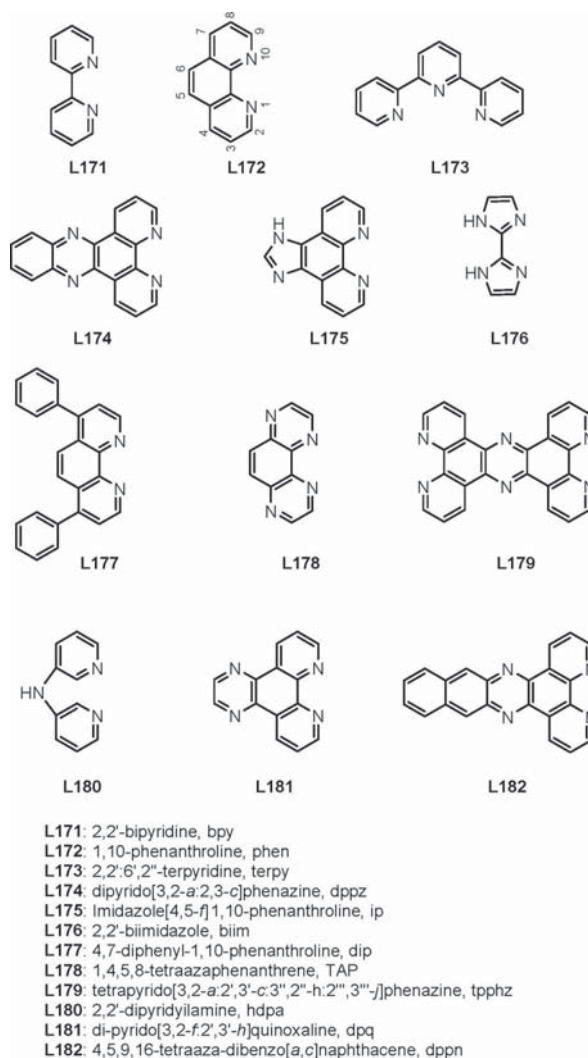


Figure 3 A library of ligands for polypyridyl ruthenium complexes with a numbering scheme for L172.

Intercalating Ruthenium Polypyridyl Complexes

One of the most powerful intercalating ligands in ruthenium polypyridyl complexes is dipyrro[3,2-a:2,3-c]phenazine, dppz, L174 (Figure 3). $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ gained attention as a molecular 'light switch' for DNA in aqueous solution, showing no photoluminescence in the absence of DNA, but emitting light in the presence of DNA at 632 nm.²⁷⁸ The same effect was observed for both Λ - and Δ - $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$, but with a six- to tenfold relative luminescence quantum yield increase for the Δ -enantiomer.^{279,280} The photoluminescence is most probably due to the stabilization of a $^3\text{MLCT}$ (metal-to-ligand charge transfer) excited state via DNA intercalation of the complex, which is otherwise quenched by protic solvents.²⁸¹ The high DNA binding affinity, measured as the intrinsic binding constant, K_b , for these complexes ($K_b > 10^6 \text{ M}^{-1}$)²⁸⁰ led to the development of new Ru-dppz complexes displayed in Figure 4 as potential anticancer drugs.

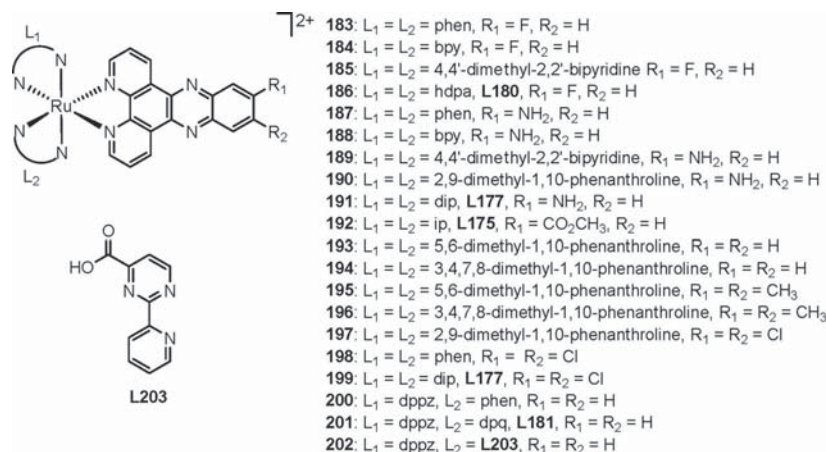


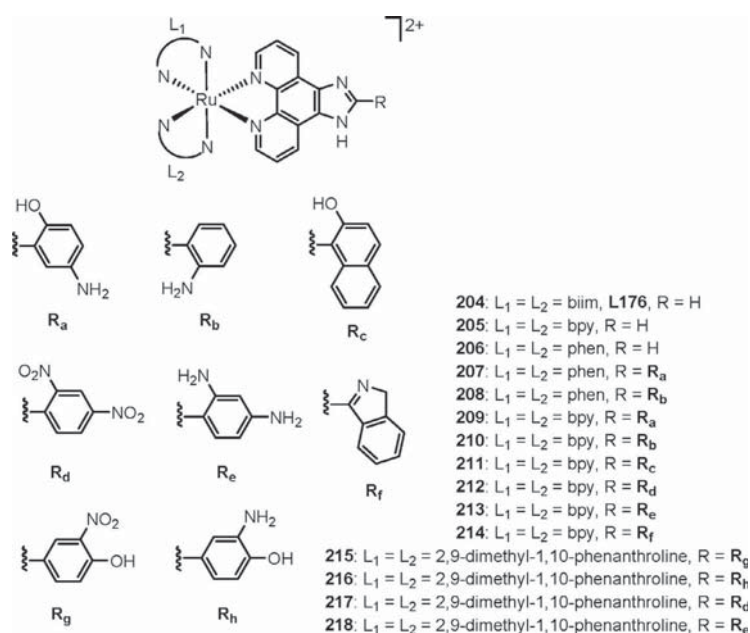
Figure 4 Ruthenium polypyridyl complexes with dppz-derived ligands.

Methylation at positions 5 and 6 of the phen coligands (**193**) improves the DNA-binding affinity with calf thymus (CT)-DNA, $K_b = 1.8 \pm 0.1 \times 10^7 \text{ M}^{-1}$ for **193** versus $1.0 \pm 0.05 \times 10^6 \text{ M}^{-1}$ for the parent complex $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$,²⁸² as well as the cytotoxicity in a non-small cell lung carcinoma NCI-H460 cell line. The IC_{50} values for **193** and $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ are 6.5 ± 1.2 and $20 \pm 1.0 \text{ }\mu\text{M}$, respectively. The bulkier 3,4,7 and 8 methylated phen ligands in **194** decrease DNA binding affinity as well as cytotoxicity. Dimethylation of the dppz ligand in **195** seems to hinder DNA intercalation and leads to a decrease in DNA binding affinity, $K_b = 3.3 \pm 0.3 \times 10^5 \text{ M}^{-1}$ for **195**, but in contrast to the unsubstituted dppz primary ligand, the DNA-binding affinity increases with bulkier coligands ($K_b = 6.0 \pm 0.3 \times 10^6 \text{ M}^{-1}$ for **196** vs. $1.0 \pm 0.09 \times 10^6 \text{ M}^{-1}$ for **194**). This is probably due to the strong DNA groove binding of **196**; however, the effect on the cytotoxicity is less pronounced: $\text{IC}_{50} = 10 \pm 0.9$, 8.0 ± 0.7 , and $7.0 \pm 0.5 \text{ }\mu\text{M}$ for **194**–**196** in the same cell line, respectively. The substitution of the dppz ligand by a dpq (**L181**) one decreases the DNA binding affinity, as well as the cytotoxicity, most likely because of the less profound DNA insertion of dpq.²⁸²

A fluoro substituent at position 7 of the dppz scaffold (**183**–**186**) reduces the cytotoxicity and DNA binding affinity in comparison with the parent complexes $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$ and $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$, presumably due to the greater steric hindrance of the substituted ligand (**183**, **184**). However, the highest DNA binding affinity as well as cytotoxicity in this series was displayed by **186**, a complex containing hdpa, **L180**, coligands. This is probably due to hydrogen bonding interactions of this ligand with DNA.²⁸³ An amino group at position 7 of the dppz ligand leads to a slight enhancement in DNA binding affinity compared to the fluoro-substituted analog, with K_b -values of 5.26×10^5 for **183** versus $6.54 \times 10^5 \text{ M}^{-1}$ for **187**. Substitution of the phen coligands with dip ligands (**L177**) with the formation of **191** leads to an increase in DNA binding affinity and cytotoxicity.²⁴¹ Introduction of sterically hindered dimethyl-substituted phen coligands, as in **190**, led to a significant increase in cytotoxicity, while introduction of dpy or dimethylated dpy derivative, as in **188** and **189**, respectively, was less effective. Complex **190** was shown to increase the ROS level and decrease the mitochondrial membrane potential leading to G_0/G_1 phase arrest and apoptosis via mitochondrial pathways.²⁸⁴ **192** with a carboxylic acid methyl ester group at position 7 of dppz and **L175** as coligands shows moderate DNA binding affinity to CT-DNA, $K_b = 1.39 \times 10^5 \text{ M}^{-1}$, that is two orders of magnitude lower than that of the parent complex $[\text{Ru}(\text{L175})_2(\text{dppz})]^{2+}$, $K_b = 2.10 \times 10^7 \text{ M}^{-1}$.²⁸⁵ Again, steric hindrance might be a reason for this decrease. However, **190** showed cytotoxicity comparable to cisplatin in a human leukemia HL-60 cell line ($\text{IC}_{50} = 8.42 \pm 1.68$ and $5.85 \pm 0.60 \text{ }\mu\text{M}$ for **190** and cisplatin, respectively).²⁸⁶

Dichlorination at positions 7 and 8 of the dppz scaffold and the use of phen as coligands lead to a lower DNA binding constant of **198** ($K_b = 2.73 \pm 1.6 \times 10^5 \text{ M}^{-1}$) when compared to that of the parent complex $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$, $K_b > 10^6$, estimated for both enantiomers.²⁸⁰ Interestingly, **198** showed similar IC_{50} values to cisplatin in two cancer cell lines: BEL-7402, 16.90 ± 1.89 versus 19.76 ± 2.18 , and HeLa, 16.38 ± 2.16 versus $16.85 \pm 2.53 \text{ }\mu\text{M}$, respectively. Complexes **197** and **199** with methyl- or phenyl-substituted phen ligands were a bit less cytotoxic in the BEL-7402 cell line but showed slightly higher cytotoxicity in the HeLa cell line.²⁸⁷ The use of two dppz ligands lowers the DNA binding ability. This was shown for $[\text{Ru}(\text{dppz})_2(\text{phen})]^{2+}$ (**200**) and $[\text{Ru}(\text{dppz})_2(\text{dpq})]^{2+}$ (**201**). The latter is a stronger DNA binder as well as cell growth inhibitor than the former, but both display higher IC_{50} values than cisplatin.²⁴³ Intriguingly, complex **202**, containing two dppz ligands and ligand **L203**, shows remarkably high cytotoxicity comparable to that of cisplatin in a series of human cancer cell lines and a superior activity to cisplatin in the cisplatin-resistant ovarian carcinoma A2780-CP70 cell line ($\text{IC}_{50} = 4.0 \pm 1.2$ for **202** vs. $13.8 \pm 3.0 \text{ }\mu\text{M}$ for cisplatin). Although **202** is a strong DNA binder with $K_b > 10^6 \text{ M}^{-1}$, DNA intercalation is not believed to be the key event in the mechanism of action. Confocal microscopy colocalization studies showed that **202** accumulated in the mitochondria. The mitochondrial membrane potential was affected by **202** without the involvement of ROS. The proposed mechanism of action implies the intercalation of **202** into the mitochondria membrane, leading to release of cytochrome c followed by apoptosis.²⁵⁵

Imidazole[4,5-*f*][1,10]phenanthroline (L175 or ip) is another important building block for DNA-binding ruthenium polypyridyl complexes. In a series of ruthenium complexes 204–206 bearing L175 as a primary ligand and biim (L176), bpy, and phen as coligands, the first one is by far the most active, undercutting IC_{50} values of cisplatin in the three cancer cell lines A375 (2.9 ± 1.3 vs. 7.3 ± 0.8), HepG2 (4.5 ± 1.1 vs. 13.6 ± 2.0), and SW480 (3.9 ± 0.9 vs. 18.3 ± 0.8 μ M). In addition, 204 is less cytotoxic than cisplatin in the nontumorigenic cell lines HS68 ($IC_{50} = 30.6 \pm 5.7$ vs. 1.8 ± 0.7) and HK-2 (74.3 ± 4.2 vs. 10.3 ± 10.3 μ M). The compound reduces the mitochondrial membrane potential ($\Delta\Psi_m$) inducing apoptosis via the mitochondrial pathway.²⁸⁸ Complexes 207–210 showed DNA binding affinity with $K_b > 10^5$ M^{-1} . However, their activity was only marginally better than that of the metal-free ip-derived ligands in three different cancer cell lines.^{242,244} Complex 211 exhibited higher affinity to yeast tRNA than to CT-DNA, $K_b = 1.61 \pm 0.07 \times 10^5$ versus 4.2 ± 0.22 M^{-1} , and demonstrated comparable activity to cisplatin in the MCF-7 and HeLa cancer cell lines.



Likewise, 214 also binds stronger to yeast tRNA than to CT-DNA.²⁸⁹ Complexes 212 and 213 are comparable to 207–210 in terms of cytotoxicity and DNA interaction.²⁹⁰ Substitution of the bpy coligands by 2,9-dimethyl-1,10-phenanthroline ligands leads to an increase in cytotoxicity, as shown by 217 and 218.²⁹¹ Complexes 215 and 216 are even more active in a panel of cancer cell lines (BEL-742, HeLa, MCF-7, and MG-63), their IC_{50} values being similar or even lower than those of cisplatin. This rise in activity cannot be explained by the DNA binding affinity, which is comparable for both 215/216 and 212/213 pairs of compounds. Enhancement of the cellular ROS level leading to apoptosis via the mitochondrial pathway was found for 215 and 216.²⁵² Other examples for ip-derived ruthenium polypyridyl complexes are well documented in the literature.^{292–297} All mentioned ip-derived complexes were synthesized and evaluated as racemic mixtures; however, overall, the Δ -enantiomer seems to be more active than the Λ isomer when the enantiopure complexes are evaluated.^{298,261,262} Curiously, selenium nanoparticles functionalized with ip-derived ruthenium polypyridyl complexes showed angiogenesis inhibition.²⁹⁹ Ruthenium polypyridyl complexes containing L179, tpphz, are strong DNA intercalators. In addition, $[\text{Ru}(\text{bpy})_2(\text{tpphz})]^{2+}$ and $[\text{Ru}(\text{phen})_2(\text{tpphz})]^{2+}$ show IC_{50} values comparable to cisplatin in *in vitro* cytotoxicity assays. These complexes act as light switches analogous to the dppz complexes, making them attractive for DNA imaging.³⁰⁰ A series of polypyridyl ruthenium complexes showed impressive cytotoxicity *in vitro*, but no clear correlation between DNA-binding and anticancer activity was observed.³⁰¹ The DNA-binding complex $[\text{Ru}(\text{phen})_2(\text{dmsO})\text{Cl}]\text{Cl}$ showed anticancer activity both *in vitro* and *in vivo*.³⁰² Oligonuclear ruthenium polypyridyl complexes are also known for their DNA-binding ability, either by noncovalent interactions^{303–306} or by covalent binding after solvolysis.^{267,307}

Mitochondria Targeting Ruthenium Polypyridyl Complexes

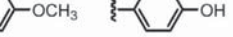
As already shown previously, mitochondria are attractive cellular targets for ruthenium polypyridyl complexes.^{252,255,288}

An ICP-MS study provided evidence that complex 219 (Figure 5) accumulates in the mitochondria. The same complex showed superior *in vitro* activity to that of cisplatin in a HeLa cancer cell line ($IC_{50} = 7.9 \pm 0.7$ for 219 vs. 17.8 ± 2.6 μ M for cisplatin) and a



G-Quadruplex DNA Targeting Ruthenium Polypyridyl Complexes, Telomerase Inhibition

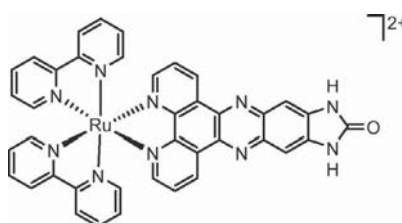
Λ -**221**: $L_1 = L_2 = \text{phen}$, $X = \text{C}$, $R = R_i$
 Λ -**222**: $L_1 = L_2 = \text{phen}$, $X = \text{C}$, $R = R_j$
223: $L_1 = L_2 = \text{bpy}$, $X = \text{C}$, $R = R_k$
224: $L_1 = L_2 = \text{phen}$, $X = \text{C}$, $R = R_k$
225: $L_1 = L_2 = \text{phen}$, $X = \text{Se}$, $R = \text{none}$



R_i R_j R_k

Chiral Λ - and Δ -221 were synthesized using corresponding tartrate salts as enantiopure catalysts.²⁶² Both complexes showed high binding affinity ($K_b > 10^6$) to human telomeric DNA (HTG21) and were shown to accumulate in the cell nucleus by fluorescence microscopy, demonstrating the possibility of DNA interaction of these complexes. Both complexes are able to stabilize G-quadruplex DNA, with the Λ -enantiomer being far more potent than its mirror counterpart. Consistently, Δ -221 is also more active in different cancer cell lines than Δ -221 and a more potent telomerase inhibitor. The Λ -complex showed interesting *in vitro* selectivity for a HepG2 liver carcinoma cell line, $IC_{50} = 4.4 \pm 0.3$ versus 13.6 ± 1.1 for Λ -40 and cisplatin, respectively.²⁶² Λ - and Δ -222 behave analogously; again, the Λ -enantiomer is the better telomerase inhibitor, which seems to be expected as 221 and 222 differ only in a terminal functional group (hydroxyl vs. methoxy); however, the *in vitro* cytotoxicity was better for the methoxy derivatives.^{261,262}

Comparative studies of 223 and 224 showed that phen coligands contribute more to G-quadruplex stabilization than bpy coligands. On the basis of CD spectra, it was also suggested that 224 may have the ability to transform parallel G-quadruplex DNA into the antiparallel form.²⁴⁸ Complex 225, containing a 1,10-phenanthroline-selenazole primary ligand, is able to stabilize G-quadruplex DNA and showed superior *in vitro* activity compared to cisplatin in a HepG2 cancer cell line.²⁶³

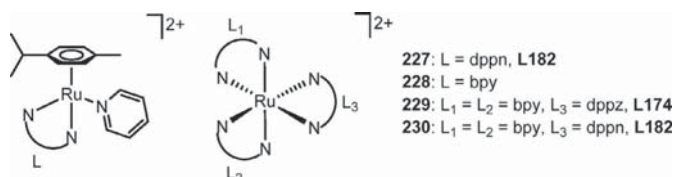


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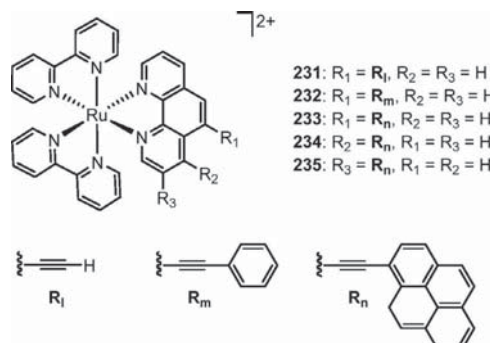
A 'light switch' effect superior to that of the known $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$, in the presence of telomeric DNA, and a more powerful stabilization of the G-quadruplex structure were shown for 226 by CD-spectroscopy. The 'light switch' mechanisms as well as the interaction with G-quadruplex DNA were further analyzed by quantum chemical calculations.³¹²

Photoactive Ruthenium Polypyridyl Complexes

It is well known that intercalating ruthenium polypyridyl complexes are able to cleave DNA under UV-vis light irradiation. This DNA damage is most probably triggered by singlet oxygen, $^1\text{O}_2$, produced upon irradiation.^{297,313}



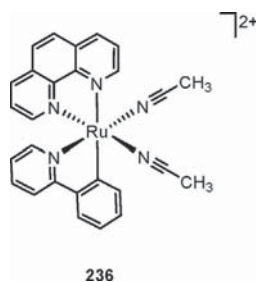
Ruthenium-arene complex 227 shows luminescence in acetonitrile with a quantum yield of 0.31, very close to that for the free dppn ligand (0.33). Time-dependent DFT calculations indicated that fluorescence mainly originates from a ^1LC (ligand centered) excited state, which is in accordance with its short lifetime (36 ns).¹⁶⁸ In contrast, the luminescence of the well-known 'light switch' complex $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$, 229, originates from a $^3\text{MLCT}$ excited state, with a much longer lifetime (750 ns).³¹⁴ The luminescence of 227 is mostly quenched in aqueous solutions (quantum yield = 0.0075), but a sixfold enhancement is observed in the presence of CT-DNA (quantum yield = 0.045).³¹⁵ This is a much lower 'on-off' ratio than for 229 ($\sim 10^4$).²⁷⁸ However, in contrast to 229, complex 227 is able to cleave CT-DNA under irradiation. The reason is, probably, that the $^3\text{MLCT}$ excited state of 229 cannot interfere with the surrounding molecules (e.g., water and oxygen) after DNA intercalation and consequently no $^1\text{O}_2$ can be produced, in contrast to 227, where the LC excited states are energetically lowered with respect to the $^3\text{MLCT}$ states. Complex 230 is an efficient DNA photo cleaver, which does not show fluorescence enhancement upon DNA binding. It seems to be generally difficult to unite 'light switch' and photo cleavage features in one ruthenium polypyridyl complex. Complex 228 is not fluorescent, as is normally the case for most ruthenium-arene complexes. The IC_{50} value for 227 is decreased by a factor of 10 upon irradiation, 23.7 (dark) versus 2.3 μM (light) in human lung carcinoma cell line A549. The dual action of 227 as 'light switch' and photo cleaver makes it suitable for the development of 'teranostic' agents, uniting diagnostic and therapeutic functions.^{168,315}



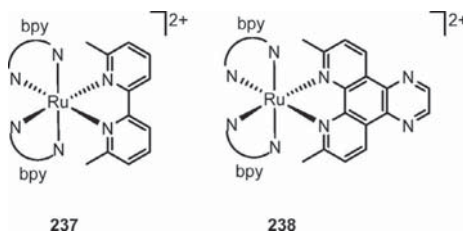
Complexes 233–235 possess astonishing long-lived triplet intraligand-centered (^3IL) excited states, which are extremely sensitive to oxygen. The excited state lifetime for the ^3IL of 233 might be the longest for this type of ruthenium complex (240 μs at room temperature in acetonitrile and 3430 μs at 77 K in 4:1 EtOH/MeOH glass) reported so far. In addition, these complexes show extremely high photoactivity. The IC_{50} value for 233 in a HL60 cancer cell line is 262 μM in the dark and 0.15 μM after a short visible light exposure, displaying the highest reported photodynamic index (PI) of 1747. Notably, cisplatin displays an IC_{50} value of 25 μM in the same cell line, being significantly less cytotoxic than light-activated 233 and much more cytotoxic than 233 in the dark.²⁶⁵ The least active of these three complexes, 235, displays a PI of 104, still being in the range of the most photoactive ruthenium polypyridyl complexes reported so far.²⁶⁹ Compounds 231 and 232 were synthesized to outline the importance of the pyrenyl unit for the photoactivity, showing rather poor PIs of 4.9 and 7.3 when compared with the other three complexes. It must also be stressed that the pyrenyl-containing complexes showed light-dependent activity in the nanomolar range in pigmented metastatic melanoma Malme-3M cells, even after short drug-to-light exposure, while the IC_{50} values in the dark were around 50 μM . This is intriguing as pigmented melanomas are believed to be less accessible for PDT because of active melanin production, a hypoxic tissue environment, and their ability to inactivate ROS.^{316,265} Several cyclometalated ruthenium(II) polypyridyl complexes with photoactive potential have been described recently as well.³¹⁷

Ruthenium Polypyridyl Complexes Containing Photolabile Ligands

Mono- and bidentate ligands can be cleaved from ruthenium polypyridyl complexes after light excitation. An intermediate for the bidentate ligand loss has been observed recently, providing further insight into the process of ligand loss and consequent substitution by solvent molecules.³¹⁸ Two histamine-containing ruthenium polypyridyl complexes showed photoinduced histamine release and acetylcholinesterase inhibition, indicating the possibility of controlled histamine delivery and release by ruthenium polypyridyl complexes.²⁷¹



A series of cyclometalated ruthenium polypyridyl complexes showing impressive anticancer activity were reported.^{319–321} One of the most active compounds of this series, complex 236, showed *in vivo* anticancer activity in a mouse model toward mouse melanoma B16F10 and human glioblastoma U87 slightly superior to that of cisplatin and with fewer side effects.³²² The observation that the *in vitro* cytotoxicity of 236 doubles upon irradiation³²⁰ prompted further investigations on the photodynamic activity of 236. It was shown that 236 exchanges its acetonitrile ligands with water when irradiated in aqueous solution, leading to a cisplatin-related diaqua species.²⁶⁸ Moreover, the ligand loss is triggered by light in the desired PDT window (600–850 nm). Light of this wavelength range shows optimal tissue penetration, while it is still energetic enough to induce ligand exchange or ROS formation. This is an advantage in comparison with *cis*-[Ru(bpy)₂(CH₃CN)₂]²⁺, where harder radiation ($\lambda < 500$ nm) is required for ligand release.³²³ A gel electrophoresis study showed that 236 binds DNA only under irradiation, another advantage for a possible use of this compound as a PDT agent.²⁶⁸ Several 2,2'-biquinoline, biq-containing ruthenium polypyridyl complexes were also activated by long-wavelength light.³²⁴



Complexes 237 and 238 contain dimethylated ligands, with the methyl substituents pointing toward the two bpy coligands. This causes a strained structure and the steric clash facilitates ligand loss. Irradiation of these two complexes with light of

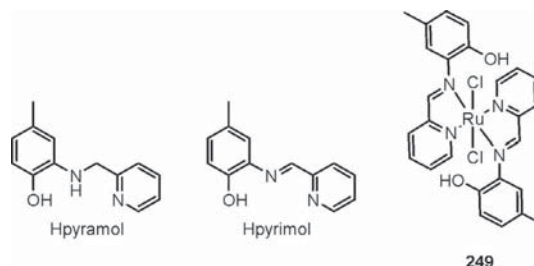
$\lambda > 450$ nm causes photoejection of the strained ligands. 237 loses its ligands with a 30 times faster rate than 238. This is probably due to the rigidity of the dimethylated dpq ligand, favoring rechelation and therefore hindering sequential Ru–N bond breakage. $[\text{Ru}(\text{bpy})_2(\text{phen})]^{2+}$, which was used as a control, is photostable under the same conditions. DNA photobinding studies were performed for both 237 and 238 and control species. DNA cleavage was observed in the case of the control complex $[\text{Ru}(\text{bpy})_2(\text{phen})]^{2+}$, while 237 formed covalent DNA bonds analogous to those of cisplatin. Note that 238, with an intercalating dpq ligand, was involved in both events, DNA cleavage and covalent binding to DNA.²⁶⁹ In addition, it was shown that 237 and 238 did not bind to glutathione (GSH), a sulfur-containing tripeptide present in cells at millimolar concentrations and largely responsible for the detoxification of cisplatin.³²⁵ In a DNA binding study in the presence of GSH, 237 and 238 were not affected, while the DNA binding ability of cisplatin was lowered. 237 and 238 show prominent light-activated cytotoxicity. The cytotoxicity of light-irradiated 237 in a HL60 cell line is $1.6 \pm 0.2 \mu\text{M}$ versus $>300 \mu\text{M}$ in the dark, resulting in a PI of >188 . 238 is more cytotoxic in the dark in the same cell line, displaying a lower PI of 42, probably due to intercalative cytotoxicity. Note that cisplatin ($\text{IC}_{50} = 3.1 \mu\text{M}$) (essentially, cisplatin exhibits identical dark and light IC_{50} -values) in the same cell line is less cytotoxic compared to light-activated 237 and 238 but much more cytotoxic compared to both substances in the dark. Interestingly, the cytotoxicity of these compounds was also tested in a three-dimensional A549 cancer cell model, consisting of cell spheroids with a diameter of about $600 \mu\text{m}$, displaying a more realistic solid cancer model in comparison with layered cells.³²⁶ The IC_{50} value of light-irradiated 237 ($21.3 \pm 2.3 \mu\text{M}$) was reduced by a factor of 19.4 in comparison with the monolayer culture of the same cell line, this ratio is called the multicellular resistance (MCR) index. The cytotoxicity reduction for 238 in the spherical cell model (light $\text{IC}_{50} = 64.6 \pm 4.7 \mu\text{M}$) was more pronounced with an MCR index of 54. However, it should be noted that MCR indices are commonly large for anticancer drugs (163 for etoposide and 6625 for vinblastine) and resemble the *in vivo* dosing, more closely. Compounds 237 and 238 do not exhibit dark cytotoxicity in the spherical model up to $300 \mu\text{M}$. Cisplatin exhibits a low MCR index (12.4) in the same model with an IC_{50} value of $42 \pm 3.6 \mu\text{M}$. $[\text{Ru}(\text{bpy})_2(\text{phen})]^{2+}$, taken as control, showed modest IC_{50} values and PIs in the same cell lines. The retention of potency of 237 in the spherical cell model has to be highlighted as a promising feature for the further development of these compounds.²⁶⁹

Mesoporous silica nanoparticles grafted with a terpy- and dppz-containing ruthenium(II) complex show photoexpulsion of the complex upon irradiation.³²⁷

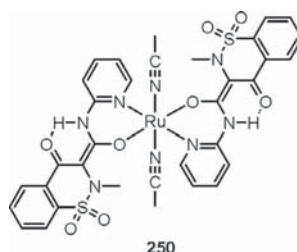
Other Mononuclear Ruthenium Complexes

Progressing preparative coordination chemistry of ruthenium provides a huge arsenal of other compounds for the development of new anticancer drugs. An overview of the complexes prepared and tested for antiproliferative activity and proposed for further development as potential anticancer drugs follows herein. We will briefly discuss the ruthenium complexes with both biologically inactive and active ligands, starting with complexes in which the ligands act as monodentate and going then to compounds with bi- and polydentate ligands. *Cis*-dichloridotetramineruthenium(III) chloride is one of the first ruthenium(III) complexes tested for anticancer potency as a close analog of cisplatin, which showed marked activity against P388 leukemia, reaching a treated/control (T/C) value of 154%.³²⁸ and which continues to attract the attention of researchers.³²⁹ A series of ruthenium(III) complexes with azole heterocycles combining features of both NAMI-A and KP1019 have been recently reported.^{330–332} The complexes *mer*- $[\text{RuCl}_3(\kappa\text{S-DMSO})(\text{Hpz})_2]$ (239) and *mer*- $[\text{RuCl}_3(\kappa\text{S-DMSO})(\text{Hdmpz})_2]$ (240), where $\text{Hpz} = 1H$ -pyrazole and $\text{Hdmpz} = 3,5$ -dimethyl- $1H$ -pyrazole, showed moderate antiproliferative activities against human breast cancer cell line MCF-7 (IC_{50} 71 ± 2 (239) and $32.2 \pm 1.1 \mu\text{M}$ (240)), while *mer*- $[\text{RuCl}_3(\kappa\text{S-DMSO})(\kappa\text{O-DMSO})(\text{Hpz})]$ (241) and *mer*- $[\text{RuCl}_3(\text{bpy})(\text{Hdmpz})]$ (242), where $\text{bpy} = 2,2'$ -bipyridine, were markedly less cytotoxic. Ruthenium methylimidazole complexes, namely, $[\text{Ru}(\text{MeIm})_4(\text{ip})](\text{ClO}_4)_2 \cdot 2\text{H}_2\text{O}$ (243), $[\text{Ru}(\text{MeIm})_4(\text{tip})](\text{ClO}_4)_2 \cdot 2\text{H}_2\text{O}$ (244), and $[\text{Ru}(\text{MeIm})_4(\text{iip})](\text{ClO}_4)_2 \cdot 2\text{H}_2\text{O}$ (245), where $\text{ip} = 1H$ -imidazol[4,5-*f*][1,10]phenanthroline (L175), $\text{tip} = 2$ -(thiophene-2-yl)- $1H$ -imidazo[4,5-*f*][1,10]phenanthroline, and $\text{iip} = 2$ -($1H$ -imidazol-4-yl)- $1H$ -imidazo[4,5-*f*][1,10]phenanthroline, showed similar cytotoxicity to that of 239 and 240 in terms of IC_{50} values in a panel of human cancer cell lines, A549 (lung), A375 (melanoma), Hep G2 (liver), HeLa (cervix), and MCF-7 (breast) cells. Moderate selectivity of 244 and 245 to cancer cell lines when compared to nontumorigenic human bronchial epithelial (HBE) cells has been observed. The cytotoxicity of 244 in A549 cells was induced by apoptosis, as evidenced by cell cycle arrest of the cells in the sub-G1 phase, and depletion of the mitochondrial membrane potential. Ruthenium(II) and ruthenium(III) complexes of 2,2'-biimidazole (biim, L176), that is, *trans,cis,cis*- $[\text{Ru}^{\text{II}}\text{Cl}_2(\kappa\text{S-DMSO})_2(\text{biim})]$ (246) and *mer*- $[\text{Ru}^{\text{III}}\text{Cl}_3(\kappa\text{S-DMSO})(\text{biim})]$ (247), did not show appreciable growth-inhibiting activity on Hep G2, MCF-7, or HeLa cells. Nevertheless, their activities were markedly higher than that of NAMI-A,³³³ while the antimetastatic activity of 247 was comparable to that of NAMI-A on 95D (metastatic lung) cells. The carboxylic acid functionalities attached at different positions of the bipyridine moiety exert a favorable effect on the cytotoxicity of bipyridine complex $[\text{RuHCl}(\text{bpy})(\text{PPh}_3)(\text{CO})]$ (248).^{334,335}

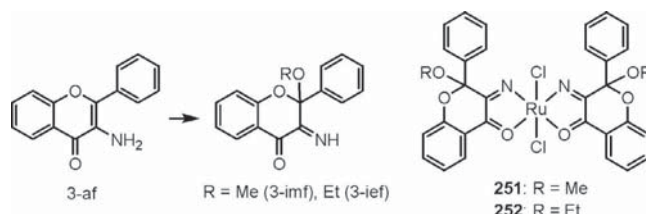
Starting from $\text{RuCl}_3 \cdot 3\text{H}_2\text{O}$ and the noninnocent ligand Hpyramol, a new ruthenium(II) complex *trans*- $[\text{Ru}^{\text{II}}(\text{Hpyrimol})_2\text{Cl}_2]$ (249) was prepared.³³⁶ The potentially tridentate ligand Hpyrimol acts as a bidentate one, binding to ruthenium(II) via the nitrogen atoms. The phenolic groups of the two ligands are involved in intramolecular hydrogen bonding to the axially bound chlorido ligands. The complex 249 showed high cytotoxicity against different cancer cell lines (A498, EVSA-T, H226, IGROV, M19-MEL, MCF-7, and WiDr) with IC_{50} values in the low micromolar concentration range, while the reduced original ligand Hpyramol was significantly less active. In addition, the complex is able to bind to and cleave DNA.



Another *trans*-configured ruthenium(II) complex *trans*-[Ru^{II}(Pir)₂(CH₃CN)₂] **250** resulted from the reaction of a sodium salt of piroxicam with RuCl₃·3H₂O in 4:1 acetonitrile/methanol mixture.³³⁷ Piroxicam exhibits anticancer activity,³³⁸ while **250** has not yet been tested for cytotoxicity.



By reaction of another noninnocent compound, 3-aminoflavone (3-af) with RuCl₃·2H₂O in a 2:1 molar ratio in methanol or ethanol, two complexes **251** and **252**, with new ligands, 3-imino-2-methoxy-flavanone (3-imf) and 3-imino-2-ethoxy-flavanone (3-ief), resulting from the dehydrogenative alcoholysis of 3-af, were synthesized.³³⁹ The complexes are cytotoxic toward both human bladder carcinoma cell sublines, sensitive (E) and resistant (EJcisR) to cisplatin, and less toxic to healthy human lymphocytes *in vitro* than cisplatin.³⁴⁰ The underlying mechanism of cytotoxicity presumably involves caspase-8 activation and DNA strand breaks, as well as generation of oxidative stress upon cells exposed to **251** and **252**, finally leading to apoptosis.



Cis-configured mixed ligand ruthenium(II) complexes *cis*-[RuCl₂(dppb)(5,5'-mebipy)] (**253**), *cis*-[RuCl₂(dppp)(5,5'-mebipy)] (**254**), and *cis*-[RuCl₂(dppb)(4,4'-mebipy)] (**255**), where dppb = 1,4-bis(diphenylphosphino)butane, dppp = 1,3-bis(diphenylphosphino)propane, 5,5'-mebipy = 5,5'-dimethyl-2,2'-dipyridyl, and 4,4'-mebipy = 4,4'-dimethyl-2,2'-dipyridyl, show remarkable cytotoxicity in human breast carcinoma cells MDA-MB-231 with IC₅₀ values of 5.41 ± 0.71, 14.6 ± 1.81, and 15.64 ± 0.44 μM, respectively.³⁴¹ Similar potency was reported for *cis,cis*-[RuCl₂(PPh₃)₂(L)] (**256**), where L is a 2:1 Schiff base of 2-chlorobenzaldehyde with 1,3-diaminopropan-2-ol, against the same cell line.³⁴²

Maltol, which is a known food additive, with antioxidative properties and an important chelating ligand in anticancer ruthenium-*p*-cymene complexes, was also used for the preparation of a series of Werner-type ruthenium(III) complexes, for example, *trans*-[Ru(ma)₂(L)₂](CF₃SO₃), where Hma = maltol = 3-hydroxy-2-methyl-4-pyrone, L = imidazole (**257**), 1(*N*)-methylimidazole (**258**), 2-methylimidazole (**259**), 4-methylimidazole (**260**), and [Ru(ma){1(*N*)-methylimidazole}₄](CF₃SO₃)₂ (**261**).³⁴³ While maltol and imidazole ligands are nontoxic, complexes **259** and **260** showed high cytotoxicity against human breast cancer cells MDA-MB-435S with IC₅₀ values of ~5 and 15 μM, respectively. In contrast, ruthenium(II) complexes with carbohydrate-based ligands, prepared by reaction of [RuCl₂(DMSO)₄] with the D-glucosaminic acid sodium salt, the 1-thiol-β-D-glucose sodium salt, or the 6'-aminolactose derivative in ethanol, did not show appreciable antiproliferative activity against the melanoma cell line A375.³⁴⁴

Coordination of dimethyldithiocarbamates, pyrrolidinedithiocarbamates, or other dithiocarbamates to ruthenium and, in particular, to ruthenium(III) afforded highly cytotoxic mono- and dinuclear complexes against human non-small cell lung cancer cells (NCI-H1975) with some of them showing IC₅₀ values in the nanomolar concentration range.^{345,346}

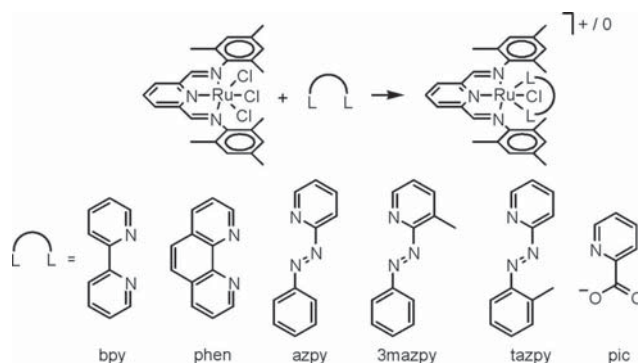
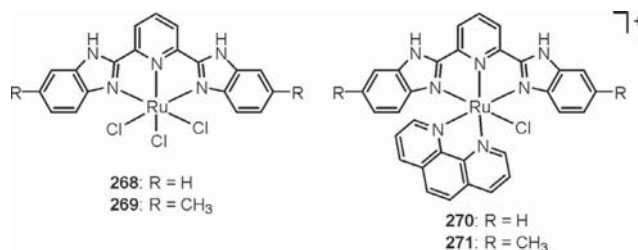


Figure 6 Ruthenium complexes with tmpimpy and a series of bidentate coligands.

A series of ruthenium(II) complexes with a tridentate Schiff base ligand 2,6-bis(2,4,6-trimethylphenyliminomethyl)pyridine (tmpimpy) able to bind meridionally to metal ions and bidentate coligands, 1,10-phenanthroline (phen), 2,2'-dipyridyl (bpy), 2-(phenylazo)pyridine (azpy), 2-(phenylazo)-3-methylpyridine (3mazpy), 2-(tolylazo)pyridine (tazpy), and 2-picolinate (pic) have been reported to show higher antiproliferative activity (IC_{50} values = 0.4–10 μ M), in a panel of seven human cancer cell lines (A498 (renal), EVSA-T (breast), H226 (lung), IGROV (ovarian), M19 (melanoma), MCF-7 (breast), and WiDr (colon)), with a drug exposure time of 120 h, than the parent ruthenium(III) complex $[RuCl_3(tmpimpy)](H_2O)$ (IC_{50} values 11–17 μ M) (see Figure 6).³⁴⁷

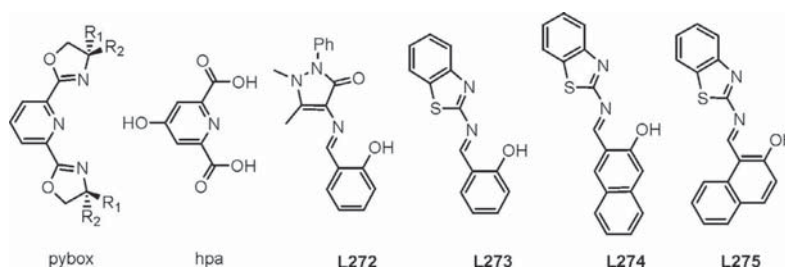
By substitution of the chlorido ligand in this type of complex of both tridentate, meridionally bound to ruthenium, and bidentate coligands, by sulfur-containing ligands supposed to build stronger bonds to ruthenium(II) and able to release these sulfur-containing molecules in a controlled way upon light irradiation, a new photochemotherapy strategy to treat cancer locally has been proposed, avoiding the coordination of competing biological ligands *in vivo* at early stages following drug administration. This concept has been validated by performing the reaction of $[Ru(terpy)(bpy)Cl]Cl$ (262) with a thioether ligand and the isolation of two complexes $[Ru(terpy)(bpy)(N\text{-acetyl-L-methionine})]Cl_2$ (263) and $[Ru(terpy)(bpy)(D\text{-biotin})]Cl_2$ (264).³⁴⁸ Complexes 263 and 264 were found to be markedly more stable toward hydrolysis under physiological conditions in the dark than the chlorido complex 262. The coordinated sulfur-protecting ligand can be efficiently cleaved from the ruthenium(II) by visible light irradiation into the MLCT band of the ruthenium–thioether complex 263 or 264, as was proved by monitoring the evolution of the UV–vis spectrum of a solution of 264 upon blue-light ($\lambda = 452$ nm) irradiation and comparison with the optical spectrum of $[Ru(terpy)(bpy)(H_2O)]^{2+}$. Thus, the ruthenium–sulfur complex is suggested as a prodrug, which can be activated upon light irradiation, inducing the release of a harmless thioether ligand. The inactive thioether ligand can presumably be replaced by a biologically active molecule, for example, 5-cyanouracil (5CNU), known as an inhibitor of pyrimidine catabolism,³⁴⁹ and, closely related to 5-fluorouracil, a clinically used drug for treatment of colorectal and breast cancers.³⁵⁰ Its cleavage from ruthenium under light irradiation would provide anticancer agents with a dual mode of action, DNA binding along with the release of a biologically active molecule with a different mechanism of action. This concept was recently proposed and verified by preparation of the complex $[Ru(tpy)(5CNU)_3]Cl_2$ (265) via treatment of $[Ru(terpy)Cl_3]$ with $AgCF_3SO_3$ and subsequent reaction with 5CNU and comparison of the binding ability and cytotoxicity of 265 upon light irradiation with those of $[Ru(terpy)(CH_3CN)_3]^{2+}$ (266) $[Ru(terpy)(CH_3CN)_2Cl]^+$ (267).³⁵¹ While all complexes bind to DNA, complex 265 shows significantly higher toxicity than 266 in HeLa cells upon light irradiation by releasing one 5CNU molecule, contributing to its cytotoxicity.

Likewise, 2,6-bis(benzimidazolyl)pyridine derivatives known as important pharmacophores were coordinated meridionally to ruthenium(III) to give complexes 268 and 269. Introduction of phen as a coligand in the coordination sphere of the latter results in the reduction of ruthenium(III) to ruthenium(II), affording complexes 270 and 271.³⁵²

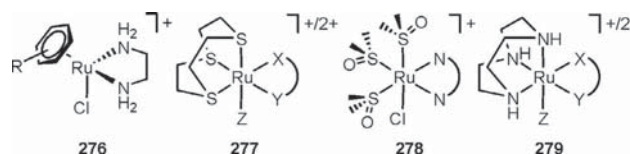


IC₅₀ assays against a panel of human cancer cell lines A375 (melanoma), HeLa (cervix), MCF-7 (breast), and PC3 (prostate) and murine Neuro2a (neuroblastoma) showed that methylation of the tridentate ligand and coordination of phen have a favorable effect on cytotoxicity. The highest potency was observed for compound 271 (IC₅₀ values ranged from 25.2 to 50.0 μ M), which, in addition, is much less toxic against normal human fibroblast cells Hs68 (IC₅₀ > 400 μ M). DNA damage via intercalation that activates p53-mediated apoptosis in A375 cells is considered as the primary underlying mechanism of cytotoxicity of 271.³⁵³

The use of the chiral tridentate ligand pybox enabled the synthesis of enantiomerically pure ruthenium(II) complexes.³⁵⁴ Although all complexes showed low cytotoxicity in HeLa cells, the effect of enantiomer identity on the cell cycle in some cases was remarkable, and while complexes with (S,S)-iPr-pybox did not exert any significant effect on the cell cycle, their enantiomers with the (R,R)-iPr-pybox induced up to 58% cell arrest at the G₂/M phase after 72 h drug exposure time. Ruthenium complexes with a series of other rigid tridentate ligands, that is, 4-hydroxypyridine-2,6-dicarboxylic acid (hpa),³⁵⁵ or with Schiff bases 272–275^{356–360} have been reported and tested for antiproliferative activity *in vitro*.

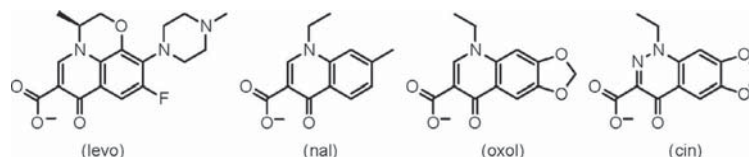


The use of tridentate ligands able to bind facially to ruthenium has been recently initiated with the aim of elucidating the importance of the η^6 -arene ligand for the anticancer activity of 276 (RM175)-type complexes and whether it can be replaced by an appropriate face-capping ligand while maintaining the other ligands intact.^{361–363}

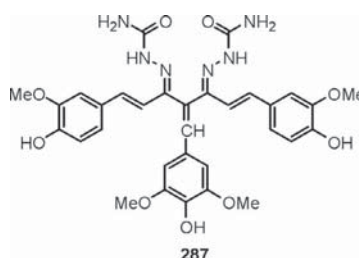


As starting materials for the synthesis of complexes 277, three different ruthenium(II) complexes were used, namely, [Ru([9]aneS₃)(κ S-DMSO)Cl₂] (280), prepared from [Ru(DMSO)₄Cl₂] and 1,4,7-trithiacyclononane ([9]aneS₃),³⁶⁴ as well as [Ru([9]aneS₃)(κ S-DMSO)₂Cl](PF₆) (281) and [Ru([9]aneS₃)(κ S-DMSO)₃](PF₆)₂ (282) resulting from treatment of 280 with 1 and 2 equiv of AgPF₆, respectively, in DMSO,³⁶¹ while for the preparation of 279, [Ru([9]aneN₃)(κ S-DMSO)₂Cl]Cl (283)³⁶⁵ or [Ru([9]aneN₃)Cl₃] (284)³⁶⁶ were exploited. By dissolving 280 in MeCN at 50 °C and allowing for slow crystallization, the complex [Ru^{III}([9]aneS₃)(MeCN)₂Cl]₂·2H₂O (281) was isolated, which exhibited low cytotoxicity at concentrations >200 μ M in the osteosarcoma cell line MG63.³⁶⁷ Mild cytotoxicity was also reported for [Ru^{III}([9]aneS₃)(glycine)Cl] (282).³⁶⁸ Reduction of ruthenium(III) to ruthenium(II) occurs in the presence of AgCF₃SO₃ in MeCN resulting in the isolation of [Ru^{II}([9]aneS₃)(MeCN)₃](CF₃SO₃)₂ (285).³⁶⁹ Reaction of 281 with 1,2-diaminoethane (en) or *trans*-1,2-diaminocyclohexane (dach) afforded complexes 277, where Z = Cl, and X–Y = en or dach, respectively, while treatment of 283 with en or dach yielded complexes 279, where Z = DMSO and X–Y = en or dach, respectively.³⁶⁵ Complexes 277 were moderately cytotoxic against the human mammary carcinoma cell line MDA-MB-231 (IC₅₀ = 81 and 124 μ M), while compounds 279 exhibited IC₅₀ values >300 μ M. This distinct activity was related to their different behavior in aqueous solution. Complexes 277 are more labile and hydrolyze binding selectively to the N7 atom of 9-methylguanine, guanosine and guanosine 5'-monophosphate,³⁷⁰ while complexes 279 basically remain intact in solution. Quite surprisingly, complexes of the type 278, resulting from the reaction of *fac*-[RuCl(κ S-DMSO)₃](κ O-DMSO)₂](PF₆) (286) with a series of bidentate ligands, for example, en, dach, and picolinate (pic), are very stable and inert in aqueous solution when compared to organoruthenium species 276 and do not display appreciable cytotoxicity against MDA-MB-231 cells.³⁷¹ By reaction of 282 with a series of quinolone compounds, known as synthetic antibacterial agents, in a 1:1 molar ratio in methanol, four compounds of the type 277 have been prepared, where Z = DMSO and X–Y = bidentate O,O'-ligand, namely, levofloxacin (levo), nalidixic acid (nal), oxolinic acid (oxol), or cinoxacin (cin), which showed very modest antiproliferative activity against HeLa (cervix) cells, and were devoid of any inhibiting activity on the proliferation of A549 (lung)

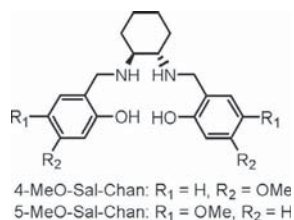
cells.³⁷² The substitution of the DMSO ligand by water in these complexes is much slower when compared to that of the chlorido ligand in the related organoruthenium compounds yielding $[\text{Ru}(\eta^6\text{-}p\text{-cymene})(O,O'\text{-quinolonato})(\text{H}_2\text{O})]^+$ species.



1,7-Bis-(4-hydroxy-3-methoxyphenyl)-2,6-heptadien-3,5-dione also referred to as curcumin (133) has been extensively studied for various medicinal applications³⁷³ and is currently being evaluated in phase I/II clinical trials for the treatment of colon cancer.³⁷⁴ Metal–curcumin complexes,²¹³ as well as copper(II) and ruthenium(III) complexes with tetradentate Schiff bases derived from curcumin and semicarbazide (287), have been recently reported as strong inhibitors of HeLa, HepG2, MDA-MB-231, and HT-29 cell proliferation.^{375,376}



Coordination of Salan-type tetradentate ligands, 4-MeO-Sal-Chan and 5-MeO-Sal-Chan, to ruthenium(III) resulted in a 2–3.5-fold enhancement of cytotoxicity against A2780 (ovarian adenocarcinoma), MCF-7 (ER α -positive noninvasive breast adenocarcinoma), and MDA-MB-231 (ER α invasive breast carcinoma).³⁷⁷ The complexes $[\text{Ru}(4\text{-MeO-Sal-Chan})(\text{PPh}_3)\text{Cl}]$ (288) and $[\text{Ru}(5\text{-MeO-Sal-Chan})(\text{PPh}_3)\text{Cl}]$ (289) showed IC_{50} values ranging from 3.3 to 27.4 μM .



Ruthenium Nitrosyl Complexes

Nitric oxide plays important roles in biochemical processes^{378–380} and, in particular, in the progression of human tumors.³⁸¹ It is produced at nanomolar or micromolar levels in the cells by at least three known isoforms of nitric oxide synthases (NOS)³⁸² by converting L-arginine into NO and L-citrulline. Lower endogenous cellular concentrations of NO (in pM–nM range) stimulate proliferation of tumor cells and angiogenesis, while higher endogenous levels (μM) than those produced in activated macrophages³⁸³ or provided by the exogenous supply of nitric oxide (NO-releasing ruthenium nitrosyl species), as well as scavenging NO before it exerts its effects, would inhibit tumor cell growth via the generation of highly reactive nitrogen species (RNS) or constriction of tumor feeder vessels³⁸⁴ and thus play a protective role. So it appears that the beneficial or deleterious role of NO in cancer development is a matter of its concentration.^{385,386} Therefore, ruthenium complexes have been prepared as nitric oxide scavengers and NOS inhibitors. From the other side, efforts by different groups are focused on the development of complexes capable of NO release in a controlled manner by reduction or photoexcitation, in addition to their significant academic interest³⁸⁷ (NO as noninnocent ligand, occurrence of structural trans effects, linkage isomerization of the N- and O-bound nitrosyl ligand, and the role of the RuNO unit as a regulator of the geometry around the central atom). The antiproliferative activity of ruthenium nitrosyl complexes supported by different organic ligands, including amino acids, both *in vitro*^{388–390} and *in vivo*³⁹¹ is well documented, and new complexes were reported recently. The complex 290 (Figure 7) has shown antiproliferative activity against human HeLa (cervix) carcinoma cells and murine Tm5 (melanoma) cells with IC_{50} values of 10.83 ± 0.33 and 17.75 ± 6.82 μM compared to 54.73 ± 33.30 μM in a nontumorigenic CHO (ovarian) cell line. In addition, inhibition of melanoma tumor growth in an *in vivo* assay in mice was observed.³⁹¹

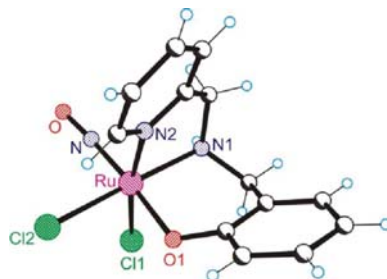
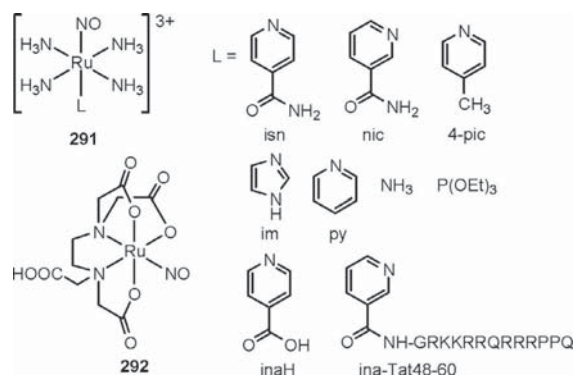


Figure 7 X-ray diffraction structure of ruthenium nitrosyl complex **290**.

Similarly, *in vitro* and *in vivo* anticancer activity was found for a series of complexes of the type **291** and **292**.³⁸⁴ Necrotic cell death induced by ruthenium complexes through DNA degradation and morphological alterations, as well as inhibition of angiogenesis, was observed. In addition, increasing animal survival in a syngeneic model of murine melanoma B16F10 upon treatment with **291** (L= isn or im) was reported.



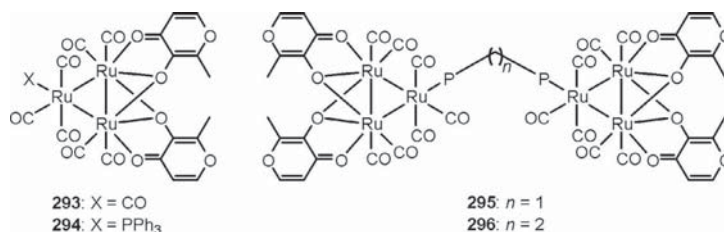
Complexes **291** (L= inaH or ina-Tat48-60) were found to release NO upon one-electron reduction with the formation of *trans*-[Ru(NH₃)₄(L)(H₂O)]³⁺.³⁹² The antiproliferative activity against the murine melanoma cell line B16-F10 was suggested to be due to NO release after complex reduction.

The effects of metal (Ru vs. Os), *cis/trans* isomerism, and azole heterocycle identity on the cytotoxic potency of ruthenium and osmium complexes of general formulas (cation)⁺[*cis*-MCl₄(NO)(Hazole)]⁻ and (cation)⁺[*trans*-MCl₄(NO)(Hazole)]⁻, where M= Ru or Os, (cation)⁺= (H₂ind)⁺, (H₂pz)⁺, (H₂bzim)⁺, (H₂im)⁺ or (*n*Bu₄N)⁺, and Hazole= 1*H*-indazole (Hind), 1*H*-pyrazole (Hpz), 1*H*-benzimidazole (Hbzim), or 1*H*-imidazole (Him), on cytotoxicity and cell line selectivity have been reported.³⁸⁹ In contrast to most pairs of analogous ruthenium and osmium complexes known, ruthenium nitrosyl complexes with azole heterocycles yielded IC₅₀ values in the low micromolar concentration range differing by factors (Os/Ru) of up to ~110 and ~410 in CH1 and SW480 cells, respectively; however, the reasons for such a striking difference are still not known.³⁸⁹

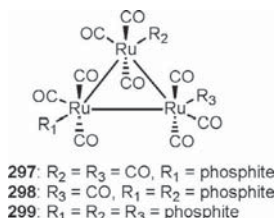
Di- and Polynuclear Ruthenium Complexes

The effect of metal–metal bonding on biological activity and interaction with biomolecular cellular targets has been little explored so far. The bridging ligands are often nontoxic, and in the case of polynuclear metal complexes, higher concentrations of biologically active metal ions might be delivered to the cellular targets because of higher kinetic stability (with respect to the mononuclear species) increasing the biological efficacy.³⁹³

Highly cytotoxic trinuclear organoruthenium(II)–maltol complexes have been quite recently reported.³⁹⁴ Reaction of Ru₃(CO)₁₂ with maltol in a 1:2 molar ratio in boiling octane afforded the complex **293** in 93% yield. One CO ligand at the ruthenium atom not involved in the coordination of the maltolate ligand can be easily substituted by a phosphine or a diphosphine ligand in the presence of trimethylamine *N*-oxide dihydrate in CH₂Cl₂ yielding complexes **294**–**296**.

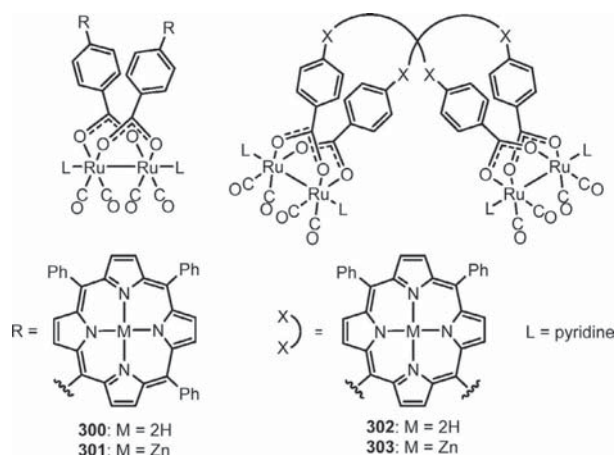


Substitution of CO by phosphine reduces the antiproliferative activity in the following order: 293 > 294 > 295 > 296. The most active species, 293, displayed IC₅₀ values of 0.4, 0.5, 0.75, 0.5, 0.3, 0.1, and 0.075 μ M against human cancer cell lines MCF-7 (breast), DU145 (prostate), H460 (lung), HT29 (colon), K562 (chronic myelogenous leukemia), HL60 (acute myeloid leukemia), and GRANTA519 (mantle cell leukemia), respectively. Stepwise replacement of one CO at each ruthenium atom by a bicyclopophosphite ligand, that is, 3,5,6-bicyclopophosphate-1,2-O-isopropylidene- α -D-glucopyranoside, yielded products 297–299.³⁹⁵ The antiproliferative activity of these compounds depends on the number of phosphite ligands in the trinuclear cluster. Compounds 297 and 298 showed very high cytotoxicity in A2780 (ovarian), A2780cisR (ovarian), HT29 (colon), A549 (alveolar basal epithelial), and ECRF24 (umbilical vein endothelial) cell lines (IC₅₀ values in the range 0.1–0.8 μ M), while 299 was devoid of cytotoxicity (IC₅₀ > 300 μ M) in the first four cell lines and displayed moderate cytotoxicity (IC₅₀ of $\sim$ 60 μ M) against endothelial cells.



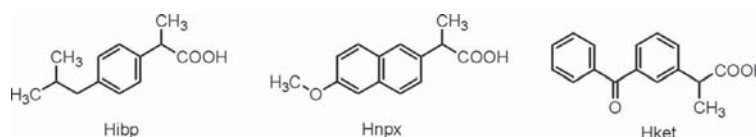
In addition, antiangiogenic activity in the *in vivo* chicken chorioallantoic membrane model was disclosed for 299. Interestingly, the cell cycle arrest in the G₁/G₀ phase induced by this compound led to senescence, and not to direct apoptosis.³⁹⁵

Treatment of Ru₃(CO)₁₂ with monocarboxy phenyl-porphyrin and dicarboxy phenyl-porphyrin derivatives in the presence of pyridine sawhorse-type diruthenium and tetraruthenium polycarbonyl complexes 300–303 was prepared to assess their potency as photosensitizing and chemotherapeutic agents.³⁹⁶ Diruthenium(II)–zinc(II) complex 301 showed the highest antiproliferative activity in the dark against HeLa (cervix), A549 (lung), Me300 (melanoma), and A2780 (ovarian) cells with IC₅₀ values of 42 $\pm$ 2, 52 $\pm$ 7, 22 $\pm$ 3, and 32 $\pm$ 3 μ M, respectively after 72 h incubation time, while the metal-free porphyrin derivative 300 displayed the highest photocytotoxicity.



The use of available pharmacological carboxylic acids, such as probenecid, indomethacin, and sulindac, known as uricosuric and as anti-inflammatory agents, and Py, PPh₃, or 5-(4-pyridyl)-10,15,20-triphenylporphyrin as potent axial ligands, resulted in sawhorse-type diruthenium tetracarbonyl complexes of poor aqueous solubility precluding the evaluation of their antiproliferative activity and/or photocytotoxicity.³⁹⁷

More successful in terms of aqueous solubility were the attempts to replace the equatorial acetate ligands in a mixed-valent diruthenium(II,III) paddle wheel complex [Ru₂Cl(CH₃COO)₄] by carboxylic pharmaceuticals, such as the nonsteroidal anti-inflammatory drugs, ibuprofen (Hibp),^{398,399} naproxen (Hnpx),⁴⁰⁰ or ketoprofen (Hket).⁴⁰¹ However, the products exhibited only a mild antiproliferative activity against colorectal cancer cells HT29 and Caco2. The thermodynamics and kinetics of axial chlorido substitution with amino acids have also been studied recently.⁴⁰² In addition, the chlorido ligand(s) can be removed in the presence of silver(I), creating available coordination sites for a variety of other ligands, including cytotoxins.



Cationic dinuclear *p*-cymene ruthenium(II) complexes containing three thiolato bridges with a variety of identical substituents at the sulfur atom^{403,404} have been prepared by reaction of [(η⁶-*p*-cymene)₂Ru₂(μ-Cl)₂Cl₂] with aromatic thiols. The formation of [(η⁶-*p*-cymene)₂Ru₂(μ-SR)₃]⁺ was found to proceed through the neutral di(ruthenium-arene) complexes with two thiolato bridging ligands, [(η⁶-*p*-cymene)₂Ru₂(μ-SR)₂Cl₂].⁴⁰⁵ The latter compounds were successfully used for the synthesis of dinuclear complexes with mixed thiolato bridges of the type [(η⁶-*p*-cymene)₂Ru₂(μ-SR¹)₂(μ-SR²)]⁺.⁴⁰⁶ Most of the compounds prepared are highly cytotoxic against human ovarian cancer cell lines A2780 and A2780cisR. For example, the complex [(η⁶-*p*-cymene)₂Ru₂(μ-SC₆H₄-*p*-t-Bu)₃]Cl displayed IC₅₀ values of 0.03 ± 0.01 μM in both cell lines. The cytotoxicity observed correlates well with the lipophilicity of the compounds expressed via log *P* values. In a similar way, cationic selenolato- and tellurophenolato-bridged diruthenium complexes of [(η⁶-*p*-cymene)₂Ru₂(μ-SePh)₃]⁺ and of [(η⁶-*p*-cymene)₂Ru₂(μ-TePh)₃]⁺, as well as complexes with mixed thiolato-selenolato and thiolato-tellurato bridges, have been synthesized, which also displayed IC₅₀ values in the sub-micromolar concentration range.⁴⁰⁷ Intriguingly, Ru₆ wheels [{Ru(S(4-*i*Pr-C₆H₄S))₂(CO)₂}]₆ resulting from treatment of Ru₃(CO)₁₂ with 4-*i*Pr-C₆H₄SH under an inert atmosphere at elevated temperature were noncytotoxic against SW480 (colon) cancer cells.⁴⁰⁸

An interesting trinuclear ruthenium(III) complex, **304**, has been reported recently, which resulted from a solvothermal reaction of Ru(acac)₃, where Hacac = pentan-2,4-dione, with relatively nontoxic 2-thiosalicylic acid (H₂TSA) in the presence of tetraethylammonium acetate.³⁹³ The complex anion in [NEt₄][Ru₃^{III}(HTSA)₂(TSA)₄] is shown in Figure 8. The complex is fairly stable in PBS-DMSO 99:1 at pH 7.4. Adducts with glutathione (GSH) were observed by mass spectrometry after 24 h incubation of **304** with GSH. The IC₅₀ values of 7.3–18.2 μM in a panel of 4 human cancer cell lines MDA-MB-231 (breast), HeLa (cervix), SUNE1 (nasopharyngeal), and HepG2 (liver) indicate appreciable cytotoxicity *in vitro*. The complex was less cytotoxic in lung fibroblast cells CCD-19Lu (IC₅₀ 32.1 ± 4.8 μM) and was reported to target the Wnt-β-catenin signaling pathway by reducing both transcriptional and protein expression levels of the main components involved.

Attempts to optimize the conditions for using complexes **305** and **306**, prepared recently,⁴⁰⁹ in the *in vivo* photodynamic therapy on mice bearing an ectopic human oral carcinoma xenograft, which is a multiparameter task, have been described.⁴¹⁰ Complex **306** proved to be more efficient for PDT treatment, although *in vitro* fluorescence microscopy studies have shown that both complexes did not reach the nucleus to target DNA accumulating only in the cytoplasm of KB cells. Slow accumulation of the

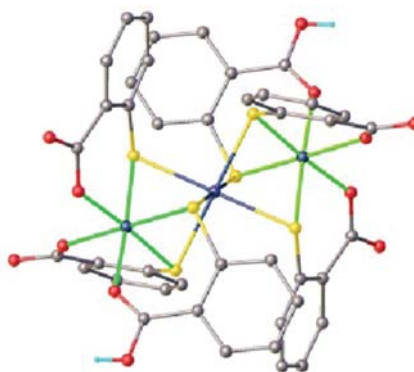
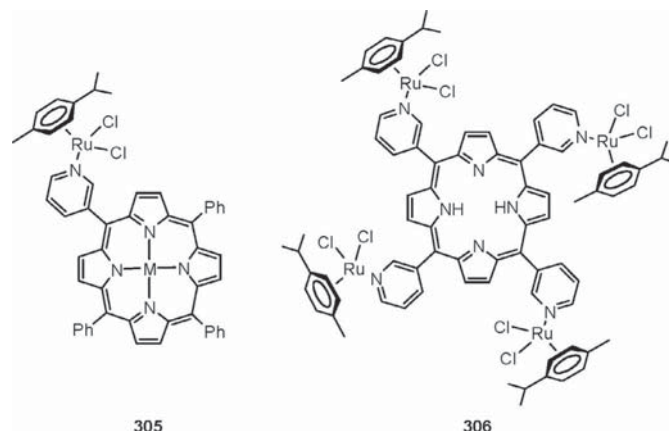
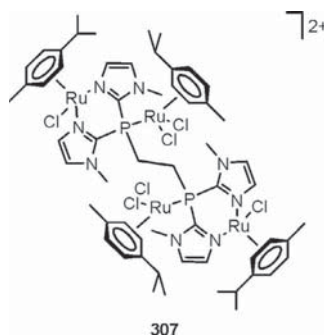


Figure 8 X-ray diffraction structure of the complex anion in **304**.

complex in the tumor, up to 1.5% of the injected dose (3 mg kg^{-1}) per gram of tumor tissue 48 h after injection with little decline on increasing the postinjection time, was observed.



A water-soluble (16 mg ml^{-1}) tetranuclear complex 307 characterized by X-ray diffraction was found to be devoid of appreciable cytotoxicity in human cancer cell lines HCT116 (colon), HUH7 (hepatoma), and A2780 (ovarian) ($\text{IC}_{50} > 100 \text{ }\mu\text{M}$).⁴¹¹



The synthesis of heterodimetallic complexes is also a promising approach to create efficient anticancer agents.⁴¹² Such complexes can provide novel modes of action via a dual mode of binding at the target site and synergistic effects resulting from the combination of different metal entities. In particular, the dichlorido-bridged hexamethylbenzene-ruthenium(II)-dimethyltin(IV) hybrid 308 (Figure 9) has shown very good antiproliferative activity against HeLa and HepG2 cancer cells (IC_{50} 5.2 and $7.4 \text{ }\mu\text{M}$, respectively). The underlying mechanism of cytotoxicity presumably involves significant DNA binding affinity and the ability to inhibit the human topoisomerase I α at a very low concentration ($8 \text{ }\mu\text{M}$).

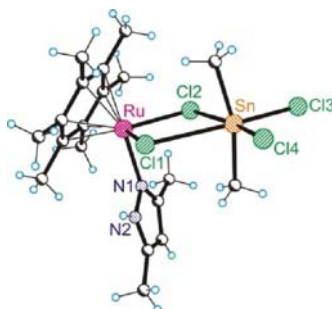
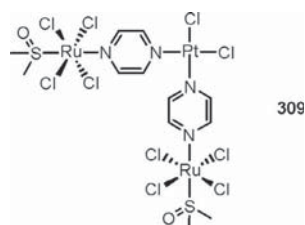


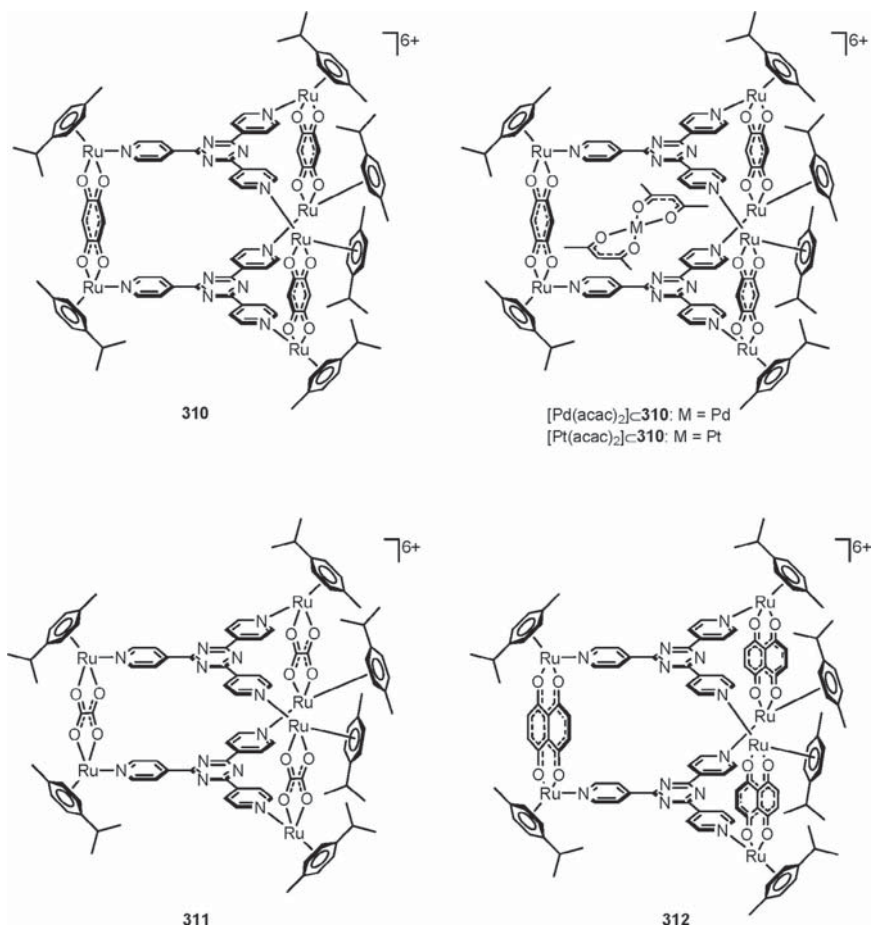
Figure 9 X-ray diffraction structure of heterodinuclear complex 308.

Another interesting trinuclear diruthenium(III)–platinum(II) hybrid **309**, prepared recently,⁴¹³ was found to be more effective in binding to RNA and to cause the inhibition of DNA migration and growth of BY4741 yeast cells than the corresponding mononuclear drugs NAMI-A and cisplatin, the structural elements of which were combined when constructing the heterotrinuclear complex.⁴¹⁴ The complex reduces the motility of breast and lung cancer cells more efficiently than NAMI-A.⁴¹⁵



Ruthenium–Arene Cages

Arene ruthenium cages are synthesized by a self-assembly approach, using appropriate dinuclear arene ruthenium–chlorido clips and a bi-, tri-, or tetradentate N-donor ligand in a certain molar ratio. The assembly is initiated by the presence of silver triflate as a chloride abstractor, and the cages are isolated as triflate salts. Tetracationic rectangles,^{416–421} hexacationic prisms,⁴²² and octacationic cubes⁴²³ are known today. An exceptional property of this class of compounds is the ability to accommodate guest molecules within their central cavity, which can be used for drug delivery. Ruthenium–arene cages have been reviewed comprehensively quite recently⁴²⁴; therefore, we will only give a short overview of the latest results reported.



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In particular, a series of prismatic metalla-cages has been developed.^{425–429} These compounds were shown to incorporate planar complexes and organic molecules and to release them after uptake into the cancer cells.⁴³⁰ Interesting features of such carceplex systems are that (i) the physical properties of the host are retained and therefore water-insoluble guests can be delivered⁴³¹; (ii) the rate of guest liberation is tunable by the size of the ligands on the side of the cage; bulky ligands lead to a small portal size, which may result in permanent encapsulation^{432,433}; (iii) due to their large particle size, the enhanced permeability and retention (EPR) effect⁴³⁴ might be exploitable.⁴²⁹ The cytotoxicity of host–guest complexes can rise dramatically compared to the single components, but also the guest-free cages display remarkable cytotoxicity. The IC_{50} value of **310** in the ovarian cancer cell line A2780 was 23 μM ; after encapsulation of platinum(II) or palladium(II) acetylacetonate, the IC_{50} values were reduced to 12 and 1 μM for $[Pt(acac)_2] \subset 310$ and $[Pd(acac)_2] \subset 310$, respectively, while $[Pt(acac)_2]$ and $[Pd(acac)_2]$ were inactive because of insolubility.⁴²⁵ Despite the promising activity of ruthenium-based cages, their mechanism of cytotoxicity is still poorly understood at the molecular level. In recent studies, the reactivity of **310–312** toward various biologically relevant molecules, that is, amino acids, GSH, and ascorbic acid, was investigated.^{435–437} Compound **310** remained intact when combined with most amino acids, but decomposition of the cage and formation of the respective ruthenium–arene–amino acid complex was observed with amino acids containing specific side chains, namely, Cys (thiol), His (imidazole), Arg, and Lys (basic amino groups). Interestingly, **310** did not react with Met, which is known to interact with platinum and ruthenium complexes.^{438,204} It was also found that **310** catalyzes the oxidation of Cys to cystine, ascorbic acid to dehydroascorbic acid, and GSH to GSSG, what may at least, in part, explain its cytotoxicity.⁴³⁵ Compound **312** exhibited a higher cytotoxicity than **310** ($IC_{50} = 3.1 \pm 1 \mu M$ in A2780 cell line).⁴²⁶ **312** reacted with the same amino acids as **310**, but the reaction is much slower and does not proceed to completion even in the presence of a large excess of amino acid. The oxidation of Cys and ascorbic acid is much slower, while that of GSH is faster than in the presence of **310**. It is worth noting that the more inert a compound is, the more cytotoxic it is. However, a negative correlation between protein binding and cytotoxicity has been previously reported.²⁰⁸ The oxidation of GSH may in part explain the higher cytotoxicity of **312**⁴³⁶ compared to **310** and **311**, which are equipotent.⁴³⁷ However, **311** is significantly more reactive than **310** and **312**. It disassembles in a 50 mM NaCl aqueous solution and reacts with amino acids that do not contain specific side chains, that is, Glu, Pro, Ser, Tyr, and Ala, indicating that **311** reacts not only with certain amino acid side chains but also with their backbones (i.e., amine and carboxylic functional groups). In context of the anticancer activity, it seems to be important that the metalla-cage enters cancer cells without degradation.^{435–437}

Pyrene can be included in metalla-cages even if it bears a functionalized side arm.⁴³⁹ This ability was exploited to develop a variety of carceplex systems as a carrier for anticancer drugs.^{100,440–442} A schematic picture of such a complex is given in Figure 10. The pyrene moiety is accommodated within the cage, while the cytotoxic moiety attached via a flexible chain is dangling out of the cage.

The cytotoxicity of **312** was increased after incorporation of cycloplatin complexes **G1** and **G2**, indicating the effective delivery of the hydrophobic platinum complexes (see Table 2). Interestingly, the cytotoxicity was not diminished in the cisplatin-resistant A2780cisR cancer cell line, implying a different mode of action compared to cisplatin. The IC_{50} values of the free platinum complexes **G1** and **G2** were not determined because of solubility problems.⁴⁴⁰ Incorporation of the RAPTA-derived complexes

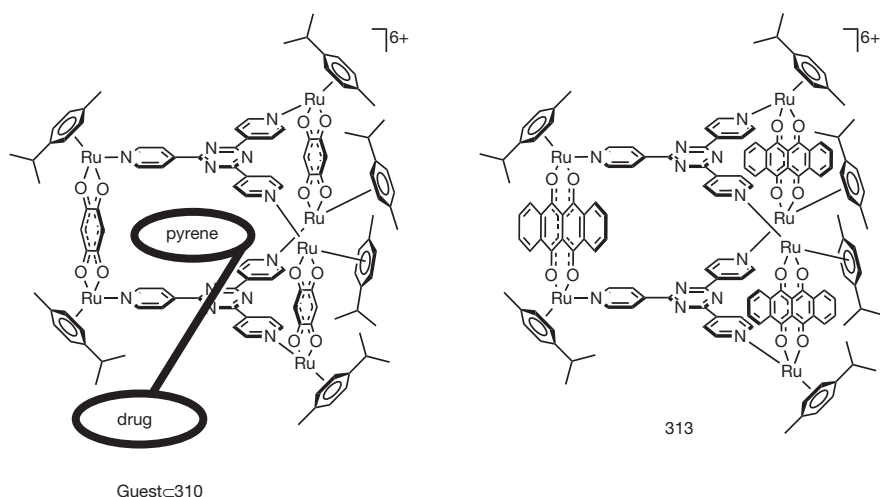


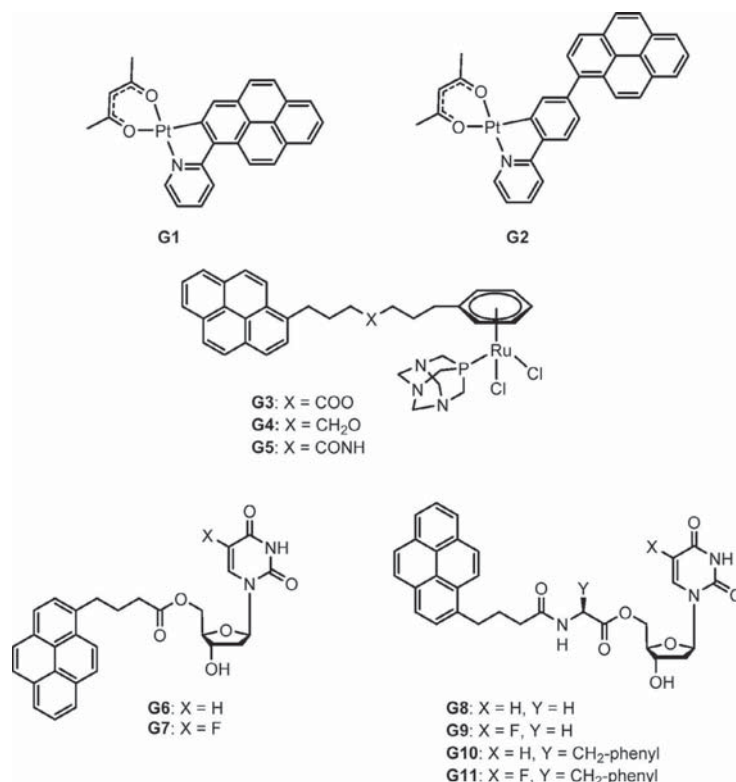
Figure 10 A schematic representation of a host–guest complex and the structure of metalla-cage **313**.

Table 2 Cytotoxicity of host ruthena-cages, guests, and host–guest complexes

Compound	A2780 (IC_{50} (μM))	A2780cisR (IC_{50} (μM))	Resistance factor (RF)
310	23 $\pm$ 2.1	25 $\pm$ 2.5	1.1
312	3.1 $\pm$ 1	4.6 $\pm$ 0.5	1.5
313	4.1 $\pm$ 0.07	6.5 $\pm$ 1.0	1.6
G1–G2	n.d.	n.d.	–
G3	>25	>25	–
G4	19.7 $\pm$ 6.5	15.8 $\pm$ 6.0	0.8
G5	>25	>25	–
G6–G14	n.d.	n.d.	–
G15	5.9 $\pm$ 0.6	7.4 $\pm$ 0.8	1.3
G16	n.d.	n.d.	–
G17	8.7 $\pm$ 0.9	8.7 $\pm$ 1.0	1.0
G1 $\subset$ 312	1.2 $\pm$ 0.2	0.9 $\pm$ 0.2	0.8
G2 $\subset$ 312	1.4 $\pm$ 0.5	0.9 $\pm$ 0.3	0.6
G3 $\subset$ 312	5.0 $\pm$ 2.9	4.1 $\pm$ 3.8	0.8
G4 $\subset$ 312	4.7 $\pm$ 2.4	6.9 $\pm$ 2.6	1.5
G5 $\subset$ 312	3.4 $\pm$ 0.6	2.6 $\pm$ 0.5	0.8
G6 $\subset$ 310	2.1 $\pm$ 0.14	2.2 $\pm$ 0.35	1.0
G6 $\subset$ 312	1.3 $\pm$ 0.05	1.2 $\pm$ 0.04	1.1
G7 $\subset$ 310	0.4 $\pm$ 0.03	1.3 $\pm$ 0.13	3.3
G7 $\subset$ 312	0.4 $\pm$ 0.10	0.4 $\pm$ 0.11	1.0
G8 $\subset$ 310	5.6 $\pm$ 0.9	6.1 $\pm$ 1.5	1.1
G8 $\subset$ 312	1.3 $\pm$ 0.3	1.4 $\pm$ 0.3	1.1
G9 $\subset$ 310	0.3 $\pm$ 0.03	0.3 $\pm$ 0.03	1.0
G9 $\subset$ 312	0.2 $\pm$ 0.02	0.4 $\pm$ 0.05	2.0
G10 $\subset$ 310	5.9 $\pm$ 0.3	5.7 $\pm$ 1.0	1.0
G10 $\subset$ 312	1.7 $\pm$ 0.06	1.4 $\pm$ 0.06	0.8
G11 $\subset$ 310	1.2 $\pm$ 0.03	1.0 $\pm$ 0.04	0.8
G11 $\subset$ 312	1.4 $\pm$ 0.18	1.7 $\pm$ 0.21	1.2
G12 $\subset$ 312	1.2 $\pm$ 0.1	0.9 $\pm$ 0.08	0.8
G12 $\subset$ 313	2.1 $\pm$ 0.4	1.6 $\pm$ 0.2	0.8
G13 $\subset$ 312	1.4 $\pm$ 0.1	0.9 $\pm$ 0.08	0.6
G13 $\subset$ 313	0.8 $\pm$ 0.3	2.2 $\pm$ 0.8	2.8
G14 $\subset$ 312	0.3 $\pm$ 0.02	0.2 $\pm$ 0.02	0.7
G14 $\subset$ 313	1.9 $\pm$ 0.4	0.9 $\pm$ 0.3	0.5
G15 $\subset$ 312	2.0 $\pm$ 0.5	1.3 $\pm$ 0.4	0.7
G15 $\subset$ 313	4.1 $\pm$ 0.6	1.9 $\pm$ 0.3	0.5
G16 $\subset$ 312	0.7 $\pm$ 0.3	0.8 $\pm$ 0.2	1.1
G16 $\subset$ 313	1.1 $\pm$ 0.3	1.5 $\pm$ 0.4	1.4
G17 $\subset$ 312	0.7 $\pm$ 0.1	0.8 $\pm$ 0.1	1.1
G17 $\subset$ 313	1.5 $\pm$ 0.03	1.5 $\pm$ 0.4	1.0
Cisplatin	1.6 $\pm$ 0.6	8.6 $\pm$ 0.6	5.4

G3–G5 led to a cytotoxicity comparable with that of the guest-free cage **312**. However, it should be noted that the parent complex RAPTA-C is relatively nontoxic *in vitro* but efficient against metastases *in vivo*.⁴⁴³ The fluorescence of the pyrenyl moiety was exploited for kinetic accumulation studies on HeLa cells, showing a twofold higher cellular accumulation of **G4** when delivered by cage **312** compared to **G4** assayed as a single agent. Since the fluorescence is quenched when the pyrenyl moiety is included within a metalla-cage, the increase in fluorescence demonstrates the transport capacity of **312**.¹⁰⁰

5-Fluoro-2'-deoxyuridine (floxuridine) is a derivative of the synthetic nucleoside 5-fluorouracil³⁵⁰ and known for its high activity against metastases.⁴⁴⁴ Pyrenyl functionalized nucleic acids are of interest because of the fluorescence and the intercalation ability of the pyrene unit.⁴⁴⁵ Floxuridine-derived carboxyl systems were synthesized in order to overcome the limitations of synthetic nucleosides, such as poor water solubility and low cellular internalization. *In vitro* studies showed a decrease of IC_{50} values for the host–guest systems compared to **310** and **312** assayed as single agents; moreover, these values were similar to or lower than those of cisplatin in the same cell lines (see Table 2). The cytotoxicity in the A2780 and cisplatin-resistant A2780cisR cell line was essentially the same, suggesting a mode of action differing from that of cisplatin. The host–guest systems involving fluoro-substituted nucleosides **G7**, **G9**, and **G11** show superior activity compared to the unsubstituted derivatives (**G6**, **G8**, and **G10**). The IC_{50} values of the free guests could not be determined because of insufficient solubility.⁴⁴¹



Dendrimers are branched molecules with a variety of properties, which were exploited as drug delivery vectors.⁴⁴⁶ The incorporation of pyrene-modified lipophilic dendrimers differing in size within **312** (Figure 11) led to water-soluble host-guest systems with enhanced cytotoxicity compared to the guest-free cage. The dendrimers, per se, were inactive because of insolubility. This example shows that pyrene derivatives, containing very large side arms, can also be incorporated within the metalla-cages and delivered to cancer cells.⁴²⁶ The role of the dendrimers in the previous example could not be fully elucidated, because insolubility made an assessment of their biological activity impossible. For this reason, water-soluble dendrimers were synthesized.⁴⁴² Hydroxyl group-containing dendrimers **G15** and **G17** are water-soluble and show high *in vitro* cytotoxicity with IC₅₀ values comparable to that of cisplatin, while their acetonide counterparts were not active in the two tested cell lines, probably because of their poor solubility in the cell culture medium. Incorporation of dendrimers **G12–G17** (Figure 11) within cages **312** and **313** led to a strong enhancement of cytotoxicity compared to host and guest tested as single agents, suggesting that the intact host-guest system is responsible for the cytotoxicity. The large particle size of these molecules may lead to a preferred accumulation within tumors and makes them attractive candidates for further development.⁴⁴²

The high cytotoxicity of thiolato-bridged dinuclear arene ruthenium complexes⁴⁴⁷ prompted the development of thiolato-bridged ruthenium-arene metalla-prisms. The IC₅₀ values for these compounds were up to one order of magnitude lower in the A2780 cell line and two orders of magnitude lower in the cisplatin-resistant A2780cisR cell line when compared to cisplatin.⁴⁴⁸ Porphyrin-based octanuclear arene ruthenium cubes show interesting photodynamic activity,^{449,450} as well as many arene ruthenium-based rectangles, and demonstrate higher cytotoxicity than cisplatin.^{424,451,452}

Ruthenium-Based Dendrimers

Dendrimers are branched polymers with a very low polydispersity. Their synthesis is well established.⁴⁵³ The divergent and the convergent methods are the two mainly used synthesis routes. The former method starts with an initiator core (generation 0) containing functionalized end groups. The building blocks are then added stepwise to generate dendrimers of the respective generation. A drawback of this synthesis is that many reactions have to occur in parallel; hence, the reaction may remain incomplete or can be accompanied by side reactions, which ultimately makes purification difficult, especially for higher-generation

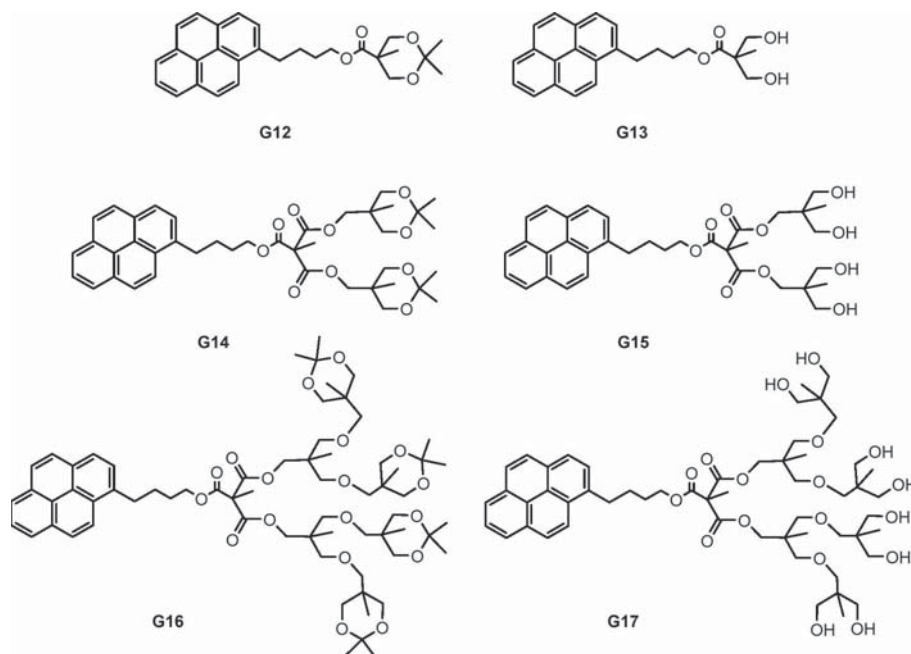
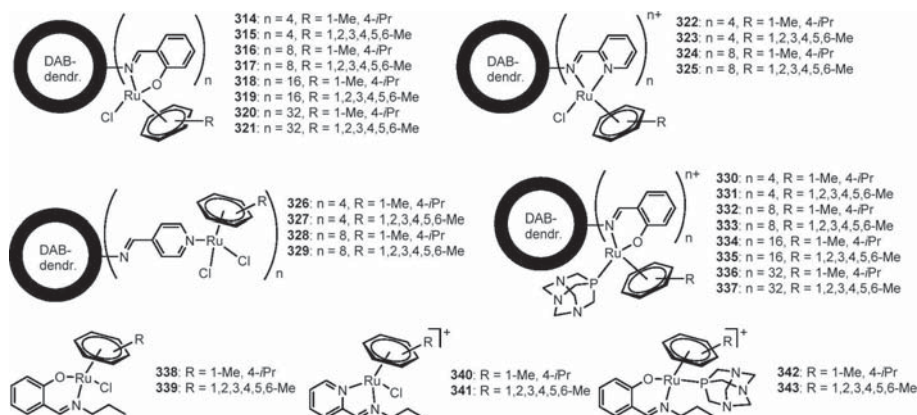


Figure 11 Pyrene-derived dendrimeric guest molecules.

dendrimers.⁴⁵⁴ The convergent method, in contrast, starts at the surface of the future dendrimer. Only two simultaneous reactions are required to gain a next-generation dendrimer. The dendrimer core is added in the last step of the synthesis. This method produces extremely uniform dendrimers.⁴⁵⁵ Figure 12 illustrates the fast enlargement of molecular size from lower to higher generations using the example of diaminobutane poly(propyleneimine) (DAB) dendrimers.

Their amphiphilic character and the ease of functionalization render dendrimers interesting for drug delivery. Some dendrimer-based drugs are already used in the clinic.⁴⁵⁶ Functionalization with coordinating moieties at the dendrimer surface leads to multinuclear complexes. Such systems often exceed the bioactivity of their mononuclear parent complexes. A heptanuclear dendrimeric copper(II) complex, for example, exhibited impressive *in vitro* anticancer activity.⁴⁵⁷ A series of arene ruthenium(II)-based dendrimers (314–337) show a clear correlation between particle size and cytotoxicity in the A2780 and the A2780cisR (cisplatin-resistant) cancer cell lines (see Table 3).^{458–460} The most cytotoxic compound in this series (336) is more active than cisplatin. The dendrimeric systems are more potent than their mononuclear analogs 338–343, also when the IC₅₀ values are calculated per metal center. The cytotoxicity in the cisplatin-resistant and cisplatin-sensitive cell line is essentially the same, indicating that these compounds act *via* a different mechanism than cisplatin.



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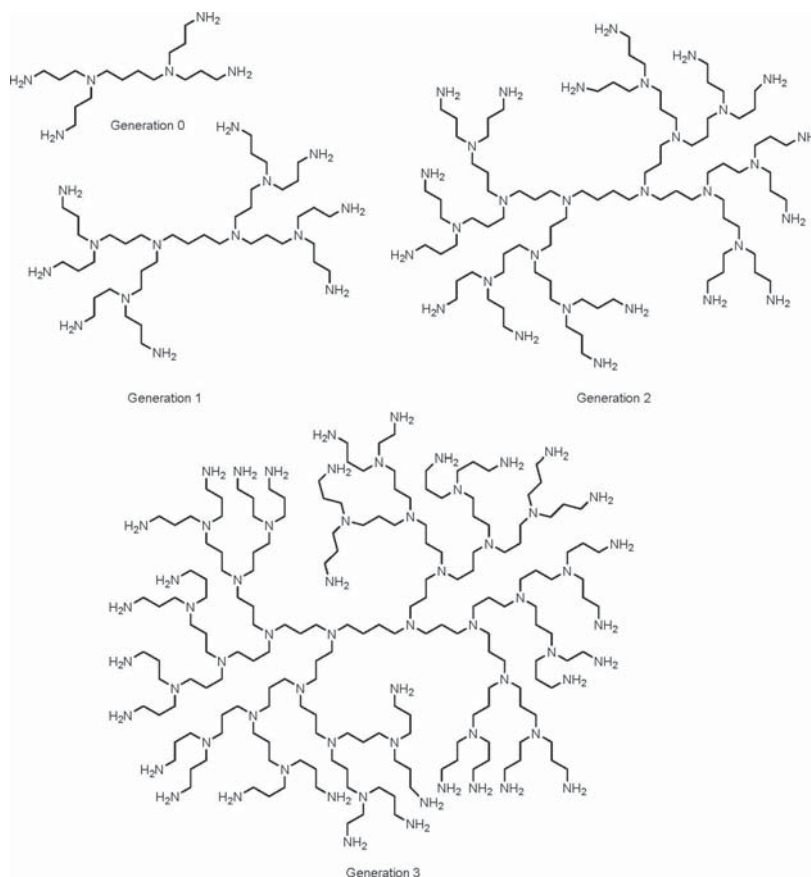


Figure 12 DAB dendrimers from generation 0 to 3.

The hexamethylbenzene-containing complexes generally exhibit a superior activity in comparison with their *p*-cymene counterparts. This is most probably due to the higher lipophilicity of the hexamethylbenzene ligand. However, this feature impairs moving from lower-generation dendrimers containing 4 and 8 metal centers to higher-generation dendrimers containing 16 and 32 metal centers. The cationic PTA-containing complexes (330–337) are more cytotoxic compared with the uncharged complexes 314–321. This is most probably due to a stronger DNA interaction of the cationic complexes with the negatively charged DNA backbone. Incubation with plasmid DNA showed a stronger DNA interaction for 331–338 than for 314–322. The finding that the cytotoxicity of dendrimeric ruthenium complexes increases (even when calculated per metal center) taken together with the fact that molecules of large size accumulate within the tumor tissue (EPR effect) makes this compound class attractive for further development.^{458–460}

Two zero-generation nitrile-functionalized DAB-derived dendrimers containing $[\text{Ru}(\eta^5\text{-C}_5\text{H}_5)(\text{PPh}_3)_2]^+$ or $[\text{RuCl}(\text{dppe})_2]^+$ (dppe = 1,2-bis(diphenylphosphino)ethane) moieties were synthesized. It was shown that the dppe-containing complex is stable in solution, while the cyclopentadienyl derivative decomposes in DMSO at 37 °C.⁴⁶¹

Conclusion

A large number of papers published in the last few years and surveyed in this review demonstrate that the search for more effective ruthenium complexes and organoruthenium compounds as chemotherapeutic agents with fewer side effects than currently applied platinum compounds continues to attract attention.

Table 3 Cytotoxicity of ruthenium(II)-arene-based dendrimers

Compound	n^i	A2780 (IC_{50} (μM))	A2780 (IC_{50} per metal center (μM))	A2780cisR (IC_{50} (μM))	A2780cisR (IC_{50} per metal center (μM))
314	4	50 ± 1.4	200	52 ± 0.8	208
315	4	27 ± 1.3	108	25 ± 1.3	100
316	8	22 ± 1.2	176	15 ± 1.4	120
317	8	10 ± 0.3	80	9 ± 0.3	72
318	16	6.0 ± 1.0	96	13.2 ± 1.4	211
319	16	2.1 ± 0.1	33.6	2.1 ± 0.1	33.6
320	32	2.9 ± 0.1	92.8	9.9 ± 1.2	316
321	32	1.6 ± 0.1	51.2	2.1 ± 0.1	67.2
322	4	>200		>200	
323	4	32 ± 1.6	128	42 ± 5.1	168
324	8	23 ± 2.1	184	76 ± 9.4	608
325	8	4 ± 1.3	32	4 ± 0.3	32
326	4	43 ± 5.0	172	n.d.	
327	4	40 ± 5.0	160	n.d.	
328	8	21 ± 5.0	168	n.d.	
329	8	20 ± 5.0	160	n.d.	
330	4	174.0 ± 40	696	72.8 ± 1.6	291.2
331	4	8.9 ± 2.8	35.6	25.0 ± 5.0	100.0
332	8	9.3 ± 0.4	74.4	19.3 ± 0.2	154.4
333	8	6.2 ± 0.3	49.6	11.8 ± 1.1	94.4
334	16	1.4 ± 0.4	22.4	3.6 ± 0.2	57.6
335	16	2.9 ± 0.1	46.4	2.0 ± 0.1	32
336	32	0.8 ± 0.1	25.6	2.7 ± 0.1	86.4
337	32	2.0 ± 0.1	64.0	1.1 ± 0.1	35.2
338	1	49 ± 2.3	49	47 ± 0.8	47
339	1	19 ± 1.8	19	18 ± 0.8	18
340	1	>200		>200	
341	1	>200		>200	
342	1	>200		82.0 ± 6.0	82.0
343	1	38.0 ± 3.4	38.0	93.0 ± 7.0	93
Cisplatin	1	1.5		25	

 n^i = number of metal centers per molecule.

Reported works are mainly directed to the synthesis of new compounds and to testing for antiproliferative activity in a panel of different cancer cell lines, accompanied in less frequent cases by comparison with antiproliferative activity in nontumorigenic cells or screening against a particular biological target, and rarely to *in vivo* assays, or establishment of structure–activity relationships. In addition, much effort is focused on the elucidation of the mechanism of action of known potential ruthenium drugs, which are in clinical or preclinical phases of development such as NAMI-A-, KP1019-, RAPTA-C-, and RM175-type complexes. In some cases, attempts to perform the synthesis of new complexes with biologically active organic compounds with known biological targets, as ligands, have been documented. The use of biologically active ligands in combination with ruthenium is an option to achieve synergistic effects.

Advances in preparative coordination and organometallic chemistry, as well as in peptide-coupling methodologies, will continue to play an important role in the further development of metal-based chemotherapy. As noted by others,²⁴ it is difficult to overestimate the role of the μ -chlorido-bridge splitting reaction of the dimeric precursor $[Ru(arene)(\mu-Cl)Cl]_2$, which can now be prepared more efficiently by microwave heating,⁴⁶² with a huge number of organic ligands, ultimately leading to a great variety of organoruthenium complexes with antiproliferative activity. By combining designed dinuclear metalla-clips, resulting from reaction of $[Ru(arene)(\mu-Cl)Cl]_2$ with certain dinucleating ligands, and bi-, tri-, or tetradentate building blocks, in the presence of $AgCF_3SO_3$ as chloride abstractor, a large number of tetracationic rectangles, hexacationic prisms, and octacationic cubes have been assembled in high yields. These polynuclear metalla-cages with interesting cytotoxicity profiles are able to accommodate guest molecules within their central cavity and serve as carriers for delivery of other cytotoxic drugs.

Compounds that mimic lead drugs, with broadly varied donor atoms and coordination environments with ligands of different denticity and chiral and achiral, biologically active or inactive, redox-active or silent, polynuclear classical ruthenium clusters and organoruthenium complexes, which show antiproliferative activity against human cancer cell lines, have been reported. In the case of polypyridyl ruthenium complexes, known as classical intercalators, new mechanisms of action are exploited. Some newly developed mitochondria-targeting complexes showed excellent cytotoxicity and pronounced selectivity for cancer cells;²⁵³ one *in vivo* animal trial showed a better tumor growth inhibition than cisplatin, a very promising result.³⁰⁸ Recently designed photoactive ruthenium complexes are by two or even three orders of magnitude more potent cytotoxins when irradiated with

light than in the dark.^{265,269} Such a distinct difference was never observed before, and the limits in this field (lower wavelength activation light and higher photodynamic indices) are still far from being reached. However, *in vivo* studies are still lacking in most of the cases and will hopefully follow soon, as this class of compounds has a strong potential for the development of new more specific and safer anticancer therapies.

The mechanism of action of ruthenium drugs is still poorly understood. Traditionally, DNA was considered the pharmacological target for ruthenium-based anticancer compounds. However, little is known about ruthenium–DNA interactions *in vivo*.⁵³ Quite recently, evidence has been provided that at least for some ruthenium compounds, proteins are possibly involved in the underlying mechanisms of their anticancer activities.^{463,464,98} For the elucidation of mode of action of metal-based drugs, it is extremely important to study the behavior of the metallodrugs in complex biological media and tissues both *in vitro* and *in vivo*. The role of traditional physical and analytical metal speciation methods, as well as of new sophisticated biophysical and bioanalytical methods, proteomics and genomics, in the investigation of underlying mechanisms of action of developing anticancer drugs increases. Exploitation of EPR spectroscopy^{72,73}; X-ray crystallography⁹³; X-ray absorption and synchrotron-based micro-XRF spectroscopy^{69,71}; fluorescence microscopy⁴⁶⁵; and mass spectrometry-based methods, for example, 2-D gel electrophoresis (2-D PAGE) combined with electrospray ionization quadrupole and time-of-flight tandem mass spectrometry (ESI-Q-TOF-MS/MS),⁴⁶⁶ surface-enhanced laser desorption/ionization-time-of-flight mass spectrometry (SELDI-TOF-MS),⁴⁶⁷ ICP-MS, off-line and coupled to separation systems, for example, size exclusion chromatography (SEC) or capillary electrophoresis,^{468,98} advanced ion mobility mass spectrometry (IM-MS), which can be used in combination with liquid chromatographic (LC) separations,⁴⁶⁹ and other techniques,⁴⁷⁰ with some of them applied for the first time to solve biological problems, has been reported. The response of human cancer cells to drug treatment on the protein level *in vitro* is being investigated by MudPIT.⁹⁷ Further extension of advanced proteomic techniques to *in vivo* samples is expected based on protein–metal drug adducts identified by *in vitro* investigations. Whether these adducts are indeed involved in the mode of action of a drug should be confirmed by additional investigations, performed only in particular cases so far.

The mechanisms of action are different, even for closely related ruthenium complexes, such as NAMI-A and KP1019, RAPTA-C, and RM-175. Of note are the insights gained into the molecular targeting and mechanism of action of two related drugs RAPTA-C and RM-175, described quite recently.^{90,471} By exploring bioanalytical, X-ray crystallographic, and computational methods, it has been demonstrated that cancer cells or nucleosome core particles treated with both RAPTA-C, the relatively noncytotoxic antimetastasis species and the cytotoxic against primary tumors compound RM-175, accumulate these drugs on chromatin consisting of DNA and proteins that make up the contents of the nucleus of the cell. However, while RAPTA-C forms mainly adducts with the histone proteins, RM-175 targets, preferentially, the DNA component. The site preferences are different, and they can be responsible at least in part for their different anticancer activity profiles. The binding site preference and anticancer activity profiles are presumably controlled by the ligands PTA (monodentate, sterically demanding) in RAPTA-C and ethylenediamine (bidentate ligand) in RM-175. The role of monofunctional versus bifunctional binding ability for RAPTA-C and RM-175, respectively, on their distinct anticancer activity remains to be elucidated. Other therapeutic protein sites are also possible, since 96% of intracellular RAPTA-C does not reach the cell nucleus.

As potential targets for ruthenium-based antiproliferative agents, protein kinases,⁴⁷² and, in particular, thioredoxin reductase, cathepsin B and PARP,^{136,92,473} mitochondrial proteins,^{474,255} and hormonal receptors⁴⁷⁵ are being considered, and recent advances in identifying new targets for metal-based drugs have been discussed.²³ By further exploring the preference of ruthenium for the octahedral or pseudo-octahedral coordination environment and the slow kinetics of ligand substitution reactions at this metal center, along with the careful design of the main organic chelating ligand,¹ highly selective glycogen synthase kinase 3 α (GSK3 α), p21-activated kinase (PAK1), proto-oncogene (Pim1), death-associated protein kinase 1 (DAPK1), and myosin light-chain kinase (MLCK) inhibitors have been developed. These were mainly based on a staurosporine-inspired ruthenium pyridocarbazole backbone.⁴⁷² In addition, nanomolar or subnanomolar inhibitors of PAK1 (MYLK or MLCK) and human repair enzyme 7,8-dihydro-8-oxoguanosine triphosphatase (8-oxo-dGTPase, NUDT1/MTH1) derived from pyridylphthalimide, pyridylphthalimide, and 8-(pyridin-2-yl)adenine ruthenium scaffolds have quite recently been reported.^{476–478} The globular shape required to fill protein pockets has been tailored by occupying the remaining four coordination sites at ruthenium with other ligands of varied denticity, which in addition form important contacts with the other sides of the protein active site. This seminal work will, of course, stimulate the discovery of organometallic inhibitors of other important proteins and their therapeutic application in the fight against cancer.

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EUROPEAN CURRICULUM VITAE



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WORK EXPERIENCE

June 2014

Assistant in the Inorganic Chemistry Practical Course, University of Vienna

May 2014

Assistant in the Basic Chemistry Practical Course, University of Vienna

Since Oktober 2013

Doctorand at the University of Vienna (Institute of Inorganic Chemistry)

April – September 2013

Project at the IST (Instituto Superior Técnico) on the topic inorganic catalysis, Lisbon, Portugal

October 2012

Tutor in the Inorganic Chemistry Practical Course, University of Vienna

July – August 2011

Internship in the residue laboratory of the Agriculture and Forestry Centre Laimburg, Auer(BZ), Italy

October/November 2010

Tutor in the Biochemical Practical Course, University of Vienna

March 2010

Tutor in the Biochemical Practical Course, University of Vienna

June/July 2005

Gavin Jones Landscaping, London

ENGLAND

Landscaping

Practical work

SCHOOL- AND PROFESSIONAL EDUCATION

April – July 2004

Baumschule Roth, tree nursery, Kesswil, Switzerland
Practical work

June/July 2003

Gärtnerei Auer, garden centre, Brixen/Bressanone, Italy (BZ)
Practical work

Since October 2013

PhD Studies in Chemistry at the University of Vienna

October 2011-March 2012

Erasmus Exchange semester at the University for
Industrial Chemistry Bologna, Italy

October 2010 – March 2013

Master Studies in Chemistry at the University of Vienna

October 2006 – June 2010

Bachelor Studies in chemistry at the University of Vienna

September 2010 – June 2006

HBLFA für Gartenbau, (Upper school for horticulture) Vienna,
taken school leaving examination

PERSONAL SKILLS

FIRST LANGUAGE

GERMAN

OTHER LANGUAGES

English

Very Good

Very Good

Very Good

Italian

Good

Good

Good

SOCIAL SKILLS

good ability to work in a team

TECHNICAL SKILLS

Working on the PC (Excel),
advanced knowledge in the laboratory

OTHER INTERESTS

nature, music, hiking
travelling, sport climbing, cooking

DRIVER LICENCE

B

PUBLICATIONS

Effects of Terminal Dimethylation and Metal Coordination of Proline- 2-formylpyridine Thiosemicarbazone Hybrids on Lipophilicity, Antiproliferative Activity, and hR2 RNR Inhibition
Bacher, F.; Dömötör, O.; Kaltenbrunner, M.; Mojović, M.; Popović-Bijelić, A.; Gräslund, A.; Ozarovski, A.; Filipović, L.; Radulović, S.; Enyedy, É. A.; Arion, V. B.
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