Dissertation

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

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Wien, 2015

Studienkennzahl lt. Studienblatt: A 796 605 419
Studienrichtung lt. Studienblatt: Chemie
Betreuer: ao. Univ.-Prof. Dr. Vladimir Arion
PhD-Thesis

Title
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intended academic degree
PhD in chemistry (PhD)

Vienna, 2015

Subject: Chemistry
Supervisor: ao. Univ.-Prof. Dr. Vladimir Arion
I want to thank the following persons for their support during my PhD-thesis:

O. Univ.-Prof. Dr. Dr. Bernhard K. Keppler, for the opportunity to work in his experienced and skilled group.

Ao. Univ.-Prof. Dr. Vladimir B. Arion for his excellent supervision.


Dr. Éva A. Enyedy for the fruitful collaboration.

Mag. Johannes Theiner and all members of the microanalytical Laboratory for performing the elemental analyses.

Mag. Elfriede Limberger for her help regarding administrative concerns.

All my colleagues from the “AG Keppler” for the great working atmosphere.
Acknowledgement
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 IIα (Topo IIα). 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


Die krebsinhinierende 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 IIα (Topo IIα). 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.

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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.\(^3\)

![Death causes in Austria 2013](image)

Figure 1: Death causes in Austria in 2013.
Introduction

The risk to die because of cancer strongly increases with the age (see Figure 2).¹

![Figure 2: Age related cancer death rate in Austria in 2013.](image)

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.² 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 exogenous or endogenetic
factors. Endogenetic factors are usually inherited and responsible only for a small fraction of cancers (e.g. some breast cancers). 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 $\gamma$- 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. 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 promotors, such 12-O-tetradecanoylphorbol 13-acetate (TPA) and alcohol accelerate this process. 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. 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 narrow, 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 ATC\(^{1}\) 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 (metalloids drugs belong to this last class).\(^{14}\)

\(^{1}\) 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.\textsuperscript{15} The first worldwide approved platinum based anticancer drug is cisplatin (cis-diamminedichloroplatinum(II), 1), which was first synthesized by Michelle Peyrone in 1844.\textsuperscript{16} However, its anticancer potential was coincidentally discovered only more than 100 years later by Barnett Rosenberg in the 1960s.\textsuperscript{17} Rosenberg studied the effect of electric fields on dividing cells. He dipped platinum electrodes in an ammonium chloride containing medium containing \textit{Escherichia coli} bacteria. Surprisingly the bacteria did not divide, but they grew to filaments up to 300 times as long as their normal size.\textsuperscript{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.\textsuperscript{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.\textsuperscript{19-21}

\begin{center}
\begin{tabular}{c}
\textbf{1} & \textbf{2} & \textbf{3} \\
\end{tabular}
\end{center}

\textbf{Chart 1:} Worldwide approved platinum drugs: 1, cisplatin, \textit{cis}-diamminedichloridoplatinum(II); 2, carboplatin, \textit{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.\textsuperscript{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

\textsuperscript{ii} FDA stands for US Food and Drug Administration.
alleviate these disadvantages second and third generation platinum based anticancer drugs were developed.\textsuperscript{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 myelosupression 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.\textsuperscript{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.\textsuperscript{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).

\begin{center}
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\end{center}

\textbf{Chart 2:} Regionally approved platinum drugs: 4, lobaplatin; 5, nedaplatin; 6, hepaplatin.

Thousands of other platinum-based anticancer drugs,\textsuperscript{iii} matching the structure-activity relationships (SARs) of cisplatin which is mononuclear and uncharged, with square-planar cis coordination geometry and oxidation state $^\text{+II}$, 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.\textsuperscript{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.\textsuperscript{30} However, satraplatin failed in clinical phase three due to the lack of activity in comparison with standard therapy. However, studies are going on.\textsuperscript{31–34}

\footnotesize\textsuperscript{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.\textsuperscript{35,31}

\begin{center}
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\end{center}

\textbf{Chart 3}: Two examples for non-classical platinum drugs: 7, satraplatin; 8, BBR3464.

\section{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).\textsuperscript{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).\textsuperscript{37}

(iii) Ruthenium stands in the 8\textsuperscript{th} 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.\textsuperscript{38,39}

Until now, three ruthenium complexes have entered clinical trials, namely NAMI-A, 9, KP1019, 10, and its sodium salt analogue KP1339, 11.
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 thiol 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. 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. The $\alpha_2$ 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. The $\beta_2$ subunit (also named R2) is significantly smaller than R1 and houses the metal cofactor which is necessary to form a thyrosyl radical ($Y^\ddagger$). 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 $\alpha_2$ subunit to generate a transient thiyl radical there. Class Ia RNR requires two iron atoms, class Ib contains two manganese atoms and class Ic contains one iron and one manganese atom.

Class II RNRs are oxygen independent. They are composed only of mono- or dimeric $\alpha$ subunits, which contain a cobalt containing adenosylcobalamin cofactor (coenzyme B12) which is located near the enzymes active center. 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.

Class III RNRs work only under anaerobic conditions. They are consisting of $\alpha_2$ and $\beta_2$ subunits. The latter bear the metal cofactor which is a 4Fe-4S cluster, needed to form an oxygen sensitive glycyl radical ($G^\ddagger$). The reductant needed for substrate turnover is formate, which is oxidized to $\text{CO}_2$, or thioredoxin in special cases, in contrast to class I and II RNRs, which use exclusively thiol dependent reductants that get oxidized upon the formation of disulfide bridges.

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.
Introduction

The biosynthesis of $Y_{122}^*$ in $\beta_2$ starts with iron(III) loading of the apo-$\beta_2$ protein. Two cytosolic monothiol glutaredoxins, bearing a Fe$_2$S$_2$ cluster, namely Grx3 and Grx4 are needed to successfully deliver iron(II) to the coordinating side chains in $\beta_2$. 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). Hydroxyurea, Triapine and other small molecules, which will be discussed in chapter 1.4.2, are able to selectively reduce the active $\beta_2$ to a stable diferric state without radical, named met-$\beta_2$. The enzyme is not able to perform catalysis in this state. 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). In vivo the issue is more complex and not understood in every detail. However, it seems likely that the ferrodoxin YfaE, containing an Fe$_2$S$_2$ cluster, together with the ferrodoxin reductase Fre is involved and largely responsible for this process. 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 turnover and allows the radical in R2 to be transported via proton-coupled
long range electron transfer involving redox active thyrosins, to generate a thyl radical in the active center 35 Å apart from the iron cluster in β2.\textsuperscript{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 thyl radical formed in R1 (C\textsubscript{439}•) is able to abstract a hydrogen from the 3’ position of the ribose ring from bound ribonucleotides and thereby generate the substrate radical B.\textsuperscript{72,73} In step 2 the 3’ hydroxyl group gets deprotonated by glutamate\textsubscript{441} and the one at the 2’ position gets protonated by cysteine\textsubscript{462}. 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 asparagine\textsubscript{437} 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.\textsuperscript{74} Computational methods using a large model, comprising the whole R1 unit of the RNR enzyme, revealed that N\textsubscript{437} 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 C\textsubscript{225} by hydrogen bond interaction with the former. Only this pinpoint orientation allows the elimination of water, otherwise the radical would return to C\textsubscript{439} and from there reform the thyrosyl radical in R2.\textsuperscript{75} In the third step the substrate radical C abstracts a hydrogen from cysteine\textsubscript{462}. 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 E_{441} mutated RNR.\textsuperscript{76} In the next step the disulfide radical anion gets oxidized and the radical migrates back via N_{437} and E_{441} to the 3'-position of the ribose ring. E_{441} acts as acid in this step by protonating the 3' oxygen.\textsuperscript{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 C_{439} completing the substrate reduction. The resulting thyl radical is then transferred back to the enzymes R2 unit reforming the thyrosyl radical there. For a complete turnover the disulfide bridge between C_{225} and C_{462} has to be reduced, this is performed via an intramolecular mechanism that involves the oxidation of two cysteins on the enzymes C-terminus.\textsuperscript{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.\textsuperscript{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\textsuperscript{-}. If C_{439}\textsuperscript{-} 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.\textsuperscript{46}

### 1.4.2 RNR inhibitors

Human RNR is especially active during S phase, when 3.2 billion base pairs need to be synthesized.\textsuperscript{80} In the late M phase the R2 protein gets degraded\textsuperscript{81} while the R1 subunit is present during the whole cell cycle.\textsuperscript{82} During G_0/G_1 phase the R2 subunit is represented by the p53-inducible p53R2 protein,\textsuperscript{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.\textsuperscript{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. 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.

(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 and gemcitabine, 13.

![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.](image)

The purine analog clofarabine is currently used in the treatment of pediatric leukemias. It is an allosteric RNR inhibitor, which changes the quaternary structure of the R1 protein. It resembles dATP (deoxyadenosine 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. 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.

Gemcitabine is a pyrimidine analog and a standard chemotherapeutic used against a variety of malignancies such as breast, pancreatic, lung and ovarian cancer. Gemcitabine gets phosphorylated within the organism, it’s 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 C_{439}^* a C3’ radical is formed which is transferred to the 2’ position of the ribose ring once one fluorine got protonated by C_{225} 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 C_{439} thiole hydrogen with the second fluorine, which also is liberated as HF and a C_{439} thiolate gets formed. This prevents radical formation at C_{439}, instead a 2’ substrate radical is formed. In the normal catalysis the C_{439} hydrogen would be donated to the 3’ radical of the ribose ring under reformation of the initial thyl radical (step 5 in Figure 4). A later model proposed covalent bond formation between substrate and protein under reductive conditions as a consequence off the above described mechanism. 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. It also has been shown that gemcitabine affects the quaternary structure of the RNR enzyme by the formation of a tight α_6β_6 complex, explaining complete RNR inhibition even at substoichiometric amounts. 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. 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. 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.

(iii) Inhibitors of the RNRs small subunit are either radical scavengers destroying its thyrosyl 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.
Hydroxyurea\textsuperscript{106} 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.\textsuperscript{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.\textsuperscript{107} Another proposed mechanism implies the formation of nitroxyl radicals after \textit{in vivo} metabolism of HU, which act as scavengers for the free thyrosyl radical in R2.\textsuperscript{108} The use of hydroxyurea against cancer is controversial because of its low \textit{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.\textsuperscript{106} Also a possible HU induced transformation of myeloproliferative neoplasms into leukemia is under debate.\textsuperscript{109} Hydroxyurea is also applied in sickle-cell anemia\textsuperscript{110} and AIDS.\textsuperscript{111}

Deferoxamine (DFO) is a potent iron chelator, which is standardly used in the treatment of iron overload disease.\textsuperscript{112} However, it also shows potential against cancer.\textsuperscript{113,114} DFO is not acting as radical scavenger, rather it depletes intracellular iron pools and prevents thereby iron loading of the apo-\(\beta_2\) enzyme.\textsuperscript{115,116}

Triapine will be discussed in chapter 1.5.

### 1.5 Thiosemicarbazones

Thiosemicarbazones (TSCs) are metal chelators with high affinity towards first row transition metals, e.g. copper(II), iron(II), iron(III), zinc(II), nickel(II).\textsuperscript{117,118} TSCs and their metal complexes bear an outstanding broad range of biological activities such as
antibacterial, antifungal, antimalarial, antiviral and anticancer.\textsuperscript{119–124} \(\alpha\)-Pyridyl thiosemicarbazones (HCTs) are especially active against cancer. The anticancer activity of HCTs was discovered in the 1950s, when 2-formylpyridine TSC, \textsuperscript{17} was able to significantly increase the life-span of leukemia bearing mice.\textsuperscript{125} The best studied HCT is Triapine, \textsuperscript{16,126,127} which has already been evaluated in several clinical phase I and II studies.\textsuperscript{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.\textsuperscript{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 thyrosyl radical in the R2 subunit of the RNR enzyme (see chapter 1.4).\textsuperscript{138,139} It has been shown that the Fe\textsuperscript{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.\textsuperscript{140,141} However, recently it was shown that the thyrosyl radical can be quenched without involvement of ROS, suggesting that the radical might be reduced directly by the iron(II)bis(triapine) complex.\textsuperscript{142}

Besides RNR, TSCs are also inhibitors of the enzyme Topoisomerase II\(\alpha\) (Topo II\(\alpha\)), 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 results in over-winding at another place. Topo II\(\alpha\) solves this issue by introducing transient double strand brakes within DNA double strands.\textsuperscript{143,144} The enzyme ATP binding site has been suggested as the possible target for TSCs.\textsuperscript{145} It was shown that square-planar copper(II)-TSC complexes are often much more potent Topo II\(\alpha\) inhibitors compared to the free ligands.\textsuperscript{146} Nuclear labeling experiments have shown that copper(II) complexes are Topo II\(\alpha\) inhibitors \textit{in vivo}.\textsuperscript{147}

\textbf{1.5.1 Structure activity relationships}
A N,N,S donor set is crucial for the anticancer activity of formylpyridine based thiosemicarbazones.\textsuperscript{148} This has been shown already more than 50 years ago when 2-formylpyridine TSC, \textsuperscript{17} was found to be active in a mice model while its isomers 3- and 4-formylpyridine (\textsuperscript{18} and \textsuperscript{19}) were lacking activity.\textsuperscript{125}
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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsubscript{α} inhibiting effect of certain TSCs as mentioned before.\textsuperscript{146} However, reaction with copper(II) has also an effect on TSCs that do not target Topo II\textsubscript{α}. For example it has been shown that the presence of copper(II) ions diminishes the \textit{in vitro} anticancer activity of Triapine,\textsuperscript{16} while the activity of its dimethylated derivative Dp44mT,\textsuperscript{20}\textsuperscript{152} increased under the same conditions. 2-Formylpyridine,\textsuperscript{17} and its dimethylated counterpart,\textsuperscript{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.\textsuperscript{153}
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, although this reaction has not been observed in a later study with Triapine, which was performed in a DMSO/water mixture. Dp44mT is about two orders of magnitude more cytotoxic than Triapine (IC\textsubscript{50} = 0.0027 vs. 0.29 μM, respectively in a HL60 leukemia cell line), while its monomethylated analogue Dp4mT, shows a similar activity to Triapine (≈ 0.3 μM). There exist contradictory reports about Dp44mT being a Topo IIα inhibitor. 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 \textit{in vivo}, especially when taking into account the high stability constant of the copper(II) complex, together with its high cytotoxicity.

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 IIα 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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Results
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

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


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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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}) and 3-methyl-((R)-pyrrolidine-2-carboxylate-2-formylpyridine thiosemicarbazone ([R-Pro-FTSC]), as well as 3-methyl-((S)-pyrrolidine-2-carboxylate-2-formylpyridine 4,4-dimethyl-thiosemicarbazone (dm-L-Pro-FTSC or ([L-Pro-FTSC-H2L]H2)), namely, [Ni([L-Pro-FTSC–2H])], [Ni([R-Pro-FTSC–2H])], [Ni(dm-L-Pro-FTSC–2H)], [Cu(dm-L-Pro-FTSC–2H)], [Cu(dm-R-Pro-FTSC–2H)], [Zn([L-Pro-FTSC–2H])], and [Zn([R-Pro-FTSC–2H])], in addition to two previously reported, [Cu([L-Pro-FTSC–2H])] and [Cu([R-Pro-FTSC–2H])], 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, 5, 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 effective 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.3–9 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 mouse model.7 The best-studied HCT to date is 3-aminoypyridine 2-carboxaldehyde thiosemicarbazone (3-AP), also referred to as Triapine, which has already been examined in several clinical phase I and II trials.10–13 Triapine was found to be safe and effective against hematologic malignancies, for example, leukemia.13,14 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.15–17 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

Received: September 13, 2014
Published: November 12, 2014


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Several mechanisms of RNR inhibition by thiosemicarbazones and especially Triapine have been proposed.15,21 Until recently, the favored mechanistic scheme was that Triapine forms an iron(III) complex within the cell, which is reduced to Fe(II)-Triapine by intracellular reductants. Then the iron(II) complex reacts with oxygen, which leads to the formation of reactive oxygen species (ROS) able to quench the RNR tyrosyl radical.22−24 However, quite recently it was found that quenching of the tyrosyl radical is not oxygen-dependent, suggesting that it might be reduced directly by the Fe(II)-Triapine complex without involvement of ROS.25

A second known target for HCTs is topoisomerase IIα (Topo IIα), an enzyme that controls the DNA topology during cell division by inducing temporary double strand breaks.26−28 A series of Topo IIα inhibiting HCTs showed high affinity for the enzyme’s ATP binding pocket, thus acting as catalytic inhibitors of Topo IIα without the generation of DNA double strand breaks.29−31 Although the structure−activity relationships (SARs) for HCTs Topo IIα inhibition are far from being completely understood, it was suggested that reaction with copper(II) leading to square-planar complexes enhances the Topo IIα inhibition rate significantly.32

Nickel(II)−TSC complexes gained attention in the past few years. The reasons for this are the versatile coordination geometry preferences of this metal ion (square-planar, octahedral, tetrahedral), the formation of monomeric and dimeric complexes, and its ability to mimic to some extent platinum(II), which is of great importance in chemotherapy.32−37 The latter, however, is characterized by quite different ligand exchange rate constants.38 Some salicylaldheyde TSC-based nickel(II) complexes exhibited higher cytotoxicity than cis-diaminedichloridoplatinum(II) (cisplatin, CDDP) in human cancer cell lines, while they were relatively nontoxic in normal kidney cells, demonstrating the potential of nickel(II)-TSC complexes for clinical development.39 Zinc(II) shows low systemic cytotoxicity and easily forms TSC complexes. Moreover, the cytotoxicity of the zinc(II) complex is often enhanced compared to the free ligand.40,41 An HCT-based zinc(II) complex showed comparable cytotoxicity to 5-florouracil in human cancer cell lines,41 and a polyhydroxybenzaldehyde TSC-based zinc(II) complex inhibited topoisomerase I.42

One of the great challenges in the design of new TSCs as possible anticancer compounds is to find the optimal balance between lipophilicity and water solubility without losing efficacy. Increased bioactivity, including anticancer activity in vivo, has been reported in the literature in many cases due to the higher lipophilicity of potential drugs.43 However, high lipophilicity often leads to low aqueous solubility, which makes administration difficult and might limit the attainment of the proper concentration of the drug needed for the desired pharmacological response. In addition, low solubility precludes studies in aqueous solution, which are of utmost importance for investigations into the mode of action of these compounds.

Low aqueous solubility of TSCs is a common feature, which explains the limited number of studies in aqueous solution reported so far.44−47 From the other side aqueous solubility has an influence on a compound’s bioavailability through solubility-limited absorption but is important for validation of in vitro antiproliferative activity assays.48,49 We reported recently on the first proline-TSC hybrids (L- and D-Pro-FTSC) and their copper(II) complexes.48 These compounds are highly water-soluble. However, they exhibited only moderate-to-low cytotoxicity (IC50 ≈ 100 μM in CH1 cell line for the copper(II) complexes, >300 μM for the free ligands), when compared with other HCTs that showed IC50 values in the nanomolar range.50−52 This low cytotoxicity is presumably caused by the very low lipophilicity of these compounds, which may hinder cell membrane passage. Nevertheless, complex formation with copper(II) had a favorable effect on antiproliferative activity. We decided to extend our work and study the effect of other metal ions on cytotoxicity of L- and D-Pro-FTSC and that of dimethylation of terminal amingroup in previously reported hybrids and their metal complexes. Dimethylation at terminal nitrogen of other HCTs was reported to increase the cytotoxicity.53,54

Herein we report on the synthesis of a new chiral ligand dm-L-Pro-FTSC or (S)-H2L1, along with two optically pure enantiomers L-Pro-FTSC and D-Pro-FTSC reported previously, and on six new nickel(II), copper(II), and zinc(II) complexes (1−3 and 6−8), in addition to two previously reported copper(II) complexes (4 and 5) (Chart 1).

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-

Chart 1. L- and D-Pro-FTSC and dm-L-Pro-FTSC and Their Metal Complexes Studied in This Work

Underlined numbers indicate complexes investigated by X-ray diffraction. In CH1 cell

Underline numbers indicate complexes investigated by X-ray diffraction.

References

1. Underlined numbers indicate complexes investigated by X-ray diffraction.

2. In CH1 cell
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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 NNR inhibiting activity of new compounds was assayed and discussed.

EXPERIMENTAL SECTION

Chemicals. 2,6-Dihydroxyxypyrrolidine and t-puleine methyl-ester hydrochloride were purchased from Alfa Aesar, while t-puleine methylster hydrochloride was purchased from Acror Organic. Solvents were dried using standard procedures if needed.29 2-Hydroxymethyl-6-chloromethylpyridine and 6-chloromethyl-2-carboddehyde were synthesized according to published procedures.30 KCl and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were Sigma-Aldrich products, and HCl, KCl, NiCl₂, and ZnCl₂ 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. t- and t-Pseudo-tFSCH₂O.H₂O.2ZECH₂OH. (S)-1: [(6-Formyl-pyridin-2-yl)-methyl]pyrrolidine-2-carboxylic acid (0.3 g, 12.8 mmol) and 4,4-dimethyl-3-thiosemicarbazide (0.1 g, 0.81 mmol) in water (15 mL) was added a solution of nickel(II) acetate tetrahydrate (0.21 g, 0.86 mmol) and nickel(II) acetate (0.19 g, 0.81 mmol), 4,4-dimethyl-3-thiosemicarbazide (0.1 g, 0.81 mmol), and nickel(II) acetate (0.3 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 washed with diethyl ether (6 mL), and the reaction mixture was stirred at room temperature for 1 h. After it cooled, the solution was subjected to preparative high-performance liquid chromatography (HPLC) (MeOH/H₂O). 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: 0.13 g, 65%. Anal. Calcd for C₁₇H₂₁N₆O₇S·0.2H₂O: C, 44.69; H, 5.89; N, 15.51; S, 7.70. Found: C, 44.55; H, 5.74; N, 15.85; S, 7.98%. Solubility in water ≥ 15.3 mg/mL. ESI-MS (methanol): positive: m/z 392 ([1/2 M + H]+). IR (ATR, selected bands, νmax): 3294, 1588, 1459, 1162, 785, 668, 605 cm⁻¹.

[Ni(t-Pseudo-tFSCH_2O.H_2O)(H_2O)_2.CH_3OH]. (2.37H_2O,MeOH). To a solution of Ni(t-Pseudo-tFSCH₂O.H₂O) (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/H₂O). 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 C₁₇H₂₁N₆O₇S·0.2H₂O: C, 44.69; H, 5.89; N, 15.51; S, 7.70. Found: C, 44.55; H, 5.74; N, 15.85; S, 7.98%. Solubility in water ≥ 15.3 mg/mL. ESI-MS (methanol): positive: m/z 392 ([1/2 M + H]+). IR (ATR, selected bands, νmax): 3294, 1588, 1459, 1162, 785, 668, 605 cm⁻¹.

[Cu(t-Pseudo-tFSCH_2O.H_2O)(H_2O)_2.OH). (S)-1: [(6-Formyl-pyridin-2-yl)-methyl]pyrrolidine-2-carboxylic acid (0.3 g, 1.28 mmol), 4,4-dimethyl-3-thiosemicarbazide (0.1 g, 0.81 mmol), and nickel(II) acetate tetrahydrate (0.3 g, 1.28 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 [Ni(4,4-dimethyl-3-thiosemicarbazide)₂] was filtered off. The filtrate was subjected to preparative HPLC (MeOH/H₂O). 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 C₁₇H₂₁N₆O₇S·0.2H₂O: C, 44.69; H, 5.89; N, 15.51; S, 7.70. Found: C, 44.55; H, 5.74; N, 15.85; S, 7.98%. Solubility in water ≥ 15.3 mg/mL. ESI-MS (methanol): positive: m/z 392 ([1/2 M + H]+). IR (ATR, selected bands, νmax): 3294, 1588, 1459, 1162, 785, 668, 605 cm⁻¹.

[Cu(t-Pseudo-tFSCH_2O.H_2O)(H_2O)_2.OH). (S)-1: [(6-Formyl-pyridin-2-yl)-methyl]pyrrolidine-2-carboxylic acid (0.3 g, 1.28 mmol), 4,4-dimethyl-3-thiosemicarbazide (0.1 g, 0.81 mmol), and nickel(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 was subjected to preparative HPLC (MeOH/H₂O). 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 C₁₇H₂₁N₆O₇S·0.2H₂O: C, 44.69; H, 5.89; N, 15.51; S, 7.70. Found: C, 44.55; H, 5.74; N, 15.85; S, 7.98%. Solubility in water ≥ 15.3 mg/mL. ESI-MS (methanol): positive: m/z 795 ([1/2 M + H]+). IR (ATR, selected bands, νmax): 3452, 3152, 2923, 1596, 1503, 1361, 1247, 1131, 910, 792, 625 cm⁻¹.

[Zn(t-Pseudo-tFSCH_2O.H_2O)(H_2O)_3.CH3OH (1.9H_2O)]. To a solution of t-Pseudo-tFSCH₂O (0.12 g, 0.39 mmol) in water (15 mL) was added a solution of zinc(II) acetate tetrahydrate (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 C₁₇H₂₁N₆O₇S·0.2H₂O: C, 58.56; H, 4.68; N, 17.29; S, 7.92%. Found: C, 58.75; H, 4.29; N, 16.89; S, 8.04%. 1H NMR (500 MHz, DMSO-d₆): δ 8.22 (s, 1H, H₁₃), 0.08 (t, J = 7.7 Hz, 2H, H₁₄), 7.62 (d, J = 7.5 Hz, 1H, H₁₇), 7.57 (s, 2H, H₁₈), 7.42 (d, J = 7.6 Hz, 1H, H₁₈), 4.34 (d, J = 16.8 Hz, 1H, H₁₀), 4.08 (d, J = 16.9 Hz, 1H, H₁₁), 3.50 (m, 1H, H₁₂), 3.25–3.12 (m, 1H, H₁₃), 2.99–2.86 (m, 1H, H₁₈), 2.32 (m, 1H, H₁₉), 1.95 (m, 1H, H₁₁), 1.79 (m, 2H, H₁₂) ppm. 13C NMR (125 MHz, DMSO-d₆): δ 183.96 (C₇), 177.11
Results

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

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<th>2·4CH₃OH</th>
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<td>C$<em>{32.88}$H$</em>{49.5}$N$_{10}$Ni$_2$O$_6.88$S$_2$</td>
<td>C$<em>{15}$H$</em>{21}$CuN$_5$O$_3$SC</td>
<td>C$<em>{14}$H$</em>{19}$N$_5$O$_3$SZn</td>
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*R$_{w}$ = \[2 \Sigma ||F$_{o}$| - |F$_{c}$||/\[\Sigma |F$_{o}$|]\] 1/2, \[wR$_{p}$ = \[\Sigma (w|F$_{o}$| - |F$_{c}$|)^{2}]^{1/2}\]/\[\Sigma (w|F$_{o}$|)^{2}\]; \[GOF = \[\Sigma (w|F$_{o}$| - |F$_{c}$|)^{2}/(n - p)\]^{1/2}\], where n is the number of reflections, and p is the total number of parameters refined.
solvent resonance. L-Pro-FTSC was dissolved in a 10% (v/v) D$_2$O/ H$_2$O mixture at a concentration of 1 mM, the (L)-ligand ratio was 1:1 and, the nickel(lI)-to-ligand ratio was 1:1 at 298 K. PSEQUAD$^{60}$ was used to calculate the PKN value of the complex [ZnLH]$^+$. $^1$H NMR spectra were recorded for the zinc(II)-L-Pro-FTSC (1:1) system at 298 K. Results are equivalent, which is not strictly correct. Different orientations of the $\langle D_0 \rangle$ and $\langle D_1 \rangle$ tensors were taken into account, as the two $\langle D \rangle$ tensors are tilted by 26° from each other according to the DFT calculations (see the DFT section below), but the corresponding components of $\langle D_0 \rangle$ and $\langle D_1 \rangle$ were assumed equal. These $\langle D \rangle$ tensor components were not fitted but were calculated from the $\langle D \rangle$ and $\langle E \rangle$ 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.

$$\chi = 3 \sum \frac{\exp(-\lambda/kT)}{\sum \exp(-\lambda/kT)}$$

The derivatives $\partial \chi/\partial B$ were evaluated numerically by calculating the energy levels slightly below and slightly above ($\pm 5$ G) the operational magnetic field of a SQUID magnetometer (1000 G in our case). The terms $\langle D_0 \rangle$ and $\langle D_1 \rangle$ represent the usual zfs of the $\text{A}_2$ ground state of Ni(II)$^{36}$

We also allowed an interdimer exchange term $(\chi_x)$ in the molecular field approximation$^{46}$ according to eq 4.

$$\chi_x = 1 - \frac{\mu_b}{\mu_b^c} \chi_f$$

The X-band (9.5 GHz) EPR spectra were recorded at 20 K on a Bruker ER-8900 helium crystal spectrometer with an Oxford Instruments ESR000 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 CuSO$_4$ 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 NHMF. 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 $\pm$ 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 ‘giann spin’ approach, in which the spectra of the coupled states $|S = 1\rangle$ and $|S = 2\rangle$ were analyzed separately in terms of the coupled-spin Hamiltonian (eq 5).

$$\hat{H} = -\mu_b g_z S_z + \hat{D}(\hat{S}_z^2 - S(S + 1)/3) + E(\hat{S}_x^2 - \hat{S}_y^2)$$

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$^{46}$ corresponding to the spin Hamiltonian (1).
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zyma (AS49), 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 Roosevelt Park Memorial Institute (RPMI) 1640 nutrient medium (Sigma Chemicals Co, USA). RPMI 1640 nutrient medium was prepared in sterile distilled water, supplemented with penicillin (192 U/mL), streptomycin (200 mg/mL), 4- (2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (25 mM), i-glutamine (3 mM), and 10% of heat-inactivated fetal calf serum (FCS) (pH 7.2). The cells were grown at 350 K in 5% CO2 in a humidified air atmosphere.

MTT Assay. Antiproliferative activity of the investigated compounds was determined using 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay. Cells were seeded into 96-well cell culture plates (Thermo Scientific Nunc) at a cell density of 3000 c/w (HeLa), 6000 c/w (AS49), 4000 c/w (MDA-MB-453), and 5000 c/w (FemX, MRC-5) 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 DMSO at a concentration of 40 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 μM). Each concentration was tested in triplicate. Serial dilutions were made in culture medium. The final concentration of DMSO per well was less than 1% (v/v) in all experiments. After incubation periods of 48 h, a 20 μL 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 μL of 10% sodium dodecyl sulfate (SDS). Absorbances were recorded after 24 h, on an enzyme-linked immunosorbent assay (ELISA) reader (Thermolabor Systems Multiskan EX 200-240 V), at the wavelength of 570 nm. The IC50 values (μM) were determined from the cell survival diagrams. The percentages of 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 μM hr2 in Tris buffer, pH 7.60/100 mM KCi/5% glycerol, 20 μM compound (1% w/v) 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 μM hr2 in Tris buffer, pH 7.60/100 mM KCi/5% glycerol, was subtracted at each time point.

RESULTS AND DISCUSSION

Synthesis and Characterization of the Metal(II) Complexes. The zinc(II) and nickel(II) complexes of l- and i-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(l-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 strong peaks at m/z 364 and 386 attributed to [Ni(l-Pro-FTSC−2H]+ H]+ or [Ni(l-Pro-FTSC−2H]+ H]+ and to [Ni(l-Pro-FTSC−2H]+ Na]+ or [Ni(l-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)]+ H]+ and [(Cu(dm-l-Pro-FTSC−2H)]+ 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 i-Pro-FTSC (4, 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 P212121, while 7 crystallized in the noncentrosymmetric monoclinic space group P21. The proline-thiosemicarbazone hybrids l-Pro-FTSC, i-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 (r = 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(l-Pro-FTSC−2H)+, Ni(dm-l-Pro-FTSC−2H)+, and 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 mononuclear 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(l-Pro-FTSC−2H)+ core, in which each nickel(II) atom is surrounded by O, N, S donor atoms of a tridentate doubly deprotonated 5-nitrobenzaldehyde 4N-
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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–N3 2.2247(16), Ni1–N4 2.1924(15), Ni1–N5 2.1946(5), Ni1–N6 2.4995(5), Ni1–O1 2.0766(15), N2–N3 1.363(2), C7–S1 1.762(2), N1–N1–N2 78.71(7), N1–N1–N5 78.08(7), N5–N1–O1 81.40(6), N1–Ni1–O1 87.41(6), N2–Ni1–O1 88.37(6), Si1–Ni1–O1 98.00(4), Si1–N1–S2 86.85(18), Si1–N1–N2 92.05(18), N1–N2–N2 92.64(18), N2–N6 1.9894(17), N2–N7 2.0496(17), N2–N10 2.1107(16), N2–S2 2.4024(5), N2–S1 2.5321(6), N2–O3 2.0417(14), N7–N8 1.367(2), C20–S2 1.770(2), N6–N2–N7 78.83(7), N6–N2–N10 79.09(7), N10–N2–O3 81.55(6), N6–N2–O3 89.72(6), N7–N2–O3 89.52(6), S2–N2–O3 93.26(4), S1–N1–S2 85.90(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 1.973(3), Ni1–N2 2.027(3), Ni1–N3 2.199(4), Ni1–N1 2.389(6)(11), Ni1–S2 2.523(9)(11), Ni1–O1 2.059(3), N2–N3 1.359(5), C7–S1 1.766(4), N1–N1–N2 79.10(14), N1–N1–N5 79.17(14), N5–N1–O1 80.98(13), N1–N1–O1 89.41(33), N2–N1–O1 89.49(13), S1–N1–O1 95.82(9), S1–N1–S2 86.12(4), N1–S1–N2 93.21(4), N1–S2–N2 93.86(4), N2–N6 1.999(3), N2–N7 2.038(3), N2–N10 2.198(3), N2–S2 2.375(8), N2–S1 2.536(8), N2–O3 2.032(3), N7–N6 1.355(3), C22–S1 1.767(4), N6–N2–N7 79.08(14), N6–N2–N10 79.01(14), N10–N2–O3 81.18(12), N6–N2–O3 91.80(13), N7–N2–O3 89.03(12), S2–N2–O3 90.87(9), S1–N1–S2 86.14(4).

methylthiosemicarbazone (H2L) and two nitrogen atoms of a bipyridine coligand, characterized by X-ray diffraction, has been found in CSD database. Upon coordination of t-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, where in most cases the nitrogen atom adopts the same configuration as the asymmetric proline carbon (S_C, R_R, or R_s), 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_R, or R_s). Documented opposite configurations that resulted from coordination to metal ion or protonation of the proline nitrogen atom are rare. 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 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 carboxylato oxygen atoms O4 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 O7ii of the neighboring methanol molecules. (Atoms marked with (i) were generated via symmetry transformation to O2ai and thiolato atom Sii bonding interactions, as can be seen in the crystal structures of the involvement of complexes in intermolecular hydrogen bonding to N3b (molecule B) and O2ai (molecule A) and to FTSC) (upper limit of 2.2 on the transformation (Atoms marked with (i) were generated via symmetry transformation to (ii) were generated via symmetry transformation to N3a and O3b, respectively (Supporting Information, Figure S5). Atoms marked with (i) were generated via symmetry transformation to oxygen atom O4 of the adjacent complex. The carboxylato oxygen atoms O2a and O2b act as proton acceptors in strong 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 to x + 1, y, z, −x + 1, y + 0.5, −y + 1, z − 0.5.)

Dimethylation of the terminal amino group reduces strongly the interaction 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 carboxylato oxygen atom O2i and thiolato atom Sii forming two strong hydrogen bonds (Supporting Information, Figure S4), while the nitrogen atom N9 of the second terminal amino group is proton acceptor in strong hydrogen bonds with O8ii, O5ii, O6ii, and O7ii of the neighboring methanol molecules.

Magnetic Properties. The magnetic properties of 2 were investigated in the temperature range of 2–300 K. At 300 K the $X_{gT}^T$ product (at 0.1 T) is 2.43 cm$^3$ K mol$^{-1}$, and it increases when the temperature is lowered indicating the presence of ferromagnetic exchange interactions in the Ni$_2$ dimer (Figure 5). The room-temperature magnitude of $X_{gT}^T$ imposes the upper limit of 2.2 on the g value. The $X_{gT}^T$ product increases continuously to reach a maximum of 3.16 cm$^3$ K mol$^{-1}$ at ~20 K (Figure 5), and subsequently drops to 2.01 cm$^3$ K mol$^{-1}$ at 2 K as a result of zfs or/and intermolecular interactions.

The fitting procedure led to $J = 10.5(3)$ cm$^{-1}$, $g = 2.13(2)$, $D_{a}=7.8$ cm$^{-1}$ (fixed), $E = 2.2$ cm$^{-1}$ (fixed) $zJ_z^T = -0.29$ cm$^{-1}$ in reasonable agreement with the experimental data ($R = 6 \times 10^{-5}$). When the restrictions on $D$ and $E$ are released, much better fit is possible even without the $zJ_z^T$ term, but the resulting $D$ and $E$ are in disagreement with the EPR data.

DFT Calculations. "Broken-symmetry" (BS) density functional theory (DFT) calculations were performed using the software package ORCA 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. The B3LYP functional was employed. 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 $J = -(E_{HS} - E_{BS})/(S_{HS} - S_{BS})$. $J = 15$ 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, which favors the ferromagnetic exchange. For comparison, in an antiferromagnetic dinuclear Ni(II) complex with $J = -19$ 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.21

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
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Figure 7. EPR spectra of 2 recorded at 3 K with the microwave frequencies as indicated. The resonance marked with * was identified as the $I=2 \leftrightarrow I=1$ 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 $I=2 \leftrightarrow I=1$ transition. When the frequency is lowered below 222 GHz, that transition moves to a higher field showing that the $\text{Ni}$ levels involved are split in zero magnetic field by slightly more than $7.41 \text{ cm}^{-1}$ (corresponding to the 222.4 GHz quantum energy). The $E$ parameter of 0.52 cm$^{-1}$ could be estimated.

Figure 8. Energy levels calculated using the spin Hamiltonian (4) with $D_1=D_3=7.8 \text{ cm}^{-1}$; $E_1=E_2=2.2 \text{ cm}^{-1}$; $J=11 \text{ cm}^{-1}$, and $g_0=2.10$. 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)$^{52}$ cannot be ruled out. High-field EPR studies on more symmetric systems were considerably more successful. $^{36}$

Solution Chemistry. Lipophilicity. The hydro-lipophilic character of dm-$L$-Pro-FTSC, its Ni(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 $D_{30}$ values (L $\approx$ -1.41(9); D $\approx$ -1.56(5)) indicating rather hydrophilic character. However, they are slightly more lipophilic than the ligands alone (log $D_{30} < -1.7$). $^{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 $D_{30} > -1.7$) as in the 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 $^1$H NMR titrations in our previous work$^{49}$, and data obtained here were found to be identical with them. Although this organic compound consists of four dissociable protons (COOH, $N_{Pyridine}H^+$, $N_{ProH}+$, $N_{Pyridine}H^+$, and $N_{ProH}+$), only three $pK_a$ values can be determined in the studied pH range. $pK_a$ (1.86) most probably belongs to the deprotonation of the COOH moiety (partly overlapped with the deprotonation of $N_{Pyridine}H^+$) and $pK_a$ (7.87) and $pK_a$ (11.08) to $N_{ProH}+$ 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. $^{50}$

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
The results show the stability of metal complexes with the ligand L-Pro-FTSC. The stability constants of nickel(II), copper(II), and zinc(II) complexes are presented in Table 2. The complexes of zinc(II) and nickel(II) were more stable than those of copper(II), as indicated by their stability constants. The complexes of zinc(II) and nickel(II) were more stable than those of copper(II), as indicated by their stability constants. The complexes of zinc(II) and nickel(II) were more stable than those of copper(II), as indicated by their stability constants.

Figure 10 shows the concentration distribution curves for the metal(II) complexes at pH 7.4. The curves are superimposed with those of the free ligand. The decomposition of [ZnL] is only 8% at 1 mM concentration at pH 7.4.

To confirm the speciation obtained by pH-potentiometry, the zinc(II) system was measured by 1H NMR spectroscopic methods. Slow ligand-exchange processes were observed separately (Figure 11 and Supporting Information, Figure S8).
Peaks belonging only to the nonbound ligand are seen at pH < 4, and a new set of signals appears additionally with increasing pH, which belongs, most probably, to the protonated complex [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. pKₐ value of [ZnLH]⁺ calculated on the basis of the charge of the signals of the CH₃=N protons of the bound ligand (Table 2) is in good agreement with that obtained from the pH-metric titration curve. The peaks of species [ZnL] have constant positions at pH 7–11.5, which strongly suggests that the formation of a mixed-dentate complex [ZnL(OH)]⁺ is not probable in this pH range. The integrated areas of the corresponding CH₃=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.

In the case of the outstanding high stability [CuL] complex of i-Pro-FTSC the coordination of the COO⁻ and proline-N functionalities of the Pro moiety in addition to the (Npyr,N,S) predominates at neutral pH even at micromolar concentrations, supports a similar binding pattern in solution. Moreover, the pentadentate (N₅pyr, N, S', COO⁻, N₉pro) coordination mode of i-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)-i-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 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 μM–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 [NiL] species in solution is more probable due to the constant ε values.

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Figure S10. The peaks of [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 $^1$H 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 human serum transferrin (Tf) has also zinc(II) binding ability compared to dm-L-Pro-FTSC hybrid alone (IC50 values of 5.5 and 69.4 μM, respectively). These results, showing that dimethylation has a favorable impact on cytotoxicity, are in accordance with the previously reported studies.65,67

Ribonucleotide Reductase Inhibition Capacity. The reduction of the tyrosyl radical content in human R2 at pH 7.4, and for L-Pro-FTSC with HSA [C(L) = 1.0 mM; C(HSA) = 0.63 mM; T = 298 K and I = 0.10 M (KCl); 10% D2O] and concentration distribution curves for the same system (b). Peaks framed up correspond exclusively to the nonbound ligand.

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 (IC50(dm-L-Pro-FTSC) < IC50(n-Pro-FTSC and L-Pro-FTSC). Complex formation of dm-L-Pro-FTSC with copper(II) (complex [Cu(dm-L-Pro-FTSC)](2)) significantly increases cytotoxicity towards HeLa and MRC5 cell lines compared to dm-L-Pro-FTSC hybrid alone (IC50 values of 98.3 ± 5.5 and 69.4 ± 4.7 μM, respectively). These results, showing that dimethylation has a favorable impact on cytotoxicity, are in accordance with the previously reported studies.65,67

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Figure 14. Low-field region of the $^1$H NMR spectra recorded for the complex [ZnL] of L-Pro-FTSC with HSA and in HEPES buffer (pH 7.40, and for L-Pro-FTSC with HSA [C(L) = 1.0 mM; C(HSA) = 0.63 mM; T = 298 K and I = 0.10 M (KCl); 10% D2O]. Peaks framed up correspond exclusively to the nonbound ligand.

Figure 15. Low-field region of the $^1$H NMR spectra of HSA, complex [ZnL] of L-Pro-FTSC with HSA and in HEPES buffer (pH 7.40, and for L-Pro-FTSC with HSA [C(L) = 1.0 mM; C(HSA) = 0.63 mM; T = 298 K and I = 0.10 M (KCl); 10% D2O]. Peaks framed up correspond exclusively to the nonbound ligand.
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Table 3. Results of MTT Assay Presented as IC_{50} (μM) Values after 48 h of Incubation Time

<table>
<thead>
<tr>
<th>compound/cell line</th>
<th>Hel a</th>
<th>A549</th>
<th>MRC-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Pro-FTSC</td>
<td>&gt;300^b</td>
<td>&gt;300^b</td>
<td>&gt;300^b</td>
</tr>
<tr>
<td>dm-L-Pro-FTSC</td>
<td>224.6 ± 6.4</td>
<td>204.3 ± 4.8</td>
<td>178.4 ± 1.5</td>
</tr>
<tr>
<td>[Ni(dm-L-Pro-FTSC−2H)]_3</td>
<td>&gt;300^b</td>
<td>&gt;300^b</td>
<td>&gt;300^b</td>
</tr>
<tr>
<td>[Cu(dm-L-Pro-FTSC−2H)]_3</td>
<td>98.3 ± 5.5</td>
<td>176.8 ± 1.7</td>
<td>69.4 ± 4.7</td>
</tr>
<tr>
<td>CDDP^a</td>
<td>7.8 ± 2.3</td>
<td>17.2 ± 0.7</td>
<td>30.3 ± 3.0</td>
</tr>
</tbody>
</table>

^a IC_{50} values were calculated as mean values obtained from two to three independent experiments and quoted with their standard deviation. IC_{50} value is not reached in the examined range of concentrations. Compounds contain cocrystallized solvent (see Experimental Section).

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. 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-formylpyrine thiosemicarbazone. Triapine was shown to be a good mR2 and hR2 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 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 = 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,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,S)Ni core in solid state, and the two metal ions are chelated by the (N_pro, 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_pro, N, S, COO−,N_pro) binding mode with a protonated noncoordinating hydrazine N^+ 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...
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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 prolinate-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, 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.

ACKNOWLEDGMENTS

This work was supported by the Hungarian Research Foundation OTKA Project No. PD103905. This research was realized in the frames of TAMOP 4.2.4. A/2-11-1-2012-0001, National Excellence Program—Elaborating and operating an inland student and researcher personal support system. A.P.-B., M.M., L.F., and S.R. acknowledge the financial support from Serbian Ministry for Education, Science and Technological Development (Grant No. III14003 and No. III14026, respectively). The high-field EPR spectra were recorded at the NHMFL, which is funded by the National Science Foundation through the Cooperative Agreement No. DMR-1157490, the State of Florida, and the Department of Energy.

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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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Keywords: Proline-thiosemicarbazone hybrids, Solution equilibrium, Stability constants, Antitumor activity, hR2 RNR
Scheme S1. Synthesis of dm-L-Pro-FTSC

Reagents 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/CH$_2$Cl$_2$ 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 $^1$H 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 1.746(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 2-4CH$_3$OH showing the intermolecular hydrogen bonding interactions. Many hydrogen atoms are omitted for clarity. Atoms marked with i have been generated via symmetry transformation $x + 0.5$, $-y$, $-z + 1$, 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···O4$^i$ 2.966(2) Å, N9···H···O4$^i$ 151.7°; N9···O8$^i$ 2.877(2) Å, N9···H···O8$^i$ 155.9°; O1···O5$^{ii}$ 2.838(2) Å, O1···H···O5$^{ii}$ 174.3°; N3···O8$^{ii}$ 2.810(2) Å, N3···H···O8$^{ii}$ 153.5°; O2···O6$^{ii}$ 2.774(2) Å, O2···H···O6$^{ii}$ 158.93°; O4···O7$^{ii}$ 2.756(2) Å, O4···H···O7$^{ii}$ 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···O2¹ 2.737(2) Å, O3–H···O2¹ 172.9°; O3···S¹ 3.495(1) Å, O3–H···S¹ 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--N3b 2.911(4) Å, N4a--H--N3b 170.1°; N4b--N3a 3.076(4) Å, N4b--H--N3a 171.5°; N4b--O3b 2.988(7) Å, N4b--H--O3b 122.7°; N4a--O2a 3.015(4) Å, N4a--H--O2a 166.7°; O2a--O3a 2.750(4) Å, O2a--H--O3a 169.3°; O2b--O3b 2.806(6) Å, O2b--H--O3b 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 $^1$H 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 [$c_L = 1.0$ mM; $T = 298$ K and $I = 0.10$ M (KCl); 10% D$_2$O]. Peaks framed up correspond exclusively to the complex [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 $^1$H 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$_2$O].
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 K and I = 0.10 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: logK' [Zn-HSA] = 7.5 taken from ref. 2, logK' [Zn-Tf] = 7.8 and logK' [Zn₂-Tf] = 6.4

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taken from ref. 3; log$K'$ [Ni-HSA] = 6.8 and log$K'$ [Ni$_2$-HSA] = 4.9 taken from ref. 4; log$K'$ [Cu-HSA] = 12.0 and log$K'$ [Cu$_2$-HSA] = 8.0 taken from ref. 4.

References

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

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*Dalton Trans.*, submitted.
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Results
Strong Effect of Copper(II) Coordination on Antiproliferative Activity of Thiosemicarbazone-Piperazine and Thiosemicarbazone-Morpholine Hybrids†

Felix Bacher,† Orsolya Dömötör, Anastasia Chugunova, Nóra V. Nagy, Lana Filipović, Siniša Radulović, Eva A. Enyedy,† Vladimir B. Arion†

Abstract. In this study, 2-formylpyridine thiosemicarbazones and three different heterocyclic pharmacophores were combined to prepare thiosemicarbazone-piperazine mPip-FTSC (HL2) and mPip-dm-FTSC (HL3), thiosemicarbazone-morpholine Morph-FTSC (HL5) and Morph-dm-FTSC (HL6), thiosemicarbazone-methylpyrrole-2-carboxylate hybrids mPyr-FTSC (HL5) and mPyr-dm-FTSC (HL6) 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 (HL1–HL6), 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 HL2 and HL4 with copper(II) was studied in aqueous solution by pH-potentiometry, UV–vis spectrophotometry and EPR spectroscopy. Proton dissociation processes of HL2 and HL4 were also characterized in detail and microscopic constants for the Z/E isomers were determined. While the hybrids HL5, HL6 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 HL1–HL4, 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 HL5 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.
Results

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 IIα (Topo IIα), 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 IIα inhibiting HCTs.31 The reaction of copper(II) with HCTs leading to square-planar complexes markedly enhances the Topo IIα 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 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 (1-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,\textsuperscript{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.\textsuperscript{40-49} Biologically active metal-based compounds containing a piperazine ring have also been reported.\textsuperscript{50-54} Morpholine is another well-known water-solubilizing unit incorporated in structures of biologically active compounds, showing often favorable pharmacologic effects.\textsuperscript{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).\textsuperscript{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.\textsuperscript{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 \( \text{HL}^{1-4} \), 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 (\(^1\)H and \(^13\)C NMR, UV–vis, IR) and X-ray diffraction (1–5). Solution equilibria of the copper(II) complexes formed with \( \text{HL}^2 \) and \( \text{HL}^4 \) 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.
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Chart 1. Hybrid Ligands and Their Copper(II) Complexes Studied in This Work.

Chemical structures are not included in the formulas (see Experimental Section). This complex was crystallized and characterized by X-ray crystallography as [CuCl(mPip-FTSC–H)]0.15CH3OH.
Results

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. 2-(Chloromethyl)-6-(dimethoxymethyl)pyridine was prepared as described previously. Solvents were dried using standard procedures, if required. 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-d₆) δ 7.82 (t, J = 7.7 Hz, 1H, CH(Ao)), 7.41 (d, J = 7.7 Hz, 1H, CH(Ao)), 7.36 (d, J = 7.7 Hz, 1H, CH(Ao)), 5.26 (s, 1H, CH(OCH₂)₃), 3.60 (s, 2H, CH₂), 3.30 (s, 6H, OCH₂Cl), 2.50 – 2.35 (m, 8H, CH₂(Pepy), overlapped with residual DMSO signal), 2.24 (s, 3H, CH₃(CH₃PP)).

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-d₆) δ 7.82 (t, J = 7.7 Hz, 1H, CH(Ao)), 7.44 (d, J = 7.7 Hz, 1H, CH(Ao)), 7.36 (d, J = 7.2 Hz,
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1H, CH$_{(A)}$, 5.26 (s, 1H, CH(OCH$_3$)$_2$), 3.63 – 3.57 (m, 6H, CH$_{2}$Morpho, CH$_2$), 3.30 (s, 6H, (OCH$_3$)$_2$), 2.44 – 2.40 (m, 4H, CH$_2$(Morpho)).

**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 x 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%. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 7.76 (t, $J = 7.8$ Hz, 1H, CH$_{(A)}$), 7.35 (d, $J = 7.7$ Hz, 1H, CH$_{(A)}$), 7.31 – 7.26 (m, 1H, CH$_{3}$Pyr), 6.97 (dd, $J = 3.9, 1.8$ Hz, 1H, CH$_{(Pyr)}$), 6.60 (d, $J = 7.6$ Hz, 1H, CH$_{(A)}$), 6.24 (dd, $J = 3.9, 2.6$ Hz, 1H, CH$_{(Pyr)}$), 5.64 (s, 2H, CH$_2$), 5.24 (s, 1H, CH$_{3}$OCH$_{(3)}$), 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 x 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%. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.97 (d, $J = 0.7$ Hz, 1H, CHO), 8.03 (t, $J = 7.7$ Hz, 1H, CH$_{(A)}$), 7.83 (dd, $J = 7.6, 0.9$ Hz, 1H, CH$_{(A)}$), 7.75 (dd, $J = 7.7, 0.9$ Hz, 1H, CH$_{(A)}$), 3.70 (s, 2H, CH$_2$), 2.48 – 2.29 (m, 8H, CH$_2$(Pyr)), 2.17 (s, 3H, CH$_3$(Pyr)).

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 x 40 mL). The united organic phases were dried over magnesium
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sulfate and the solvent was removed under reduced pressure. The product was obtained as a yellow oil. Yield: 0.62 g, 82 %. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.98 (d, $J = 0.7$ Hz, 1H, CHO), 8.04 (t, $J = 7.7$ Hz, 1H, CH$_2$(α)), 7.87 – 7.81 (m, 1H, CH$_2$(β)), 7.78 (dd, $J = 7.7$, 1.1 Hz, 1H, CH$_2$(β)), 3.72 (s, 2H, CH$_2$), 3.64 – 3.58 (m, 4H, CH$_2$(Morph)), 2.47 – 2.42 (m, 4H, CH$_2$(Morph)).

Methyl 1-((6-formylpyridin-2-yl)methyl)-1H-pyrole-2-carboxylate. To a solution of methyl 1-((6-dimethoxymethyl)pyridin-2-yl)methyl)-1H-pyrole-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%. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.95 (d, $J = 0.7$ Hz, 1H, CHO), 7.99 (td, $J = 7.8$, 0.6 Hz, 1H, CH$_2$(α)), 7.82 (dd, $J = 7.7$, 0.9 Hz, 1H, CH$_2$(α)), 7.76 (dd, $J = 2.5$, 1.9 Hz, 1H, CH$_2$(pyr)), 7.02 – 6.92 (m, 2H, CH$_2$(pyr), CH$_2$(α)), 6.27 (dd, $J = 3.9$, 2.6 Hz, 1H, CH$_2$(pyr)), 5.76 (s, 2H, CH$_2$), 3.66 (s, 3H, COOCH$_3$).

mPip-FTSC·0.2CH$_3$OH (HL·0.2CH$_3$OH). A suspension of 6-((4-methylpiperazin-1-yl)methyl) piconaldehyde (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. Caled for C$_{13}$H$_{20}$NaNS·0.2CH$_3$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: $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 11.63 (s, 1H, H$^1$), 8.33 (s, 1H, H$^2$), 8.19 – 8.09 (m, 2H, H$^3$, H$^4$), 8.06 – 8.01 (m, 1H, H$^{12}$), 7.79 (t, $J = 7.8$ Hz, 1H, H$^7$), 7.39 (m, 1H, H$^8$), 3.57 (s, 2H, H$^9$), 2.35 (m, 8H, H$^6$, H$^5$, H$^{10}$, H$^{11}$), 2.15 (s, 3H, H$^{14}$). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 178.80 (Cq, C$^1$), 158.80 (Cq, C$^3$), 153.13 (Cq, C$^5$), 143.07 (CH, C$^{12}$), 137.35 (CH, C$^6$), 123.44 (CH, C$^8$), 118.97 (CH, C$^7$), 64.02 (CH$_2$, C$^9$), 55.18 (2CH$_2$, C$^4$, C$^{10}$), 53.21 (2CH$_2$, C$^5$, C$^{11}$), 46.22 (CH$_3$, C$^{14}$). Z-isomer: $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 14.27 (s, 1H, H$^1$), 8.51 (s, 1H, H$^2$), 8.19 – (m, 1H, H$^3$), 8.06 – 8.01 (m, 1H, H$^4$), 7.64 (d, $J = 7.5$ Hz, 1H, H$^7$), 7.55 (d, $J = 7.6$ Hz, 1H, H$^8$), 7.41 – 7.35 (m, 1H, H$^{12}$), 3.70 (s, 2H, H$^9$), 2.49 – 2.21 (m, 8H, H$^6$, H$^5$, H$^{10}$, H$^{11}$), 2.15 (s, 3H, H$^{14}$). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta$ 179.34 (Cq, C$^1$), 157.93 (Cq, C$^3$), 151.62 (Cq, C$^5$), 139.16 (CH, C$^6$), 133.91 (CH, C$^{12}$), 125.26 (CH, C$^8$), 124.18 (CH, C$^7$), 63.79 (CH$_2$, C$^9$), 55.18 (2CH$_2$, C$^4$, C$^{10}$), 53.08 (2CH$_2$, C$^5$, 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 $\geq$ 3.3 mg/mL. Electrospray ionization mass spectrometry (ESI-MS, methanol), positive: m/z 293 ([M + H]$^+$). IR (attenuated total reflectance (ATR), selected bands,
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\( \nu_{\text{max}} \): 3420, 3258, 3162, 2940, 2802, 1599, 1546, 1446, 1342, 1280, 1146, 983, 924, 832, 789, 732, 684 cm\(^{-1} \). UV–vis in water (52 \( \mu \)M) most intense bands \( \lambda, \text{nm} (\epsilon, \text{M}^{-1}\text{cm}^{-1}) \): 315 (30681).

**mPip-dm-FTSC-0.25CH\(_2\)OH** (HL\(^2\)-0.25CH\(_2\)OH). A suspension of 6-(4-(methyl)piperazin-1-yl)methyl)picinaldehyde (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. Caled for C\(_{15}\)H\(_{22}\)N\(_2\)S.0.25CH\(_2\)OH (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: \(^1\)H NMR (500 MHz, DMSO-\(d_6 \)) \( \delta \) 11.16 (s, 1H, H\(^2\)), 8.18 (s, 1H, H\(^3\)), 7.84 – 7.71 (m, 2H, H\(^4\), H\(^5\)), 7.39 (d, \( J = 7.3 \) Hz, 1H, H\(^6\)), 3.57 (s, 2H, H\(^7\)), 3.31 (s, 6H, H\(^8\), H\(^9\), H\(^10\)), 2.47 – 2.19 (m, 8H, H\(^1\), H\(^2\), H\(^11\)), 2.15 (s, 3H, H\(^12\)). \(^{13}\)C NMR (126 MHz, DMSO-\(d_6 \)) \( \delta \) 181.10 (Cq, C\(^13\)), 158.93 (Cq, C\(^3\)), 153.36 (Cq, C\(^5\)), 144.32 (CH, C\(^7\)), 137.55 (CH, C\(^8\)), 123.22 (CH, C\(^9\)), 118.25 (CH, C\(^10\)), 64.04 (CH\(_2\), C\(^11\)), 55.19 (2CH\(_2\), C\(^12\), C\(^13\)), 53.20 (2CH\(_2\), C\(^8\), C\(^11\)), 46.22 (CH\(_3\), C\(^16\)), 42.83 (2CH\(_3\), C\(^14\), C\(^15\)). Z-isomer: \(^1\)H NMR (500 MHz, DMSO-\(d_6 \)) \( \delta \) 14.89 (s, 1H, H\(^2\)), 8.05 (t, \( J = 7.8 \) Hz, 1H, H\(^3\)), 7.65 (d, \( J = 7.7 \) Hz, 1H, H\(^4\)), 7.59 (s, 1H, H\(^5\)), 7.53 (d, \( J = 7.7 \) Hz, 1H, H\(^6\)), 3.62 (s, 2H, H\(^7\)), 3.42 (s, 6H, H\(^8\), H\(^9\)), 2.47 – 2.19 (m, 8H, H\(^1\), H\(^2\), H\(^11\)), 2.15 (s, 3H, H\(^12\)). \(^{13}\)C NMR (126 MHz, DMSO-\(d_6 \)) \( \delta \) 180.62 (Cq, C\(^13\)), 157.65 (Cq, C\(^3\)), 151.94 (Cq, C\(^5\)), 139.25 (CH, C\(^7\)), 136.62 (CH, C\(^8\)), 124.94 (CH, C\(^9\)), 124.30 (CH, C\(^10\)), 63.66 (CH\(_2\), C\(^11\)), 55.08 (2CH\(_2\), C\(^8\), C\(^10\)), 53.25 (2CH\(_2\), C\(^8\), C\(^11\)), 46.18 (CH\(_3\), C\(^16\)), 40.59 (2CH\(_3\), C\(^14\), C\(^15\)), overlapped with residual DMSO signal). For atom numbering and structures of E and Z isomers see Supporting Information (SI), Scheme S1. Solubility in water ≥ 11.5 mg/mL. ESI-MS (methanol), positive: \( m/z \) 321 ([M + H]\(^+\)). IR (ATR, selected bands, \( \nu_{\text{max}} \)): 3039, 2929, 2801, 1597, 1542, 1446, 1361, 1156, 821, 711, 618 cm\(^{-1} \). UV–vis in water (51 \( \mu \)M), \( \lambda, \text{nm} (\epsilon, \text{M}^{-1}\text{cm}^{-1}) \): 216 (20784), 271sh (14510), 314 (31569).

**Morph-FTSC.0.3CH\(_2\)OH.0.1H\(_2\)O** (HL\(^2\)-0.3CH\(_2\)OH.0.1H\(_2\)O). A suspension of 6-(morpholinomethyl)picinaldehyde (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. Caled for C\(_{12}\)H\(_2\)N\(_2\)O\(_2\)S.0.3CH\(_2\)OH.0.1H\(_2\)O (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: \(^1\)H NMR
(500 MHz, DMSO-\(d_6\)) \(\delta\) 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). \(^1\)C NMR (126 MHz, DMSO-\(d_6\)) \(\delta\) 178.81 (Cq, C\(^{13}\)), 158.35 (Cq, C\(^3\)), 153.20 (Cq, C\(^1\)), 143.03 (CH, C\(^{15}\)), 137.37 (CH, C\(^5\)), 123.58 (CH, C\(^8\)), 119.05 (CH, C\(^6\)), 66.66 (2CH\(_2\), C\(^8\), C\(^{10}\)), 64.37 (CH\(_2\), C\(^7\)), 53.79 (2CH\(_3\), C\(^9\), C\(^{11}\)). Z-isomer: \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 14.26 (s, 1H, H\(^\delta\)), 8.52 (s, 1H, H\(^\gamma\)), 8.16 (m, 1H, H\(^\zeta\)), 8.08 – 7.99 (m, 1H, H\(^\zeta\)), 7.65 (d, \(J = 7.5\) Hz, 1H, H\(^\zeta\)), 7.58 (d, \(J = 7.8\) Hz, 1H, H\(^\delta\)), 7.39 (s, 1H, H\(^3\)), 3.71 (s, 2H, H\(^3\)), 3.66 – 3.52 (m, 4H, H\(^8\), H\(^9\)), 2.48 (m, 4H, H\(^8\), H\(^9\)). \(^1\)C NMR (126 MHz, DMSO-\(d_6\)) \(\delta\) 179.37 (Cq, C\(^{13}\)), 157.51 (Cq, C\(^3\)), 151.68 (Cq, C\(^\zeta\)), 139.19 (CH, C\(^7\)), 133.91 (CH, C\(^\zeta\)), 125.31 (CH, C\(^6\)), 124.30 (CH, C\(^4\)), 66.66 (2CH\(_2\), C\(^8\), C\(^{10}\)), 64.10 (CH\(_2\), C\(^7\)), 53.69 (2CH\(_3\), C\(^9\), C\(^{11}\)). 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 ([M + 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 \(\mu\)M), \(\lambda\) nm (\(\varepsilon\), M\(^{-1}\)cm\(^{-1}\)): 316 (26923).

Morph-dm-FTSC (HL\(^4\)). 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. Caled for C\(_{14}\)H\(_{12}\)N\(_2\)O\(_3\): C, 54.70; H, 6.87; N, 11.52. Found: C, 54.85; H, 6.92; N, 11.51. E-isomer: \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 11.16 (s, 1H, H\(^\gamma\)), 8.18 (s, 1H, H\(^\zeta\)), 7.84 – 7.74 (m, 2H, H\(^\delta\), H\(^9\)), 7.44 – 7.39 (m, 1H, H\(^\zeta\)), 3.62 – 3.53 (m, 6H, H\(^\zeta\), H\(^8\), H\(^6\)), 3.31 (s, 2H, H\(^3\)), 2.45 – 2.40 (m, 4H, H\(^8\), H\(^9\)). \(^1\)C NMR (126 MHz, DMSO-\(d_6\)) \(\delta\) 181.07 (Cq, C\(^{13}\)), 158.49 (Cq, C\(^3\)), 153.45 (Cq, C\(^1\)), 144.30 (CH, C\(^{15}\)), 137.58 (CH, C\(^8\)), 123.34 (CH, C\(^4\)), 118.32 (CH, C\(^6\)), 66.67 (2CH\(_2\), C\(^9\), C\(^{10}\)), 64.38 (CH\(_2\), C\(^7\)), 53.78 (2CH\(_2\), C\(^8\), C\(^{10}\)), 42.84 (2CH\(_2\), C\(^{14}\), C\(^{15}\)). Z-isomer: \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) 14.88 (s, 1H, H\(^\gamma\)), 8.05 (t, \(J = 7.8\) Hz, 1H, H\(^\zeta\)), 7.66 (d, \(J = 7.7\) Hz, 1H, H\(^\zeta\)), 7.59 (s, 1H, H\(^3\)), 7.56 (d, \(J = 7.7\) Hz, 1H, H\(^\zeta\)), 3.63 (s, 2H, H\(^3\)), 3.62 – 3.53 (m, 4H, H\(^\delta\), H\(^9\)), 3.42 (s, 6H, H\(^8\), H\(^9\)), 2.40 – 2.35 (m, 4H, H\(^1\)), 2.40 – 2.35 (m, 4H, H\(^1\)). \(^1\)C NMR (126 MHz, DMSO-\(d_6\)) \(\delta\) 180.61 (Cq, C\(^{13}\)), 157.21 (Cq, C\(^3\)), 152.00 (Cq, C\(^1\)), 139.27 (CH, C\(^{15}\)), 136.59 (CH, C\(^{15}\)), 125.02 (CH\(_2\), C\(^4\)), 124.41 (CH, C\(^6\)), 66.58 (2CH\(_2\), C\(^8\), C\(^{10}\)), 64.01 (CH\(_2\), C\(^7\)), 53.78 (2CH\(_2\), C\(^8\), C\(^{10}\)), 40.63 (2CH\(_2\), C\(^{14}\), C\(^{15}\), 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 ([M + 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, M\(^{-1}\)cm\(^{-1}\)): 216 (19628), 273 (13430), 315 (21488).

\textbf{mPyr-FTSC (H\textsubscript{L}⁵)}. A suspension of methyl 1-[(6-formylpyridin-2-yl)methyl]-1H-pyrole-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 C\(_{16}\)H\(_{15}\)N\(_{2}\)O\(_{4}\)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: \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) δ 11.66 (s, 1H, H\(^3\)), 8.35 (s, 1H, H\(^1\)), 8.20 – 8.11 (m, 2H, H\(^4\), H\(^5\)), 8.04 (s, 1H, H\(^13\)), 7.75 (t, J = 7.9 Hz, 1H, H\(^15\)), 7.33 – 7.29 (m, 1H, H\(^6\)), 7.00 – 6.92 (m, 1H, H\(^11\)), 6.60 (d, J = 7.7 Hz, 1H, H\(^8\)), 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, DMSO-\(d_6\)) δ 178.84 (Cq, C\(^{15}\)), 161.08 (Cq, C\(^{15}\)), 158.54 (Cq, C\(^3\)), 153.30 (Cq, C\(^1\)), 142.66 (CH, C\(^{13}\)), 137.99 (CH, C\(^5\)), 131.10 (CH, C\(^8\)), 121.65 (Cq, C\(^6\)), 120.41 (CH, C\(^4\)), 119.08 (CH, C\(^9\)), 118.61 (CH, C\(^1\)), 109.05 (CH, C\(^10\)), 53.37 (CH\(_2\), C\(^7\)), 51.39 (CH\(_3\), C\(^15\)). Z-isomer: \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) δ 14.05 (s, 1H, H\(^3\)), 8.60 (s, 1H, H\(^1\)), 8.24 (s, 1H, H\(^1\)), 8.00 (t, J = 7.8 Hz, 1H, H\(^8\)), 7.66 (d, J = 7.6 Hz, 1H, H\(^5\)), 7.64 – 7.59 (m, 1H, H\(^8\)), 7.40 (s, 1H, H\(^13\)), 6.98 – 6.91 (m, 2H, H\(^8\), H\(^13\)), 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, DMSO-\(d_6\)) δ 179.40 (Cq, C\(^{15}\)), 161.08 (Cq, C\(^{15}\)), 157.34 (Cq, C\(^3\)), 151.83 (Cq, C\(^1\)), 139.84 (CH, C\(^5\)), 133.66 (CH, C\(^{13}\)), 131.63 (CH, C\(^8\)), 125.65 (CH, C\(^9\)), 122.07 (CH, C\(^4\)), 121.30 (Cq, C\(^6\)), 118.95 (CH, C\(^1\)), 109.37 (CH, C\(^{10}\)), 53.37 (CH\(_2\), C\(^7\)), 51.47 (CH\(_3\), C\(^15\)). ESI-MS (methanol, positive): m/z 340 ([M + Na\(^+\)], 317 ([M + H\(^+\)])). IR (ATR, selected bands, \(\tilde{\nu}_{\text{max}}\)): 3561, 3353, 3243, 2972, 2706, 1612, 1530, 1443, 1245, 723, 653, 608 cm\(^{-1}\). UV–vis in methanol (22 μM), λ, nm (ε, M\(^{-1}\)cm\(^{-1}\)): 204 (13636), 237 (12182), 266 (17227), 324 (25545), 388 (1227).

\textbf{mPyr-dm-FTSC (H\textsubscript{L}⁵)}. A solution of methyl 1-[(6-formylpyridin-2-yl)methyl]-1H-pyrole-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 C\(_{16}\)H\(_{20}\)N\(_2\)O\(_4\)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: \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) δ 11.18 (s, 1H, H\(^3\)), 8.17 (s, 1H, H\(^13\)), 7.79 – 7.73 (m, 2H, H\(^8\), H\(^5\)), 7.33 – 7.27 (m, 1H, H\(^11\)), 6.96
(dd, $J = 3.9, 1.8$ Hz, 1H, $H^{15}$), $6.69 - 6.62$ (m, 1H, $H^1$), $6.23$ (dd, $J = 3.9, 2.6$ Hz, 1H, $H^{10}$), $5.65$ (s, 2H, $H^3$), $3.66$ (s, 3H, $H^{13}$), $3.31$ (s, 6H, $H^{15}$, $H^{16}$). $^{13}C$ NMR (126 MHz, DMSO-$d_6$) $\delta$ 180.99 (Cq, C$^{14}$), 161.06 (Cq, C$^{15}$), 158.57 (Cq, C$^{3}$), 153.55 (Cq, C$^{1}$), 144.09 (CH, C$^{13}$), 138.14 (CH, C$^{6}$), 131.12 (CH, C$^{8}$), 121.66 (Cq, C$^{5}$), 120.34 (CH, C$^{4}$), 118.56 (CH, C$^{11}$), 118.35 (CH, C$^{9}$), 108.96 (CH, C$^{15}$), 53.40 (CH$_3$, C$^{5}$), 51.38 (CH$_3$, C$^{17}$), 42.76 (2CH$_3$, C$^{13}$, C$^{16}$). Z-isomer: $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 14.71 (s, 1H, $H^1$), 7.99 (t, $J = 7.9$ Hz, 1H, $H^5$), 7.65 (d, $J = 7.7$ Hz, 1H, $H^8$), 7.62 (s, 1H, $H^{11}$), 7.40 – 7.35 (m, 1H, $H^9$), 7.02 (dd, $J = 4.0, 1.8$ Hz, 1H, $H^{13}$), 6.49 (d, $J = 7.8$ Hz, 1H, $H^6$), 6.30 (dd, $J = 3.9, 2.6$ Hz, 1H, $H^{10}$), 5.73 (s, 2H, $H^3$), 3.64 (s, 3H, $H^{15}$), 3.44 (s, 6H, $H^{14}$, $H^{16}$). $^{13}C$ NMR (126 MHz, DMSO-$d_6$) $\delta$ 180.60 (Cq, C$^{14}$), 161.01 (Cq, C$^{15}$), 158.01 (Cq, C$^{3}$), 151.49 (Cq, C$^{1}$), 140.11 (CH, C$^{8}$), 136.28 (CH, C$^{15}$), 131.12 (CH, C$^{6}$), 124.83 (CH, C$^{4}$), 121.66 (Cq, C$^{5}$), 120.10 (CH, C$^{9}$), 118.94 (CH, C$^{11}$), 109.49 (CH, C$^{12}$), 53.40 (CH$_3$, C$^{5}$), 51.51 (CH$_3$, C$^{17}$), 40.60 (C$^{15}$, C$^{16}$, overlapped with residual DMSO signal). For atom numbering and structures of Z and Z isomers see SI, Scheme S1. ESI-MS (methanol), positive: $m/z$ 368 ([M + Na]$^+$), 346 ([M + H$^+$]). IR (ATR, selected bands, $\tilde{\nu}_{max}$): 2837, 1693, 1541, 1316, 1252, 1106, 899, 775, 622 cm$^{-1}$. UV–vis in methanol (28 $\mu$M), $\lambda$, nm ($\varepsilon$, M$^{-1}$cm$^{-1}$): 235 (23929), 266 (27321), 321 (21786), 405 (2757).

Synthesis of the copper(II) complexes

$[\text{CuCl}(\text{mPipH-FTSC-H})\text{Cl}\cdot 0.1\text{H}_2\text{O} ] (1+\text{H})\text{Cl}\cdot 0.1\text{H}_2\text{O})$. 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. Calcld for C$_{11}$H$_{10}$Cl$_{2}$CuN$_{10}$S: 0.1H$_2$O (M 428.66 g/mol): C, 36.43; H, 4.75; N, 19.61; S, 4.78. Found: C, 36.49; H, 4.74; N, 19.24; S, 7.48. Solubility in water $\geq 13.2$ mg/mL. ESI-MS (methanol), positive: $m/z$ 354 ([M – Cl$^-$]). IR (ATR, selected bands, $\tilde{\nu}_{max}$): 3266, 3094, 1613, 1459, 1418, 1158, 1025, 978, 825, 787, 653 cm$^{-1}$. UV–vis in water, $\lambda$, nm ($\varepsilon$, M$^{-1}$cm$^{-1}$): 286 (19545), 392 (10063) (measured at 44 $\mu$M); 607 (265) (measured at 1.88 mM). X-ray diffraction quality crystals of the composition 1.05CH$_3$OH 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.

$[\text{CuCl}(\text{mPipH-dm-FTSC-H})\text{Cl}\cdot 0.9\text{H}_2\text{O} \cdot 0.5\text{CH}_3\text{OH} ] (2+\text{H})\text{Cl}\cdot 0.9\text{H}_2\text{O} \cdot 0.5\text{CH}_3\text{OH})$. 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 $\mu$L; 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. Caled for C_{13}H_{23}ClCuN_{2}O_{2}·1.5H_{2}O·0.5CH_{3}OH (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]²). IR (ATR, selected bands, ν_max): 3458, 3394, 3037, 2690, 1492, 1369, 1311, 1249, 1130, 908, 612 cm⁻¹. UV–vis in water, λ, nm (ε, M⁻¹cm⁻¹): 254 (11463), 299 (17561), 405 (15366) (measured at 41 μM); 574 (145) (measured at 1.79 mM).

[CuCl(Morph-FTSC–H)]·2H_{2}O·0.2C_{2}H_{4}OH (3·2H_{2}O·0.2C_{2}H_{4}OH). To a solution of Morph-FTSC (HL) (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 ≈ 5 mg/mL). Yield: 0.20 g, 88%. Anal. Caled for C_{14}H_{25}ClCuN_{2}O_{4}·2H_{2}O·0.2C_{2}H_{4}OH (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]²). IR (ATR, selected bands, ν_max): 3431, 3367, 3109, 1677, 1640, 1462, 1419, 1166, 1114, 783, 630 cm⁻¹. UV–vis in water, λ, nm (ε, M⁻¹cm⁻¹): 284 (20179), 389 (10893) (measured at 56 μM); 598 (252) (measured at 2.83 mM).

[CuCl(Morph-dm-FTSC–H)]·0.2H_{2}O·0.6CH_{3}OH (4·0.2H_{2}O·0.6CH_{3}OH). To a solution of Morph-dm-FTSC (HL) (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. Caled for C_{14}H_{25}ClCuN_{2}O_{4}·0.2H_{2}O·0.6CH_{3}OH (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]²). IR (ATR, selected bands, ν_max): 3499, 2859, 1593, 1359, 1242, 1123, 909, 869, 789 cm⁻¹. UV–vis in water, λ, nm (ε, M⁻¹cm⁻¹): 255 (10474), 299 (17207), 405 (15212) (measured at 40 μM); 575 (293) (measured at 1.78 mM).
[CuCl(mPyrr-FTSC–H)(H$_2$O)]·0.2H$_2$O (5·0.2H$_2$O). To a solution of mPyrr-FTSC (HL$_3^-$) (0.05 g, 0.16 mmol) in methanol (15 mL) was added a solution of copper(II) chloride dihydrate (0.03g, 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. Calc’d for C$_{14}$H$_{16}$ClCuN$_3$O$_3$·0.2H$_2$O (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 – H$_2$O]$^+$). 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$^{-1}$cm$^{-1}$): 298 (16447), 326 (13026, sh), 422 (11474) (measured at 76 μM); 514 (274, sh), 684 (225) (measured at 2.29 mM).

[CuCl(mPyrr-dm-FTSC–H)(H$_2$O)] (6). To a warm solution of mPyrr-dm-FTSC (HL$_4^-$) (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. Calc’d for C$_{16}$H$_{20}$ClCuN$_3$O$_3$·0.2H$_2$O (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 – H$_2$O]$^+$). 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$^{-1}$cm$^{-1}$): 306 (13851), 426 (15946) (measured at 74 μM); 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$_3^-$) and Morph-dm-FTSC (HL$_4^-$) were verified and the exact concentrations of the stock solutions prepared were determined by the Gran method.$^{35}$ 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 pH = −log[H+] scale in water according to the method suggested by Irving et al. The average water ionization constant pKw is 13.76 ± 0.01, which corresponds well to the literature data. 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. PSEQUAD was utilized to establish the stoichiometry of the complexes and to calculate the stability constants (logβ(M,L,Hx)). β(M,L,Hx) is defined for the general equilibrium pM + qL + rH ⇌ MxLqHy as β(M,L,Hx) = [MxLqHy]/[M][L]p[H]q, 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. The spectrophotometric titrations were performed on samples of the copper(II) complexes 2 and 4 of ligands nPip-dm-FTSC (HL2) and Morph-dm-FTSC (HL4) 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 [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 HL2 and HL4. Samples contained 50 μM copper(II) ion and 50 μM EDTA, and the concentration of the ligands HL2 and HL4 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$_5^-$) and Morph-dm-FTSC (HL$_5^+$) 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 μM 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 $^1$H NMR studies were carried out on a Bruker Ultrasound 500 Plus instrument. 4,4-Dimethyl-4-silapentane-1-sulfonic acid was used as an internal NMR standard. Ligands mPip-dm-FTSC (HL$_5^-$) and Morph-dm-FTSC (HL$_5^+$) were dissolved in a 10% (v/v) D$_2$O /H$_2$O 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. 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. Two parallel experiments were performed for each sample. The ligands and the complexes were dissolved at 100 μM (~30 μM 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 (0.1 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 Pumdrive 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.
Results

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$_2^+$) and $-8$ degree for the mPip-dm-FTSC (HL$_4^-$) 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. The parameters $g_\alpha$, $A_{\text{Cu}}$, copper hyperfine ($I_{\text{Cu}}=3/2$) and $A_{\text{N}}$, nitrogen ($I_N = 1$) superhyperfine couplings have been taken into account to describe each component curve. The relaxation parameters, $\alpha$, $\beta$, and $\gamma$ defined the linewidths through the equation $\sigma_M = \alpha + \beta M^2 + \gamma M^4$, 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 $K(M_\text{d,L,H})$ 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 (SOD) 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 SOD. 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. The anisotropic EPR spectra, recorded at 77 K, were analyzed individually with the aid of the EPR program. In case of copper(II) complexes, the anisotropic EPR parameters: rhombic $g$-tensor ($g_x$, $g_y$, $g_z$), rhombic copper(II) hyperfine tensor ($A_{x\text{Cu}}$, $A_{y\text{Cu}}$, $A_{z\text{Cu}}$) and rhombic nitrogen hyperfine tensor ($a_x$, $a_y$, $a_z$, for which $x$, $y$ and $z$ denotes the directions of the $g$-tensor) were fitted. For the description of the linewidth the orientation dependent $\alpha$, $\beta$ and $\gamma$ 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. 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, molecular diagrams, ORTEP. CCDC1052906-1052910.
Table 1. Crystal data and details of data collection for 1-0.15CH₃OH, 2·2H₂O, 3-0.5(C₂H₃)₂O, 4·0.93CH₃OH and 5.

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<td>1·0.15CH₃OH</td>
<td>C₁₆H₁₃Cl₂Cu₂N₂O₇S</td>
<td>395.20</td>
<td>P2₁/n</td>
<td>7.6944(5)</td>
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<td>72.114(1)</td>
<td>102.615(2)</td>
<td>73.613(1)</td>
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<td>0.26 × 0.02 × 0.02</td>
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<td>0.0753</td>
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<td>2·2H₂O</td>
<td>C₁₅H₁₄Cl₂Cu₂N₂O₇S</td>
<td>490.93</td>
<td>P-1</td>
<td>7.6629(3)</td>
<td>11.7864(3)</td>
<td>13.0949(4)</td>
<td>73.673(1)</td>
<td>74.015(3)</td>
<td>73.673(1)</td>
<td>1734.0(2)</td>
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<td>0.17 × 0.10 × 0.04</td>
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<td>1.407</td>
<td>0.0303</td>
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<td>3·0.5(C₂H₃)₂O</td>
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<td>414.42</td>
<td>P2₁/n</td>
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<td>17.4139(14)</td>
<td>13.145(1)</td>
<td>86.450(3)</td>
<td>74.015(3)</td>
<td>103.700(2)</td>
<td>1858.2(2)</td>
<td>4</td>
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<td>0.09 × 0.04 × 0.03</td>
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<td>1.548</td>
<td>0.0424</td>
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<td>4·0.93CH₃OH</td>
<td>C₁₄H₁₂Cl₂Cu₂N₂O₇S</td>
<td>435.04</td>
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<td>9.8676(7)</td>
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<td>63.866(17)</td>
<td>74.015(3)</td>
<td>77.176(3)</td>
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<td>5</td>
<td>C₁₅H₁₁Cl₂Cu₂N₂O₇S</td>
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<td>P-1</td>
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<td>12.356(5)</td>
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<td>83.407(18)</td>
<td>73.077(17)</td>
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<td>1.310</td>
<td>0.0326</td>
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[a] R₁ = Σ||F₀| - |Fₑ||ΣF₀|. [b] wR₂ = {Σ[w(F₀² - Fₑ²)²]}/L{Σ[w(F₀²)²]}]. [c] GOF = {Σ[w(F₀² - Fₑ²)²]/(n - p)}(n-p). 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-dimethylthiazolyl)-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 HL¹ and HL² and 5 mM HL⁴, while ligand HL⁴ 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\(^4\).** 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\(^4\) in 47–91% yields. One- and two-dimensional \(^1\)H and \(^{13}\)C NMR spectra confirmed the expected structures for HL\(^1\)–HL\(^4\) 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\(^4\) 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 \(\alpha\)-pyridyl-TSCs.\(^{78}\) The purity of HL\(^1\)–HL\(^4\) was further evidenced by elemental analysis. The positive-ion ESI mass spectra of HL\(^1\)–HL\(^4\) showed strong peaks at \(m/z\) 293, 321, 280, 308, 317 and 346, respectively, which were assigned to the [M+H]\(^+\) ion.

The lipo-hydrophilic character of the ligands (HL\(^1\)–HL\(^4\)) is discussed in the section Solution Chemistry.

**Synthesis and Characterization of Copper(II) Complexes.** By reaction of CuCl\(_2\)·2H\(_2\)O with HL\(^1\) and HL\(^2\) in methanol two complexes [CuCl\((m\text{PipH-FTSC–H})\)]Cl·0.1H\(_2\)O ((I+H)Cl·0.1H\(_2\)O) and [CuCl\((m\text{PipH-dm-FTSC–H})\)]Cl·0.9H\(_2\)O·0.5CH\(_3\)OH ((2+H)Cl·0.9H\(_2\)O·0.5CH\(_3\)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 [CuCl\((m\text{Pip-FTSC–H})\)]·0.15CH\(_3\)OH (1·0.15CH\(_3\)OH), 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 \([\text{CuCl}(\text{Morph-FTSC–H})] \cdot 2\text{H}_2\text{O} \cdot 0.2\text{C}_2\text{H}_5\text{OH}\) (3.2\text{H}_2\text{O} \cdot 0.2\text{C}_2\text{H}_5\text{OH}) and \([\text{CuCl}(\text{Morph-dm-FTSC–H})] \cdot 0.2\text{H}_2\text{O} \cdot 0.6\text{C}_2\text{H}_5\text{OH} \cdot 4.0\text{H}_2\text{O} \cdot 0.6\text{C}_2\text{H}_5\text{OH})\) were prepared in 88 and 98% yields, by reaction of copper(II) chloride with \( \text{HL}^3 \) and \( \text{HL}^4 \) 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 \([M – Cl – \text{H}_2\text{O}]^+\) ion. Starting from copper(II) chloride and \( \text{HL}^3 \) and \( \text{HL}^4 \) in methanol the complexes \([\text{CuCl}(\text{mPyrr–FTSC–H})(\text{H}_2\text{O})] \cdot 0.2\text{H}_2\text{O} \cdot 5.0\text{H}_2\text{O} \) and \([\text{CuCl}(\text{mPyrr–dm–FTSC–H})(\text{H}_2\text{O})] \cdot 6\text{H}_2\text{O} \) 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 \([M – Cl – \text{H}_2\text{O}]^+\) ion. Re-crystallization of \([\text{CuCl}(\text{mPyrr–FTSC–H})(\text{H}_2\text{O})] \cdot 0.2\text{H}_2\text{O} \) from DMF afforded the complex \([\text{CuCl}(\text{mPyrr–FTSC–H})(\text{DMF})] \cdot 5\text{H}_2\text{O} \) 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 1-0.15\text{C}_2\text{H}_5\text{OH}, [2+H]Cl \cdot 2\text{H}_2\text{O}, 3-0.5(\text{C}_2\text{H}_5\text{OH}), 4-0.93\text{C}_2\text{H}_5\text{OH} and 5 are shown in Figures 1–5. The complexes 1-0.15\text{C}_2\text{H}_5\text{OH} and 4-0.93\text{C}_2\text{H}_5\text{OH} crystallized in the centrosymmetric monoclinic space group \( P2_1/n \), while 2 \cdot 2\text{H}_2\text{O}, 3-0.5(\text{C}_2\text{H}_5\text{OH}) and 5 crystallized in the centrosymmetric triclinic space group \( P\text{-1} \). The piperazine-thiosemicarbazone and morpholine-thiosemicarbazone hybrid ligands \( \text{HL}^1 \), \( \text{HL}^3 \) and \( \text{HL}^4 \) 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 \cdot 2\text{H}_2\text{O} the organic ligand \( \text{HL}^2 \) 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 polyhedra can be described as a square-pyramid \( (\tau = 0.13, 0.11, 0.12 \) and 0.10 (0.07 for another crystallographically independent complex), respectively. The apical position is occupied by a chlorido ligand. Three five-membered metalloccycles 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 N3\(^1\) of a neighboring molecule of 1 forming pairs of molecules as displayed in Figure S1 and in hydrogen bonding to Cl\(^{1i}\), where i and ii denote the atoms generated by symmetry transformations $-x + 2, -y + 1, -z + 1$ and $x + 1, y, z$, respectively.
**Results**

**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 101.60(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⋯Cl$^-$ at 3.0837(14) Å, where i denotes atom positions generated by symmetry transformation $x - 1, y + 1, z,$ and N6–H⋯Cl$^-$ 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–S1 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 HLL$_5$ 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$^1$-hybridized proline nitrogen atom in proline-thiosemicarbazole conjugates was involved in binding to first-row transition metals and became a chiral center upon coordination.$^{30}$
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–O3 94.701(16), Cl–Cu–O3 107.32(4), N1–Cu–S 163.45(4).

Solution Chemistry: Proton Dissociation Processes of Ligands HL\(^2\) and HL\(^4\), Lipophilicity of ligands HL\(^1\) – HL\(^5\), Morph-dm-FTSC (HL\(^3\)), 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 methylpiperezine analogue, mPip-dm-FTSC (HL\(^4\)) (Chart 1) for comparison. Deprotonation processes of these ligands were followed in aqueous solution by pH-potentiometric and \(^1\)H 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 (HL\(^3\)) possesses three, while mPip-dm-FTSC (HL\(^4\)) 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 p\(K_a\) value for the N\(\text{pyridyl}\)H\(^+\) and a significantly higher value for the N\(\text{hydrazino}\)H functional group based on the proton dissociation constants of structurally similar HCTs, such as 2-formylpyridine N\(^4\),N\(^4\)-dimethylthiosemicarbazone (PTSC, p\(K_1\): 3.38 and p\(K_2\): 10.54) or 3-aminopyridine-2-carboxaldehyde N\(^3\),N\(^3\)-dimethylthiosemicarbazone (APTSC, p\(K_1\): 4.31 and p\(K_2\): 10.29). Taking into account these data we attributed the p\(K_2\) 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 $^1$H 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-potentimetry and $^1$H NMR titrations ($T = 298$ K, $I = 0.10$ M (KCl))

<table>
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<tr>
<th>method</th>
<th>$pK_1$</th>
<th>$pK_2$</th>
<th>$pK_3$</th>
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<td>pH-metry</td>
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<td>10.23±0.01</td>
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<td>1H NMR isomer $Z$</td>
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<td></td>
<td>1H NMR isomer $Z$</td>
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<tr>
<td></td>
<td>1H NMR$^a$</td>
<td>2.21</td>
<td>5.90</td>
<td>10.30</td>
</tr>
</tbody>
</table>

$^a$Estimated from the summed concentration distribution curves of the $E/Z$ isomers in Figures 6B ad S4B.

**Scheme 1.** Deprotonation steps of the H$_2$L$_2^{2+}$ form of ligand Morph-dm-FTSC (HL$_4^+$) for its $E$ (A) and $Z$ (B) isomers
The pH-dependent $^1$H 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 ($t_{1/2}<$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 $\sim 9$, where the neutral HL form predominates. Based on the pH-dependence of the $^1$H 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 $^1$H pyridine ring proton and $^{14,15}$H$_2$ terminal methyl protons. The morpholine ($^{8,11}$H$_2$, $^{9,10}$H$_2$) and $^{17}$H$_2$ 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 ($^{12}$H, $^{14,15}$H$_2$) 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 $N^1_{\text{pyridyl}}H^+$, $N^5_{\text{morpholinium}}H^+$ and $N^2_{\text{hydrazine}}H$ 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 $N^2_{\text{hydrazine}}H$ 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 $N^2_{\text{hydrazine}}H$ functional group participating in the hydrogen bonding is more difficult.

![Figure 6](image_url)

**Figure 6.** pH-Dependence of the molar fraction of the $E$ (red symbols) and $Z$ (blue symbols) isomers of the ligand Morph-dm-FTSC ($H_2L^+$) 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$_2$O]
Figure 7. Low- (A) and high-field (B) regions of the $^1$H NMR spectra of Morph-dm-FTSC (HL$^4$) at different pH values, red and blue symbols denote the peaks belonging to the protons of the major E and minor Z isomers, respectively. [$c_L = 1.5$ mM; $T = 298$ K; $I = 0.10$ M (KCl); 10% D$_2$O]

The pH-dependent $^1$H NMR spectra of mPip-dm-FTSC (Figure S3) and the changes of the chemical shifts of the various protons (Figure S4) were analyzed similarly. Data revealed that $pK_1$ corresponds to the deprotonation of pyridinium nitrogen. However only the macroscopic constant could be determined by pH-potentiometry (Table 2) as the $^1$H NMR signals were fairly broadened in the pH range where this process takes place and data were not appropriate for calculation. The second deprotonation step is accompanied by significant electronic shielding effects in the case of the pyridine ring protons and a large upfield shift of the C$^{14}$H$_3$ protons. The signals belonging only to the C$^{14}$H$_3$ methyl protons are sensitive to the third proton dissociation process. These changes strongly indicate that $pK_2$ and $pK_3$ can be assigned to the deprotonation of the N$^2$-piperaziniumH$^+$ and N$^1$-piperaziniumH$^+$ groups, respectively (Scheme S3). Protons of the thiosemicarbazone moiety were found to be sensitive to the last deprotonation step in which the N$^2_{hydrazinic}$H releases the proton. Comparing the microscopic constants of the E and Z isomers of the methylpiperazine-thiosemicarbazone hybrid (Table 2) it can be concluded that the lower $pK_2$ (N$^3$-piperaziniumH$^+$) and higher $pK_4$ (N$^2_{hydrazinic}$H) values of the Z isomer are due to the presence of the hydrogen bond in the H$_2$L$^2$ and HL forms (see the explanations in the case of Morph-dm-FTSC vide supra). At the same time the isomerization has no effect on the $pK_3$ value since the N$^6$-piperaziniumH$^+$ group is quite far from the CH$^{12}$=N$^3$
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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$_{pyridyl}$H$^+$ functional group of the studied thiosemicarbazone-based hybrids are significantly lower compared to those of ligands PTSC, APTSC$^{41}$ due to the electron withdrawing effect of the charged morpholinium and methylpiperazinium moieties.

Both ligands mPip-dm-FTSC ($\text{HL}^2$) and Morph-dm-FTSC ($\text{HL}^4$) 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 $\text{HL}^2$.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>log $D_{r,4}$</th>
<th>complex</th>
<th>log $D_{r,4}$</th>
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<tbody>
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<td>mPip-FTSC</td>
<td>$\text{HL}^1$</td>
<td>1</td>
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<td>mPip-dm-FTSC</td>
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<tr>
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<td>$-1.15 \pm 0.09$</td>
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<tr>
<td>Morph-dm-FTSC</td>
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<td>$-0.90 \pm 0.09$</td>
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<tr>
<td>mPyrr-FTSC</td>
<td>$\text{HL}^5$</td>
<td>5</td>
<td>$&gt;1.8$</td>
</tr>
</tbody>
</table>

Table 3. Log $D_{r,4}$ values ($n$-octanol/water) for the ligands $\text{HL}^1$ – $\text{HL}^5$ and for the copper(II) complexes 1 – 5 [$T = 298$ K, pH = 7.40 (10 mM HEPES) and $I = 0.10$ M (KCl)].

The lipo-hydrophilic character of the ligands $\text{HL}^1$ – $\text{HL}^5$ was studied at pH 7.4 via the partitioning between $n$-octanol and water. The log $D_{r,4}$ 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 ($\text{HL}^2$ and $\text{HL}^4$) compared to that of the corresponding non-methylated ligands ($\text{HL}^1$ and $\text{HL}^3$). Compounds containing the morpholine moiety ($\text{HL}^3$ and $\text{HL}^4$) possess significantly higher log $D_{r,4}$ values compared to those of the methylpiperazine derivatives ($\text{HL}^1$ and $\text{HL}^2$) 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 ($\text{HL}^2$) is partly protonated (74% H$_2$L$^+$, 26% HL), while Morph-dm-FTSC ($\text{HL}^4$) is mainly neutral (97% HL,
3% H$_2$L$^3$) at pH 7.4. On the other hand, the methyl ester mPyr-$r$-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 $D_{7,4} < -1.7$). It should be also noted that all the ligands studied except mPyr-$r$-FTSC (HL$^5$) are more hydrophilic than Triapine (log $D_{7,4} = +0.85$) 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 7A,B. The simulation of the EPR spectra resulted in the individual isotropic and anisotropic EPR spectra and parameters of the various species (Figures 8C, 7C and 8; 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 [CuL]$^\text{Cu}$, which predominates in a wide pH-range, were determined by competition reactions with EDTA. The displacement of EDTA from the [Cu(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 [CuL]$^\text{Cu}$, the only species contributing to the measured absorbance. The cumulative stability constants (log$\beta$) of [CuL]$^\text{Cu}$ 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 \([T = 298\ \text{K}, \ I = 0.10\ \text{M (KCl)}]\)

<table>
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<tr>
<th>pH-metry</th>
<th>UV–vis</th>
<th>EPR</th>
</tr>
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<tbody>
<tr>
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<tr>
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<tr>
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<td>–</td>
</tr>
<tr>
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<td>8.4±0.1</td>
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<tr>
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</tr>
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<td>(pK_a[\text{CuL}_2]^{1+})</td>
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<td>(\log K[\text{CuL}_2]^{0})</td>
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</table>

\(^a\) Determined via the EDTA displacement reactions by the ligand HL\(^2\) or HL\(^4\) by UV–vis spectrophotometry. Data for the \(pK_a\) values of EDTA \((0.9; 1.6; 2.0; 2.66; 6.16; 10.24)\) and \(\log\beta\) of the \([\text{Cu(EDTA)}]^2\) complex \((18.82)\) taken from ref. 61 and conditional stability constants of \([\text{Cu(EDTA)}]^2\) 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 \((10\ \text{mM HEPES})\) and 13.79 ± 0.03 \((HL\(^4\)) at pH 5.6 \((10\ \text{mM MES})\). \(\beta\) values of \([\text{CuL}]^+\) are calculated as \(\beta = \beta' \times a_0^\text{H}^\text{L}\), where \(a_0^\text{H}^\text{L} = 1 + \Sigma \beta(H\text{L}) \times [\text{H}^+]^\beta\).
Figure 8. Experimental (black) and simulated (red) solution EPR spectra recorded for the copper(II) – mPip-dm-FTSC (HL₂) 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 (HL₂) 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 (S₁,N₁,N₄,N₃) 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 < ~3 indicate the alteration of the coordination mode. \( \lambda_{\text{max}} \) 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 → 690 nm, 406 nm → 410 nm, 302 nm → 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.
Results

compared to that of [CuL]$^+$ (Table 5). On the other hand the deprotonation of [CuL]$^+$ observed at pH $> -10$, is accompanied by only minor changes of the UV–vis spectra (see changes at $\sim 256$ nm in Figure S10B). However, the decreasing ligand field (lower $A_{0s}$) supports the formation of a mixed hydroxido complex, [CuL(OH)], in which the ligand binds through ($S^-, N^1, N^3$) donor atoms. A fairly similar deprotonation process of [CuL]$^+$ is characteristic for mPip-dm-FTSC. The formation of [CuLH$_{1-}$] ($= [CuL(OH)]$) 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 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 [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 [CuLH]$^{2+}$ and [CuL]$^+$ (Table 5) indicate the same coordination mode of mPip-dm-FTSC in these complexes via a ($S^-, N^1, N^3, N^5$) 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 [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 pH $< -3$ were found to be similar to those found for the Morph-dm-FTSC system, thus the ($S^-, N^1, N^3$) coordination is suggested for [CuLH$_3$]$^{3+}$ 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. \( [c_{\text{complex}} = 2.5 \text{ mM}; T = 298 \text{ K}; l = 0.10 \text{ M (KCl)}; l = 1 \text{ cm}] \)

Formation of merely mono-ligand copper(II) complexes for \( \text{HL}^2 \) and \( \text{HL}^4 \) was expected. However at ligand excess \( (c_L/c_{b}>2) \) 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 \( (S^-;N^1;N^4) \) and \( (S_{equatorial};N^1_{ axial}) \) 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 \( \text{[CuL}_2H_2]^{3+} \) (Morph-dm-FTSC) and \( \text{[CuL}_2H]^- \) (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 \( (g_{0,\text{calc}} \text{ and } A_{0,\text{calc}} \text{ in Table 5}) \) 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 \( \text{(HL}^2 \) and copper(II) – Morph-dm-FTSC \( \text{(HL}^4 \) 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)]; $c_\text{Cu}/c_\text{Cu} = 10$; $c_\text{Cu} = 1 \mu\text{M}$) 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/H$_2$O$^{55}$ and are comparable to that of the pentadentate L-Pro-FTSC conjugate (17.5) in pure water.$^{35}$

![Figure 10](https://example.com/figure10.png)

**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. [$c_\text{L} = 1.0 \text{ mM}; c_\text{Cu} = 1.0 \text{ mM}; T = 298 \text{ K}; I = 0.10 \text{ M (KCl)}$]

Log $D_{3.4}$ 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 $[\text{CuL}]^{+}$ species predominate at physiological pH. Complex 5 is much more lipophilic than the other complexes, although its log $D_{3.4}$ value cannot be determined exactly and compared to that of mPyrr-FTSC (HL$^5$).
Table 5. EPR parameters of the components obtained for the copper(II) complexes of mPip-dmSC ([HL]) and Morph-dm-FTSC ([HL])

<table>
<thead>
<tr>
<th>Isoelectric parameters</th>
<th>Anisotropic parameters</th>
<th>Calculated parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$g_e$, $A_e$ / G</td>
<td>$a_x$, $a_y$, $a_z$ / G</td>
</tr>
<tr>
<td>[CuL]$^+$</td>
<td>2.1057(2) 68.6(4)</td>
<td>13.8(5) 10.7(7)</td>
</tr>
<tr>
<td></td>
<td>2.035, 2.058, 2.216</td>
<td>18.7, 30.4, 155.9</td>
</tr>
<tr>
<td>[CuL]$^-$</td>
<td>2.0885(1) 63.3(1)</td>
<td>17.8(1) 11.9(1)</td>
</tr>
<tr>
<td></td>
<td>2.032, 2.053, 2.176</td>
<td>8.9, 98, 160.0</td>
</tr>
<tr>
<td>[CuLH$_2$]$^+$</td>
<td>2.0953(6) 62.1(8)</td>
<td>9.0(8) 2.05, 2.07, 2.249</td>
</tr>
<tr>
<td></td>
<td>16.9, 156</td>
<td></td>
</tr>
<tr>
<td>[Cu$_2$L$_2$]$^-$</td>
<td>2.0745(5) 85.5(6)</td>
<td>12.3(8)</td>
</tr>
<tr>
<td></td>
<td>17(1) 14(1)</td>
<td></td>
</tr>
<tr>
<td>[CuL$_2$]</td>
<td>2.106(2) 53(2)</td>
<td>17(1) 14(1)</td>
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<tr>
<td></td>
<td>18.4, 14, 12</td>
<td></td>
</tr>
<tr>
<td>[CuLH$_3$]$^+$</td>
<td>2.1026(4) 58.0(5)</td>
<td>17.4(4) 10.3(9)</td>
</tr>
<tr>
<td></td>
<td>2.035, 2.059, 2.214</td>
<td>-18.9, 30.4, 155.7</td>
</tr>
<tr>
<td>[CuLH]$^+$</td>
<td>2.0899(1) 58.3(1)</td>
<td>18.2(1) 11.5(2)</td>
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<td></td>
<td>2.031, 2.055, 2.176</td>
<td>4.4, 83, 155.9</td>
</tr>
<tr>
<td>[CuLH$_2$]$^+$</td>
<td>2.0894(1) 64.2(1)</td>
<td>17.9(1) 12.1(2)</td>
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<td>2.033, 2.053, 2.177</td>
<td>6.7, 10.9, 159.2</td>
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<tr>
<td>[CuL$_2$]$^-$</td>
<td>2.1059(7) 59(1)</td>
<td>10(1) 2.05, 2.07, 2.249</td>
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<td>16.9, 156</td>
<td></td>
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<tr>
<td>[CuLH$_3$]$^+$</td>
<td>2.1144(6) 54(1)</td>
<td>15(1) 12(2)</td>
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<tr>
<td></td>
<td>2.03, 2.05, 2.200</td>
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<tr>
<td>[CuL]$^+$</td>
<td>2.1118(6) 53(1)</td>
<td>16(1) 12(2)</td>
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<td></td>
<td>25.0, 11, 150</td>
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<tr>
<td>[Cu$_2$L$_2$]$^-$</td>
<td>17(1) 10(1)</td>
<td></td>
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<tr>
<td></td>
<td>10, 17, 10</td>
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</table>

$^a$ Uncertainties (SD) are shown in parentheses. $^b$ The experimental errors were $\pm 0.002$ for $g_e$ and $g_z$ and $\pm 0.001$ for $g_x$, $\pm 2$ G for $A_x$ and $A_z$, and $\pm 1$ G for $A_y$. $^c$ Isotropic values calculated via the equation $g_i = (g_x+g_y+g_z)/3$, and $A_i[MHz] = (A_x+A_y+A_z)/3$. $^d$ The signs of the couplings were derived from a comparison of $A_{calc}$ with the experimental $A_{obs}$ values. $^e$ Higher uncertainties of anisotropic parameters were obtained for minor species.
Cytotoxicity in Cancer Cell Lines. The antiproliferative activity of the ligands HL\textsuperscript{1}–HL\textsuperscript{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 \textit{in vitro} cytotoxicity evaluation. The results for the ligands and their copper(II) complexes are summarized in Table 6 in terms of IC\textsubscript{50} values with their standard deviations. The results revealed that compounds 1–4 exhibited significant antiproliferative activity (IC\textsubscript{50} < 100 \textmu M) 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 IC\textsubscript{50} values varying from 13.1 to 17.5 \textmu M. In contrast, the ligands showed significantly lower activity than the parent 2-formyl- and/or 2-acetylpyridine thiosemicarbazones, except ligand HL\textsuperscript{4}, which exhibited significant cytotoxic activity against both the MRC5 and LS174 cell lines (63.2±4.2 and 15.9±0.6 \textmu M, respectively).

Table 6. Results of the MTT assay presented as IC\textsubscript{50} values obtained after 48 h treatment.

<table>
<thead>
<tr>
<th>Complex</th>
<th>HeLa</th>
<th>A549</th>
<th>LS174</th>
<th>MRC5</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>38.3±1.7</td>
<td>62.7±4.7</td>
<td>16.4±4.2</td>
<td>50.6±3.5</td>
</tr>
<tr>
<td>2</td>
<td>65.1±5.7</td>
<td>131.3±3.9</td>
<td>17.4±0.2</td>
<td>38.6±5.9</td>
</tr>
<tr>
<td>3</td>
<td>63.3±2.7</td>
<td>208.0±0.1</td>
<td>17.5±1.6</td>
<td>132.1±9.2</td>
</tr>
<tr>
<td>4</td>
<td>25.5±5.3</td>
<td>42.8±3.7</td>
<td>13.1±2.1</td>
<td>28.3±3.8</td>
</tr>
<tr>
<td>HL\textsuperscript{1}</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>HL\textsuperscript{2}</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>HL\textsuperscript{3}</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>HL\textsuperscript{4}</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>15.9±0.6</td>
<td>63.2±4.2</td>
</tr>
</tbody>
</table>

The sign > (in front of the maximum value of the concentration) indicates that IC\textsubscript{50} 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\textsubscript{50} values of 2.9 ± 0.6, 3.2 ± 0.6, 10.6 ± 0.1 and 3.3 ± 0.5 μM, respectively. \cite{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 (APTS) in the first two cancer cell lines, a considerable increase was observed against the other two cell lines (IC\textsubscript{50} = 0.4 ± 0.01 and 0.2 ± 0.02 μM). Terminal N\textsuperscript{4}-dimethylation of FTSC resulted in a very strong enhancement of antiproliferative activity reaching IC\textsubscript{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. \cite{85} The favorable effect of N\textsuperscript{4}-dimethylation is also well-documented for other related \( \alpha \)-heterocyclic thiosemicarbazones. \cite{31} The coordination of FTSC to copper(II) was reported to increase or decrease the activity depending on the cell type. \cite{85, 88} In particular, [Cu(FTSC)Cl\textsubscript{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. \cite{84} The proline-FTSC hybrids, we synthesized previously, \cite{80} showed a different activity compared to the compounds reported herein. Hybrids that were not methylated at N\textsuperscript{4} (L- and D-Pro-FTSC) and their nickel(II), copper(II) and zinc(II) complexes lacked activity (IC\textsubscript{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 (fetal human fibroblast). The terminally dimethylated hybrid dm-L-Pro-FTSC showed moderate to low anticancer activity with IC\textsubscript{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\textsubscript{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. \cite{80} Comparison of IC\textsubscript{50} values for 3 and 4 indicates that terminal N\textsuperscript{4}-dimethylation enhances the cytotoxicity in accord with the general trend observed in the literature. \cite{81, 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\textsuperscript{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 H$_{1}$$^{5}$ and H$_{1}$$^{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 potencies of new compounds are undergoing in our laboratory. In contrast, the hybrids H$_{1}$$^{3}$–H$_{1}$$^{4}$ possess excellent water solubility. The solution speciation of copper(II) complexes of H$_{1}$$^{2}$ and H$_{1}$$^{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$_{py}$,S$_{thio}$) donor atoms. This binding mode was confirmed by X-ray crystallography in the case of complexes 1–4. Predominant formation of highly stable [CuL]$^{2-}$ 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 H$_{1}$$^{5}$ and H$_{1}$$^{6}$ possess markedly higher log $D_{7,4}$ values compared to those of the piperazine counterparts H$_{1}$$^{1}$ and H$_{1}$$^{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 H$_{1}$$^{3}$–H$_{1}$$^{4}$ to copper(II) significantly increased the cytotoxicity in vitro. While H$_{1}$$^{3}$–H$_{1}$$^{4}$ possess low cytotoxicity with IC$_{50}$ > 300 µM, their copper(II) complexes revealed high antiproliferative activity. The most active compound 4 exhibited IC$_{50}$ 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 IC$_{50}$ 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.

Acknowledgments. This work was supported by the Hungarian Research Foundation OTKA project PD103905. We are thankful to the Ministry of Science and Technology of Serbia for financial support from Grant No. III141026. We thank Prof. M. Galanski for measuring the two-dimensional NMR spectra and Dr. Michael Malarek for reading the manuscript and his comments.

Notes and References


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† Electronic Supplementary Information (ESI) available: NMR numbering scheme for HL1–HL6 (Scheme S1), synthesis scheme for HL1–HL6 (Scheme S2), deprotonation steps of HL2 (Scheme S3), part of the crystal structure of I showing complex pairing via intermolecular hydrogen bonding interactions (Figure S1), pH-dependence of the chemical shifts of various protons of HL4 (Figure S2), low- (A) and high-field (B) regions of the 1H NMR spectra HL2 at different pH values (Figure S3), pH-dependence of the chemical shifts of various protons of HL2 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 HL2 (Figure S6), 3-dimensional fluorescence spectra of HL2 and HL4 (Figure S6), experimental and simulated solution EPR spectra recorded for the copper(II) – HL4 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 HL2 and HL4 in frozen solution (Figure S8), UV–vis spectra of [Cu(EDTA)]2– in the presence of HL2.
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(Figure S9), UV–vis spectra of the copper(II) – $\text{HL}_2$ and $\text{HL}_4$ systems at 1:1 metal-to-ligand ratio (Figure S10).


Results

Results

65. SCQuery, The IUPAC Stability Constants Database, Academic Software (Version 5.5)
72. SAINT-Plus, version 7.06a and APEX2; Bruker-Nomius AXS Inc.: Madison, WI, 2004.
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 $^1$H and $^{13}$C NMR data for HL$^1$ – HL$^6$. HL$^2$ is depicted as Z-isomer for clarity reasons.


Reagents and conditions: (i) and (ii) see ref.1; (iii) trimethyl orthoformate, methanesulfonic acid, methanol, 78 °C, 3 h; (iv) E: methylpiperazine, triethylamine, THF/CH$_2$Cl$_2$ 1:1, 40 °C, 12 h, purification by column chromatography; F: morpholine, triethylamine, THF/CH$_2$Cl$_2$ 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 NaHCO$_3$, extraction with CH$_2$Cl$_2$; J: water/acetone 5:1, reflux, 12 h; (vi) HL$^1$: thiosemicarbazide, EtOH, 78 °C, 12 h, purification on prep. HPLC; HL$^2$: 4,4-dimethyl-3-thiosemicarbazide, EtOH, room temperature, 12 h, purification on prep. HPLC; HL$^3$: thiosemicarbazide, EtOH, 78 °C, 12 h, recrystallization from water/methanol 5:1; HL$^4$: 4,4-dimethyl-3-thiosemicarbazide, EtOH, room temperature, 12 h; HL$^5$: thiosemicarbazide, EtOH/MEOH 1:1, 78 °C, 12 h, recrystallization from water/methanol 5:1. HL$^6$: 4,4-dimethyl-3-thiosemicarbazide, EtOH/MEOH 1:1, room temperature, 6 h.
Scheme S3. Deprotonation steps of the $\text{H}_4\text{L}^3^+$ form of ligand mPip-dm-FTSC ($\text{HL}_2^+$) 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 (Å) and donor-hydrogen–acceptor ($D\cdots H\cdots A$) angles (deg) of the intermolecular hydrogen bonds: $\text{N}4\cdots\text{N}3^i$ 2.946(3) Å, $\text{N}4\cdots\text{H}\cdots\text{N}3^i$ 173.7°; atoms marked with $i$ are at symmetry position $-x + 2, -y + 1, -z + 1$. 

Results
Results

Figure S2. pH-Dependence of the chemical shifts of the various protons of the ligand Morph-dm-FTSC (HL₄) 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. \([c_L= 1.5 \text{ mM}; \ T = 298 \text{ K}; \ I = 0.10 \text{ M (KCl); 10\% D}_2\text{O}]\)

Figure S3. Low- (A) and high-field (B) regions of the \(^1\text{H} \text{NMR spectra of ligand mPip-dm-FTSC (HL₃)}\) at different pH values, red and blue symbols denote the peaks belonging to the protons of the major E and minor Z isomers, respectively. \([c_L= 3 \text{ mM}; \ T = 298 \text{ K}; \ I = 0.10 \text{ M (KCl); 10\% D}_2\text{O}]\)
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 mM; $T$ = 298 K; $I$ = 0.10 M (KCl); 10% D$_2$O]

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 mM; $T$ = 298 K; $I$ = 0.10 M (KCl); 10% D$_2$O]
Figure S6. The 3-dimensional fluorescence spectrum of mPip-dm-FTSC (HL²⁻) (A) and Morph-dm-FTSC (HL⁴⁻) (B) at physiological pH in water. [cL = 10 μM; T = 298 K; I = 0.10 M (KCl); pH = 7.4 (10 mM HEPES)].

Figure S7. Experimental (black) and simulated (red) solution EPR spectra recorded for the copper(II) – Morph-dm-FTSC (HL⁴⁻) system at 1:1 (A) and 1:2 (B) metal-to-ligand ratio. Calculated component EPR spectra obtained for the different copper(II) – Morph-dm-FTSC (HL⁴⁻) species (C). [c_ligand = 1.0 mM; c_Cu = 1.0 mM (A) or c_Cu = 0.5 mM (B); T = 298 K; I = 0.10 M (KCl)].
Figure S8. Calculated component EPR spectra obtained for the different copper(II) – mPip-dm-FTSC (HL₂) (A) and Morph-dm-FTSC (HL₄) (B) complexes in frozen solution. [T = 77 K; I = 0.10 M (KCl)]

Figure S9. UV–vis spectra of the [Cu(EDTA)]²⁻ complex (grey solid line, c = 50 μM) in the presence of ligand mPip-dm-FTSC (HL₂) at increasing concentrations (solid lines in color, c = 7.5 - 167.6 μM). UV–vis spectra of EDTA (grey dashed line, c = 50 μM), mPip-dm-FTSC (black dashed line, c = 167.6 μM) and complex 2 (black solid line, c = 50 μM) are shown for comparison. Inset shows the absorbance values recorded at 420 nm for two parallel sets of measurements on the ternary copper(II) – EDTA – ligand system in dependence of the concentration of ligand mPip-dm-FTSC. [T = 298 K; I = 0.10 M (KCl); l = 1 cm; pH = 7.4 (10 mM HEPES)]
**Figure S10.** UV–vis spectra of the copper(II) – mPip-dm-FTSC ([H]L2) (A) and copper(II) – Morph-dm-FTSC ([H]L4) (B) systems at 1:1 metal-to-ligand ratio. (Grey dashed lines show the spectra of the ligands alone at chosen pH values.) \([c_L]= 153 \mu M\) (A) and 157 \mu M\) (B); \(T = 298 K\); \(I = 0.10 M\) (KCl); \(l = 0.5 \text{ cm}\]

**References**

2.3 Ruthenium Compounds as Antitumor Agents: New Developments

Bacher, F.; Arion, V. B.

Results
Results


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Introduction

Coordination and organometallic chemistry offers additional options for the design and synthesis of chemotherapeutics when compared to organic chemistry. 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) with controls of both the thermodynamics and kinetics of these substitution reactions, redox activity, and the ability of metal-containing species to bind reversibly to biomolecular targets, for example, enzymes, 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. 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. Table 1 summarizes the anticancer products launched worldwide from 2011 to 2013 with exception of biological drugs. 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. Although platinum complexes dominate the field of metal-based cancer chemotherapy, 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. Examples of current clinical interest involving metal-based compounds and chelators as theranostics have also been listed in the literature.

Table 1 summarizes the anticancer products launched worldwide from 2011 to 2013 with exception of biological drugs. 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.
<table>
<thead>
<tr>
<th>Trade name/country/year</th>
<th>Company (developing/marking)</th>
<th>Active ingredient</th>
<th>Indication</th>
<th>Underlying mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zytiga®/USA/2011</td>
<td>Cougar Biotechnology/Johnson &amp; Johnson</td>
<td>Abiraterone acetate</td>
<td>Treatment of castration-resistant prostate cancer</td>
<td>Inhibition of CYP17A1 enzyme expressed in testicular, adrenal, and prostatic tumor tissues</td>
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<tr>
<td>Giotrif™/USA/2013</td>
<td>Boehringer Ingelheim</td>
<td>Afatinib</td>
<td>Treatment of metastatic non-small cell lung cancer (NSCLC) with common epidermal growth factor receptor (EGFR) mutations</td>
<td>Inhibition of tyrosine kinase and of vascular endothelial growth factor receptor (VEGFR-1, -2, and -3)</td>
</tr>
<tr>
<td>Inlyta®/USA/2012</td>
<td>Pfizer</td>
<td>Axitinib</td>
<td>Treatment of advanced renal cell carcinoma</td>
<td>Inhibition of tyrosine kinase, including Ret, Met, VEGFR-1, VEGFR-2 and VEGFR-3, Trk-B, FLT-3, AKI, and TIE-2 involved in oncogenesis, metastasis, angiogenesis, and maintenance of the tumor microenvironment</td>
</tr>
<tr>
<td>Cometriq™/USA/2013</td>
<td>Exelixis</td>
<td>Cabozantinib S-malate</td>
<td>Treatment of progressive, metastatic medullary thyroid cancer</td>
<td>Inhibition of multiple receptor tyrosine kinases, including Ret, Met, VEGFR-1, VEGFR-2 and VEGFR-3, Trk-B, FLT-3, AKI, and TIE-2 involved in oncogenesis, metastasis, angiogenesis, and maintenance of the tumor microenvironment</td>
</tr>
<tr>
<td>Kyprolis™/USA/2012</td>
<td>Onyx Pharmaceuticals</td>
<td>Carfilzomib</td>
<td>Treatment of multiple myeloma</td>
<td>Inhibition of proteasome</td>
</tr>
<tr>
<td>Xalkori®/USA/2011</td>
<td>Pfizer</td>
<td>Crizotinib</td>
<td>Treatment of metastatic NSCLC that is caused by the echinoderm microtubule associated protein-like 4 (EML4) mutation of anaplastic lymphoma kinase (ALK)</td>
<td>Inhibition of mesenchymal epithelial transition factor/anaplastic lymphoma kinase (cMET/ALK)</td>
</tr>
<tr>
<td>Tafinlar®/USA/2013</td>
<td>GlaxoSmithKline</td>
<td>Dabrafenib</td>
<td>Treatment of adult patients with unresectable or metastatic melanoma with BRAF V600E mutation</td>
<td>Inhibition of Raf kinase B</td>
</tr>
<tr>
<td>Dacogen®/EU/2012</td>
<td>Aastex/Janssen-Digal</td>
<td>Decitabine</td>
<td>Treatment of adult patients with newly diagnosed de novo or secondary acute myeloid leukemia (AML)</td>
<td>Inhibition of DNA methyltransferase</td>
</tr>
<tr>
<td>Xland®/USA/2012</td>
<td>AstellasPharmaceuticals/ AstraZeneca</td>
<td>Enzalutamide</td>
<td>Treatment of metastatic castration-resistant prostate cancer (mCRPC)</td>
<td>Inhibition of androgen binding to androgen receptors</td>
</tr>
<tr>
<td>Commana®/China/2011</td>
<td>Zhejiang Bata Pharma Inc.</td>
<td>Icotinib</td>
<td>Treatment of mantle cell lymphoma (MCL)</td>
<td>Inhibition of Bruton tyrosine kinase (BTK)</td>
</tr>
<tr>
<td>Pauver®/EU/2012</td>
<td>Cell Therapeutics</td>
<td>Pixantrone maleate</td>
<td>Treatment of multiple relapsed or refractory aggressive non-Hodgkin’s B-cell lymphomas</td>
<td>DNA intercalation</td>
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<tr>
<td>Pomalyst®/USA/2013</td>
<td>Celgene</td>
<td>Pomalidomide</td>
<td>Treatment of multiple myeloma</td>
<td>Immunomodulation</td>
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<tr>
<td>Iclusig®/USA/2013</td>
<td>Ariad Pharmaceuticals</td>
<td>Ponatinib</td>
<td>Treatment of chronic, accelerated, or blast phase Philadelphia chromosome-positive (Ph+) chronic myelogenous leukemia (CML)</td>
<td>Inhibition of tyrosine kinases with antiangiogenesis effects</td>
</tr>
<tr>
<td>Xofogo®/USA/2013</td>
<td>Ageta/Bayer Scheving Pharma</td>
<td>Radium Ra223 dichloride</td>
<td>Treatment of CRPC, with symptomatic bone metastases and without known visceral metastases</td>
<td>Alpha particle-emitting radioactive therapy</td>
</tr>
<tr>
<td>Sitang®/USA/2012</td>
<td>Bayer/Onyx Pharmaceuticals</td>
<td>Regorafenib</td>
<td>Treatment of metastatic colorectal cancer (mCRC)</td>
<td>Multikinase inhibition</td>
</tr>
<tr>
<td>Jakafi®/USA/2011</td>
<td>Icynite</td>
<td>Ruxolitinib</td>
<td>Treatment of myelofibrosis (MF), post-polycythemia vera MF, and post-essential thrombocytemia MF</td>
<td>ATP competitive inhibition of tyrosine-protein kinases JAK1 and JAK2</td>
</tr>
<tr>
<td>Marqibo®/USA/2013</td>
<td>Talon Therapeutics/ Spectrum Pharmaceuticals</td>
<td>Sphingosmal vincristine</td>
<td>Treatment of Philadelphia chromosome-negative acute lymphoblastic leukemia (ALL)</td>
<td>Inhibition of mitosis</td>
</tr>
</tbody>
</table>
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.  However, their efficacy can be confirmed only by in vivo studies.  Interest in and the enormous importance of the topic motivate more and more researchers for the synthesis of new metal-based drugs 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. 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-A and KP1019 and discussed. 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.

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 complex, also named KP1339, whose aqueous solubility is 35 times higher than that of indazolium salt. Further, disease-specific studies are under way to elucidate the safety and efficacy of this compound in patients. It has been claimed recently that sodium trans-[tetrachloridobis(1H-indazole)ruthenate(III)] is particularly effective in inhibiting the growth of glioma cells, prostate cancer cells, and pancreatic cancer, 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 (differenctiated 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 chemotherapy) other than KP1019 or, alternatively, relapses after responding first positively to the treatment) gastric cancer. Similar efficacy by application of KP1339 against refractory colorectal cancer, melanoma, and lung cancers has also been claimed. 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, 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.

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, 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. 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. 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, 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 analog have revived the literature debate. In particular, by reaction of [DMSO]2RuCl3 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-methylbenzylamino)purine was found to undergo a metal-centered quasireversible one-electron reduction (E1/2 = –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-TL–ruthenium adducts has also been suggested. Exposure of KP1019 or KP1339 adducts with holo-transferrin to glutathione at simulated cancer cytosol concentration was claimed to result in ruthenium(II) species, [RuCl(1-Hind)(GSH)]2– and [RuCl(1-Hind)(GSSG)]3–, based on tandem electrospray ionization mass spectrometry data. In addition, this adduct was found to release both ruthenium and iron when treated with glutathione and ascorbic acid.

In the case of KP1019, the induction of apoptosis via the mitochondrial pathway and oxidative stress is considered to play a major role in the mechanism contributing to cytotoxicity, while interaction with DNA and topoisoquen Unary II 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. 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. 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 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. Dibenzyyl-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, a somewhat subordinate role for ruthenium–DNA interactions in vivo was recently suggested. However, coadministration of KP1019 with ascorbic acid, the main reducing agent in blood, was demonstrated to enhance the interaction of the ruthenium species with DNA in SW480 cells and with plasmid DNA under cell-free conditions 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 chemoprotective agent against kidney and bladder toxicity, a combination chemotherapy regimen involving NAMI-A and an anticancer drug inducing bladder and kidney cytotoxicity was proposed.

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 (SRF) spectroscopy for the investigation of biotransformations of anticancer ruthenium(III) complexes 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(V) clusters and binding of ruthenium(III) to S-donors, amine/imine, and
carboxylate groups of proteins. 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. 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.

The formation μ-oxido-bridged ruthenium(III) oligomers from NAMI-A was suggested in physiological medium at pH 7.4 24 h after dissolution. The addition of ascorbic acid to NAMI-A in physiological buffer results in quantitative reduction of paramagnetic ruthenium(III) ($d^5, S = ½$) to diamagnetic ruthenium(II) ($d^6, S = 0$). The reduction is prevented when the complex binds covalently to HSA. 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. 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, 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. 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. 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.

RAPTA compounds were shown to form adducts with histone proteins. 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, while one other RAPTA-C-related arene-modified compound was shown to inhibit the glutathione transferase enzyme. The reactivity of RAPTA-C in the presence of proteins differs from that of cisplatin. The antimetastasis effect of RAPTA-C is presumably realized by inhibition of angiogenesis and induction of apoptosis in endothelial cells via DNA fragmentation. 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.

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), providing evidence for the significance of histones to the RAPTA-T-mediated mechanism of antimetastasis activity.

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. Both complexes are generally
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. 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. The compound induces apoptosis in the cisplatin-resistant MCF-7 (breast) cancer cell line via multiple pathways. 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. 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. Substitution of PTA in RAPTA-type complexes by fluorescent BODIPY–phosphane, phosphinoferrocene amino acid conjugates, α-diphenylphosphino-functionalized allyl phenyl sulfides, or oligocatenic triarylphosphate ligands 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. 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 LNZ308 with IC50 values of 6.4 ± 2.1, 8.3 ± 2.4, and 5.7 ± 0.9 μM, respectively, which is superior to that found in primary neuronal cultures of the cerebral cortex (IC50 = 20.2 ± 5.1 μM) after 72 h drug exposure.110

A series of \( \rho \)-cymene ruthenium(II) complexes with amino derivatives of biologically active ligands such as flavone, chromone, benzofuran, and \( \rho \)-, meta-, and \( \rho \)-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 \( \mu \)-chlorido-bridge splitting reaction of [(\( \rho \)-cymene)RuCl(\( \mu \)-Cl)]2 with amine or pyridine-derived ligands.111–115 Complex 24 has shown the highest antiproliferative activity with IC50 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 IC50 values in the submicromolar concentration range in an A2780 cell line.

Another series of arenne 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 (ERs), 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 fibroplasts.116 The complexes did not show any affinity for the estrogen receptor ERs, 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 \([\text{p-cymene}]{\text{RuCl}_2}(k\text{N},\text{S-heterocycle})]\), where N,S-heterocycle is 2-mercaptobenzothiazole, 5-chloro-2-mercaptobenzothiazole, 3-methyl-2-mercaptobenzothiazolthion, thiouracil, mercaptonicotinic acid, 2-mercaptobenzoxazole, 2-mercaptobenzimidazole, 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 (IC\(_{50}\) = 2.97 mM) 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 \([\{n^2\text{-benzene}\}\text{RuCl(\text{letrozole})}(\text{PPh}_3)\}\text{BF}_4\) has shown antiproliferative activity in MCF-7 cells superior to that of metal-free letrozole with an IC\(_{50}\) value of 36 ± 6 \(\mu\)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 \(\text{PTA}\) in \(53\), complexes \(54\text{–}56\) 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 \(\mu\)M compared to > 100 \(\mu\)M for the parent complex.

Reaction of \([\text{CpRuCl(PPh}_3)_2]\) with trifluoromethanesulfonate salt of \(\text{N},\text{N}^0\text{-dimethylPTA (dmPTA)}\) in refluxing acetone afforded \(57\), closely related to \(53\), in 87% yield, containing a new ligand, 3,7-\(\text{H},3,7\text{-dimethyl}-1,3,7\text{-triaza-5-phosphabicyclo[3.3.1]}\))monane (HdmoPTA).\(^{123}\) Note that the same ligand can be obtained by saponification of trifluoromethanesulfonate salt of \(\text{N},\text{N}^0\text{-dimethylPTA}\) in methanol in the presence of KOH in 65% yield.\(^{124}\) Complex \(57\) was then used for the preparation of heterodinuclear complexes \(58\text{–}63\).\(^{43,125}\) The antiproliferative activity of \(57\text{–}60\) 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 \(\mu\)M. 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 \([\text{Ni}_8(\mu_4\text{-OH})(\mu_4\text{-OH}_2)(\mu_4\text{-4,4\prime-(buta-1,3-diyne-1,4-diyl)bispyrazolato})_6]_n\) \((64)\) 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. A tenfold increase of cytotoxicity for the macromolecular drugs compared to RAPTA-C in ovarian cancer cell lines A2780, A2780cisR, and Ovcar3 has been claimed.

Thermodynamic and spectroscopic properties of RAPTA-C and other closely related complexes have been studied using DFT methods, and the effect of bidentate carboxylate ligands on the molecular properties of these systems has been described. Theoretical basis for activation of RAPTA-type anticancer complexes has been provided, and attempts to find a correlation between hydrolysis transformations and the anticancer activity were reported. Molecular docking simulations for a series of RAPTA complexes with cathepsin B and kinases have also been performed, but the absolute energies were in separate cases too large and the results very sensitive to calculation details. By using B3LYP hybrid functional and IEF-PCM solvation models, the binding of the hydrolyzed drug \( \left[ (\text{t}^3\text{r}^-\text{C}_6\text{H}_5(\text{CH}_2)_2\text{OH})\text{RuCl}_2(\text{DAPTA}) \right] \) (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. The diaqua complex proved to be slightly more reactive than the chloridoaqua species. The order of the activation free energy was \( G > His > A > Cys > C \) for the chloridoaqua complex and \( G > C > A > His > Cys \) 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 benzimidazolylidene, as well as imidazolylidene carbene, have been synthesized quite recently 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 \( \mu \)M in both cell lines (65) to 2.07 ± 0.93 and 2.40 ± 0.97 \( \mu \)M (68), respectively.

![Image](image-url)
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 μM for 69 to 13 ± 2 and 2.4 ± 0.7 μM 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),¹³⁹ as well as damaging the male reproductive system.¹³⁹

\[
\text{RuCl}_2(\text{bpy})_2\text{Cl}\] \text{PF}_6 (RM175/ONCO4417) 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

\[
\text{RuCl}_2(\text{bpy})_2\text{Cl}\] \text{PF}_6

RM175

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.¹⁴³,¹⁴⁴ 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 formation of thermodynamically more stable adducts with the guanine.¹⁴⁵ 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,¹⁴⁶ 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 \([\text{Ru}^{II}\text{bipy}]^{2+}\) 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.¹⁴⁷ In this context, the ability of \([\text{Ru}^{II}\text{bipy}]^{2+}\) to inhibit human glutathione-S-transferase π (GSTπ), 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.¹⁴⁸ 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.¹⁴⁹ The competition between GSH and the single-stranded 14-mer oligonucleotide for \([\text{Ru}^{II}\text{bipy}]^{2+}\) has been studied under physiologically relevant conditions by ESI-MS and high-resolution ESI-FTICR-MS coupled to HPLC.¹⁵⁰ The data indicated that the only resulting product is the mono-ruthenated 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.¹⁵¹ 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 study¹⁵² 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-H1 and could be internalized via THR-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 documented¹⁵³ that the antiproliferative activity of \([\text{Ru}^{II}\text{arene}]^{2+}\) 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 shown¹⁵⁴ 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.¹⁵⁵ Induction of apoptosis and G_0/G_1 cell cycle arrest mediated by tumor suppressor protein p53 were the main cellular responses observed on treatment of A2780 cells with the para-isomer.¹⁵⁶ The complex did not show mutagenicity, which increases its suitability for further development as an 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.\textsuperscript{158,159} Rapid hydrolysis and loss of cytotoxicity were also found for related cis-dichlorido ruthenium ammine complexes, namely, \([(\eta^6-p\text{-cymene})Ru(NH}_3)Cl_2]\) and \([(\eta^6-biphenyl)Ru(NH}_3)Cl_2]\).\textsuperscript{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.\textsuperscript{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 \((\eta^6\text{-arene})Ru(en)^+\) 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 \((\eta^6-p\text{-cymene})Ru^{12+}\) 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.\textsuperscript{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 \textit{in vitro}, with some exceptions for particular cell lines.\textsuperscript{163,164} Lack of biological activity was noted for the ruthenium-\(p\text{-cymene complex with 2,4-diamino-6-(2-pyridyl)1,3,5-triazine isolated as tetrafluoroborate salt, despite its propensity to interact with DNA.}\textsuperscript{165} The complexes 86 and 87 were shown by a series of biophysical methods to react with DNA oligonucleotides 5\textsuperscript{’}-G3-(T2AG3)3-3\textsuperscript{’}(HTG21) and stabilize G-quadruplex DNA. A change in G-quadruplex conformation led to inhibition of telomerase activity and \textit{in vitro} cytotoxicity.\textsuperscript{166} Telomerase enzyme activity is reported in 80–90\% of tumors, and therefore, G-quadruplex DNA is considered an important target for anticancer chemotherapy.\textsuperscript{167}

Binding a fluorescent ligand derived from phenanthroline, namely, 4,5,9,16-tetraazadibenzo\([3,2,3]\)naphthacene, to a \(p\text{-cymene) Ru^{12+}\) moiety resulted in two complexes, 88 and 89, which retained fluorescence and showed antiproliferative activity in MCF-7 cells with \(IC_{50}\) values of 5.5 ± 2 and 60 ± 5 \(\mu\text{M}\) and accumulation in the nucleus.\textsuperscript{168}

A combination of \(\beta\text{-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.}\textsuperscript{169} Interestingly, another ruthenium–benzene complex with 193, isolated as the hexafluorophosphate salt, \([(\eta^6\text{-benzene})Ru(L93)Cl]PF_6\), showed excellent antitumor activity in Dalton’s lymphoma ascites (DLA)-bearing albino mice with a T/C value of 211\%.\textsuperscript{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 \( \text{2,2',2''-tris(4-pyridyl)cyclohexa[2,1-b:3,4-b']dipyrromethene} \) \( \text{(L56)} \), both mono- and dinuclear, were active in the human breast (MCF-7), lung (AS49), and colon (HCT116) cancer cells.173 On the other hand, \([\text{(p-cymene)Ru(L97)Cl}]\text{Cl}\) and \([\text{(p-cymene)Ru(L98)Cl}]\text{Cl}\), in which the ligands adopt a \( \text{kN, kN} \) 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 \( \text{2,2'-bipyrimidine} \),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 \( \text{A}_1 \) 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 iodido 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 iodido 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, AS49, 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 iodido 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.
Substitution of the coordinated chlorido ligand by neutral monodentate nitrogen- or phosphorus-containing ligands while retaining the $N,N$-chelated ligand and the variation of the arene has also been explored in the search for more effective anticancer drugs, in particular, by generating reactive monoaqua species via photoactivation and selective dissociation of the monodentate ligand and subsequent covalent DNA binding. Conjugation of a potentially photoactive ruthenium(II)-arene complex to homing peptides (HP), such as the dicarba analog of octreotide and the Arg-Gly-Asp (RGD) tripeptide to give conjugates of the type 110, does not affect the photoactivation pathway. The reactive species generated upon irradiation with visible light might react preferably with DNA than with proteins.

Ruthenium–arene complexes with a series of bidentate $N,O$-ligands, such as ketopyridine derivatives, pyridine carboxylic acids, amino alcohols, $\beta$-ketoamines, hydrazides, and hydrazones, were prepared, and most of them exhibited excellent cytotoxicity in vitro. A few organoruthenium complexes with an additional Ru–C bond emerged via coordination of $N,C$-ligands and in particular, neutral and ionic cycloruthenated benzimidazoles and indoles have quite recently been reported. Compounds 111–113 prepared in good yields by reaction of benzimidazole ligands and [(η⁶-p-cymene)RuCl₂]₂ in dichloromethane were tested for cytotoxicity against HT29 (colon), T47D (breast), A2780, and A2780cisR (ovarian) cells. Complex 111 was found to be considerably more potent in the inhibition of cell proliferation than the metal-free benzimidazole ligand, and together with 113 superior to cisplatin in the first two cancer cell lines. The IC₅₀ values for 113 in these two cancer cell lines are at 2.40±0.13 and 4.37±0.11 μM compared to 9.5±0.2 and 38±2 μM for cisplatin. In addition, all complexes showed non-cross-resistance with resistance factor (RF) < 2, defined as the ratio of the IC₅₀ for A2780cisR to the IC₅₀ for parent line A2780. The compounds affect the cell cycle causing the arrest of cells in the S phase, induce early apoptosis, and strongly bind to HSA.

Excellent candidates for further in vivo studies are the highly cytotoxic neutral and ionic complexes 114–118 and 119–123, respectively. Most of them delivered IC₅₀ values <2 μM in breast cancer cell lines MCF-7 and MDA-MB-231, while the corresponding ligands were significantly less cytotoxic (IC₅₀ from 12 to ~100 μM).

With the aim of targeting the steroidal receptors, a steroidal conjugate 17-$\alpha$-[2-phenylpyridyld-4-ethynyl]19-nortestosterone 124 and the ruthenium-p-cymene species 125, in which the conjugate acts as a $kC,kN$-ligand, were prepared. Complex 125 showed by a factor of 8 higher antiproliferative potency (IC₅₀ value of 7.4±0.1 μM) than cisplatin and by a factor of >14 than both the metal-free conjugate L124 and complex 126 in T47D (breast) cells, circumventing resistance to cisplatin in A2780cisR cells. Clear synergism between the ruthenium center and the steroidal component has to be noted. Combination of inactive components 124 and 126 in terms of cytotoxicity resulted in a highly potent ruthenium anticancer agent 125.
In contrast, similar complexes with bidentate O,O- ligands, derived from the biomolecule maltol, such as hydroxypyrones\(^{199}\) 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 hydroxypyrone and hydroxypyridone ligands by pH potentiometry\(^ {1}\), \(^ {1}H\) 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 \([\text{Ru}^\text{II}(\text{p-cymene})_2(\text{OH})_4]^+\) was observed accompanied by release of metal-free ligand (HL = hydroxypyrone or hydroxypyridone), along with generation of a hydroxido complex \([\text{Ru}^\text{II}(\text{p-cymene})(\text{L})(\text{OH})]\). The hydroxythiopyrone and hydroxythiopyridone ligands form significantly more stable complexes with the Ru\(^{II}(\text{p-cymene})\) 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 \([\text{Ub} + \text{Ru}^\text{II}(\text{p-cymene})]^+\) adducts, while complexes with thiothiopyronato 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 \([\text{Leu}^5]-\text{enkephalin}\) to a hydroxypyrone and further complexation to a Ru\(^{II}(\text{p-cymene})\) 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 ± 5 \(\mu\)M compared to >640 \(\mu\)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 ± 52 \(\mu\)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 \textit{in vitro} with IC\(_{50}\) values in the low \(\mu\)M concentration range, which correlated with their potency to inhibit topoisomerase IIα 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 [(η6-arene)-Ru(curcuminato)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 κ2P,S-chelating ligands 134–138 were prepared from [(η6-p-cymene)RuCl2] and o-diphenylphosphino-functionalized alkyl phenyl sulfide and sulfoxide ligands Ph2P(CH2)nS(O)xPh (n = 1–3, 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 [Ru(η6-p-arene)Cl2] 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 59.6/5.6, 168/9, 205/4, and 46.7 ± 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.
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. 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. Lower cytotoxicity was found for semicarbazonate 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. A potential dinucleating ligand L158 was allowed to react with [((Z)-6-arene)RuCl2]2, where arene = p-cymene or C6H5C3H6COOH, to give two dinuclear Z6-arene ruthenium thiosemicarbazone complexes, [(Ru(p-cymene)Cl)2(L158)]Cl2 and [(Ru(C6H5C3H6COOH)Cl)2(L158)]Cl2. The dinucleating ligand L158 was considerably more active against esophageal cancer cells WHCO1 than benzaldehyde thiosemicarbazonate (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)Cl)2(L158)]Cl2 and [(Ru(C6H5C3H6COOH)Cl)2(L158)]Cl2, respectively.

A series of another type of diruthenium arene complexes with thiosemicarbazones (159–162) was prepared starting from [Ru(η5-arene)Cl2], and 5-nitrofuraldehyde thiosemicarbazones. Complexation of 5-nitrofuraldehyde 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)(EPh3)3] or [RuCl3(EPh3)3] (E = P or As). For example, 2-hydroxybenzaldehyde thiosemicarbazono reacts with [RuHCl(CO)(PPh3)3] in benzene under reflux to form two different products, 163 and 164, which were separated by column chromatography. 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 HeLa (cervical) 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 A549 (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).

Classical ruthenium thiosemicarbazonates with or without ancillary organic coligands, which do not contain a Ru–C bond, have also been reported as potential anticancer agents. Ruthenium(III) complex 168 was prepared by reaction of
[RuCl3(PPh3)3] with 2-hydroxybenzaldehyde N-ethylthiosemicarbazone in a 1:1 or 1:2 molar ratio in boiling benzene. 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. An important finding of those first studies was that the intact complex cations were responsible for their biological activity. 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. Today, polypyridyl ruthenium complexes are promising anticancer drug candidates.

The classical mechanism of anticancer action of ruthenium polypyridyl complexes is DNA intercalation, the first X-ray structures with atomic resolution were published recently, showing intercalative/semi-intercalative interaction of Α1[Bu(TAP)](dipps)3+ (TAP = L178 in Figure 3) with d(TCGGCGCCGA) DNA strands. Along with that, other mechanisms are assumed to be operative, namely, stabilization of G-quadruplex DNA and reactive oxygen species (ROS) formation, followed by cell death via the mitochondrial pathway, inhibition of ROS formation, mitochondria targeting without involvement of ROS, and inhibition of enzymes such as histone deacetylase, pancreatic phospholipase A2, and telomerase (due to G-quadruplex DNA stabilization). 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 O2, when falling back from a photon-induced excited state to a state with lower energy. In particular cases, photoinduced covalent ligand–peptide bond formation may also play a role in the mechanism of action. 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 interaction. The ligand loss is often photoinduced, referring to the previously mentioned PDT principle. The unique photophysical properties of ruthenium polypyridyl complexes also renders them interesting for analytical purposes.

Figure 2 Stereoisomers of tris(1,10-phenanthroline)ruthenium(II).
Intercalating Ruthenium Polypyridyl Complexes

One of the most powerful intercalating ligands in ruthenium polypyridyl complexes is dipyrido[3,2-a:2,3-c]phenazine, dppz, L174 (Figure 3). [Ru(bpy)2(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 L- and D-[Ru(phen)2(dppz)]2+, but with a six- to tenfold relative luminescence quantum yield increase for the D-enantiomer.[279,280] The photoluminescence is most probably due to the stabilization of a 3MLCT (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 ($K_b > 10^6$ M$^{-1}$)[280] led to the development of new Ru-dppz complexes displayed in Figure 4 as potential anticancer drugs.

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

Intercalating Ruthenium Polypyridyl Complexes

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Results

Methylation at positions 5 and 6 of the phen coligands (193) improves the DNA-binding affinity with calf thymus (CT)-DNA, $K_b = \text{1.8} \pm \text{0.1} \times \text{10}^{5} \text{M}^{-1}$ for 193 versus $1.0 \pm 0.05 \times \text{10}^{5} \text{M}^{-1}$ for the parent complex $[\text{Ru(phen)}_2(\text{dppz})]^2+$.286 as well as the cytotoxicity in a non-small cell lung carcinoma NCI-H460 cell line. The IC$_{50}$ values for 193 and $[\text{Ru(phen)}_2(\text{dppz})]^2+$, are 6.5 ± 1.2 and 20 ± 1 μM, respectively. The bulkier 3,4,7,8 methyalted phen ligands in 194 decrease DNA binding affinity as well as cytotoxicity. Dimethylation of the dppe ligand in 195 seems to hinder DNA intercalation and leads to a decrease in DNA binding affinity, $K_b = \text{3.3} \pm \text{0.3} \times \text{10}^{5} \text{M}^{-1}$ for 195, but in contrast to the unsubstituted dppe primary ligand, the DNA-binding affinity increases with bulkier coligands ($K_b = \text{6.0} \pm \text{0.3} \times \text{10}^{5} \text{M}^{-1}$ for 196 vs. 1.0 ± 0.09 × 10$^5$ 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: IC$_{50} = \text{10.0} \pm \text{0.9}$, 8.0 ± 0.7, and 7.0 ± 0.5 μM for 194–196 in the same cell line, respectively. The substitution of the dppe 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 dppe scaffold (183–186) reduces the cytotoxicity and DNA binding affinity in comparison with the parent complexes $[\text{Ru(phen)}_2(\text{dppz})]^2+$ and $[\text{Ru(bpy)}_2(\text{dppz})]^2+$, 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 hdppe, L180, coligands. This is probably due to hydrogen bonding interactions of this ligand with DNA.283 An amino group at position 7 of the dppe 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 ± 10$^5$ 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 dpf 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 C0/G1 phase arrest and apoptosis via mitochondrial pathways.284 192 with a carboxylic acid methyl ester group at position 7 of dppe and L175 as coligands shows moderate DNA binding affinity to CT-DNA, $K_b = \text{1.39} \times \text{10}^{5} \text{M}^{-1}$, that is two orders of magnitude lower than that of the parent complex $[\text{Ru(L175)}(\text{dppe})]^2+$, $K_b = \text{2.10} \times \text{10}^{5} \text{M}^{-1}$.241 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 (IC$_{50}$= 8.42 ± 0.68 and 5.85 ± 0.60 μM for 190 and cisplatin, respectively).286

Dichlorination at positions 7 and 8 of the dppe scaffold and the use of phen as coligands lead to a lower DNA binding constant of 198 ($K_b = \text{2.74} \pm \text{1.6} \times \text{10}^{5} \text{M}^{-1}$) when compared to that of the parent complex $[\text{Ru(phen)}_2(\text{dppz})]^2+$, $K_b > \text{10}^{5}$, estimated for both enantiomers.286 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 ± 2.53 μM, 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.285 The use of two dppe ligands lowers the DNA binding ability. This was shown for $[\text{Ru(dppe)}_2(\text{phen})]^2+$ (200) and $[\text{Ru(dppe)}_2(\text{dppe})]^2+$ (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 dppe 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 (IC$_{50}$= 4.0 ± 1.2 for 202 vs. 13.8 ± 3.0 μM for cisplatin). Although 202 is a strong DNA binder with $K_b > \text{10}^{5}$ 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.243

Figure 4  Ruthenium polypyridyl complexes with dppz-derived ligands.
Imidazole[4,5-f][1,10]phenanthroline (L175 or ip) is another important building block for DNA-binding ruthenium polypyr-ridyl 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 IC50 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 μM). In addition, 204 is less cytotoxic than cisplatin in the nonmammogenic cell lines H568 (IC50 = 30.6 ± 5.7 vs. 1.8 ± 0.7) and HeK-2 (74.3 ± 4.2 vs. 10.3 ± 0.3 μM). The compound reduces the mitochondrial membrane potential (ΔΨm) inducing apoptosis via the mitochondrial pathway.288 Complexes 207–210 showed DNA binding affinity with Kb > 105 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, Kb = 1.61 ± 0.07 × 105 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.299 Complexes 212 and 213 are comparable to 207–210 in terms of cytotoxicity and DNA interaction.292 Substitution of the bpy coligands by 2,9-dimethyl-1,10-phenanthroline ligands leads to an increase in cytotoxicity, as shown by 217 and 218.289 Complexes 215 and 216 are even more active in a panel of cancer cell lines (BEL-742, HeLa, MCF-7, and MG-63), their IC50 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 L-enantiomer seems to be more active than the D 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, qphhz, are strong DNA intercalators. In addition, [Ru(bpy)(qphh)]2+ and [Ru(phen)(qphh)]2+ show IC50 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.300 The DNA-binding complex [Ru(phen)]2(dmso)ClCl 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 interactions293–295 or by covalent binding after solvolysis.297,298

Mitochondria Targeting Ruthenium Polypyridyl Complexes

As already shown previously, mitochondria are attractive cellular targets for ruthenium polypyridyl complexes.292,293,294 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 (IC50 = 7.9 ± 0.7 for 219 vs. 17.8 ± 2.6 μM for cisplatin) and a
BEL-7402 cancer cell line (IC$_{50}$ = 10.3 ± 0.9 for 219 vs. 20.1 ± 2.1 µM for cisplatin). Importantly, this complex was far less toxic than cisplatin in a non-tumorigenic LO2 cell line (IC$_{50}$ = 77.4 ± 4.3 for 219 vs. 11.2 ± 0.6 for cisplatin). Complex 220 bearing three β-carboline (norharman) ligands and one tridentate terpy ligand is up to 30 times as active as cisplatin in a series of cancer cell lines and less toxic than cisplatin in a normal kidney cell line, displaying high selectivity for cancer cells. Although this complex is a strong CT-DNA binder (K$_{b}$ = 9.49 × 10$^{10}$ M$^{-1}$), this is presumably not the main reason for its high biological activity. Instead, increase of the ROS level in the cells and the lowering of the mitochondria membrane potential, with subsequent release of cytochrome c and p53-mediated apoptosis, may be involved in the drug mechanism of cytotoxicity.

In vivo studies showed a better tumor inhibition rate for 220 than for cisplatin in mice bearing MCF-7 (52.1% vs. 47.9%) and HepG2 (58.2% vs. 39.3%) xenografts. Norharman as a metal-free ligand was far less active. All this taken together, 220 is a good candidate for further development as an anticancer drug.

G-Quadruplex DNA Targeting Ruthenium Polypyridyl Complexes, Telomerase Inhibition

Telomerase, an enzyme protecting the telomeric ends of chromosomes, is highly active in cancer cells, while in most normal somatic cells, it is not detectable, making it an attractive anticancer target. Telomeric DNA contains a 3' single-stranded overhang consisting of repeated d(TTAGGG) sequences. This overhang is not accessible for telomerase; consequently, molecules that stabilize G-quadruplex DNA are able to inhibit telomerase by depletion of its substrate, leading to senescence and telomere shortening in cancer cells. Chiral L- and D-221 were synthesized using corresponding tartrate salts as enantiopure catalysts. Both complexes showed high binding affinity (K$_{b}$ > 10$^{10}$) 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 L-enantiomer being far more potent than its mirror counterpart. Consistently, L-221 is also more active in different cancer cell lines than Δ-221 and a more potent telomerase inhibitor. The L-complex showed interesting in vitro selectivity for a HepG2 liver carcinoma cell line, IC$_{50}$ = 4.4 ± 0.3 versus 13.6 ± 1.1 for L-40 and cisplatin, respectively. A- and Δ-222 behave analogously; again, the A-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.
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. 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.

A ‘light switch’ effect superior to that of the known [Ru(bpy)2(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.

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). In contrast, the luminescence of the well-known ‘light switch’ complex [Ru(bpy)2(dppz)]2+, 229, originates from a 3MLCT excited state, with a much longer lifetime (750 ns). 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). This is a much lower ‘on–off’ ratio than for 229 (~106). However, in contrast to 229, complex 227 is able to cleave CT-DNA under irradiation. The reason is, probably, that the 3MLCT excited state of 229 cannot interfere with the surrounding molecules (e.g., water and oxygen) after DNA intercalation and consequently no 1O2 can be produced, in contrast to 227, where the LC excited states are energetically lowered with respect to the 3MLCT 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 IC50 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.
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 IC50 value for 233 in a HL60 cancer cell line is 262 µM after a short visible light exposure, displaying the highest reported photodynamic index (PI) of 1747. Notably, cisplatin displays an IC50 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. The least active of these three complexes, 235, displays a PI of 104, still being in the range of the most phototoxic ruthenium polypyridyl complexes reported so far. 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 IC50 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. Several cyclometalated ruthenium(II) polypyridyl complexes with photoactive potential have been described recently as well.

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. 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.

A series of cyclometalated ruthenium polypyridyl complexes showing impressive anticancer activity were reported. One of the most active compounds of this series, complex 236, showed in vitro 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. The observation that the in vitro cytotoxicity of 236 doubles upon irradiation 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. 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+, where harder radiation (λ < 500 nm) is required for ligand release. 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. Several 2,2′-biquinoline, biq-containing ruthenium polypyridyl complexes were also activated by long-wavelength light.

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
Results

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Ruthenium Compounds as Antitumor Agents: New Developments

3 > 450 nm causes photoexcitation 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. [Ru(dpq)2(phf)]77, 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 [Ru(dpq)2(phf)]77, 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. 454 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. 235 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 10.60 cell line is 1.6 ± 0.2 µM versus >300 µM 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 (IC50 = 3.1 µM) (essentially, cisplatin exhibits identical dark and light IC50 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 also seems to be in a three-dimensional A549 cancer cell model, consisting of cell spheroids with a diameter of about 600 µm, displaying a more realistic solid cancer model in comparison with layered cells. 455 The IC50 value of light-irradiated 237 (21.3 ± 2.3 µM) 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 IC50 = 64.6 ± 4.7 µM) 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 µM. Cisplatin exhibits a low MCR index (12.4) in the same model with an IC50 value of 42 ± 3.6 µM. [Ru(dpq)2(phf)]77, taken as control, showed modest IC50 values and Pt(VI) 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. 232 Mesoporous silica nanoparticles grafted with a terpy- and dppz-containing ruthenium(II) complex show photodisruption of the complex upon irradiation. 444

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 bidentate and polydentate ligands. 

cis-Dichloroditetramethylenediaminuruthenium(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%, 239 which and polydentate ligands. Other Mononuclear Ruthenium Complexes


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Another trans-configured ruthenium(II) complex trans-[RuII(Pir)2(CH3CN)2] 250 resulted from the reaction of a sodium salt of piroxicam with RuCl3·H2O 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·C12H2O 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 (EJ) 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-[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-α-cymene complexes, was also used for the preparation of a series of Werner-type ruthenium(II) 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)]$_2$] (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-435 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-glucoside sodium salt, or the 6′-aminolactose derivative in ethanol, did not show appreciable antiproliferative activity against the melanoma cell line A375.344

Coordination of dimethylthiocarbamates, 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 (NCl-H1975) with some of them showing IC50 values in the nanomolar concentration range.345,346
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 (tatpy), and 2-picolinate (pic) have been reported to show higher antiproliferative activity (IC$_{50}$ values ≈ 0.4–10 $\mu$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$_2$O) (IC$_{50}$ values 11–17 $\mu$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 photothermochemotherapy 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 ($\text{262}$) with a thioether ligand and the isolation of two complexes [Ru(terpy)(bpy)](N-acetyl-L-methionine)]Cl$_2$ ($\text{263}$) and [Ru(terpy)(bpy)](o-biotin)]Cl$_2$ ($\text{264}$). 348 Complexes $\text{263}$ and $\text{264}$ were found to be markedly more stable toward hydrolysis under physiological conditions in the dark than the chlorido complex $\text{262}$. 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 $\text{263}$ or $\text{264}$, as was proved by monitoring the evolution of the UV–vis spectrum of a solution of $\text{264}$ upon blue-light ($\lambda = 452$ nm) irradiation and comparison with the optical spectrum of [Ru(terpy)(bpy)](H$_2$O)]$^{2+}$. 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-cyanoauracil (SCNUI), known as an inhibitor of pyrimidine catabolism, and, closely related to 5-fluourouracil, a clinically used drug for treatment of colorectal and breast cancers. 346 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(terpy)](SCNUI)]Cl$_2$ ($\text{265}$) via treatment of [Ru(terpy)]Cl$_2$ with AgCF$_3$SO$_3$ and subsequent reaction with SCNUI and comparison of the binding ability and cytotoxicity of 265 upon light irradiation with those of [Ru(terpy)](CH$_3$CN)$_2$]+ ($\text{266}$) and [Ru(terpy)] (CH$_3$CN)$_2$Cl]$^{+}$ ($\text{267}$). While all complexes bind to DNA, complex $\text{265}$ shows significantly higher toxicity than $\text{266}$ in HeLa cells upon light irradiation by releasing one SCNUI 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 $\text{268}$ and $\text{269}$. 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 $\text{270}$ and $\text{271}$.

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

Results

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IC_{50} 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 (IC_{50} values ranged from 25.2 to 50.0 \mu M), which, in addition, is much less toxic against normal human fibroblast cells Hs68 (IC_{50}> 400 \mu 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 enables 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-hydroxypridine-2,6-dicarboxylic acid (hpa),355 or with Schiff bases 272–275356–360 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 \( \delta \)-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)(DMSO)Cl2] (280), prepared from [Ru(DMSO)4Cl2] and 1,4,7-triethylcyclononane ([9]aneS3), as well as [Ru([9]aneS3)2Cl2]PF6 (281) and [Ru([9]aneS3)(DMSO)Cl2]PF6 (282) resulting from treatment of 280 with 1 and 2 equiv of AgPF6, respectively, in DMSO.369 While for the preparation of 279, [Ru([9]aneS3)(DMSO)Cl2]Cl2 (283)369 or [Ru([9]aneS3)Cl2] (284)367 were exploited. By dissolving 280 in MeCN at 50°C and allowing for slow crystallization, the complex [Ru([9]aneS3)(MeCN)2][Cl2PF6] (281) was isolated, which exhibited low cytotoxicity at concentrations >200 \mu M in the osteosarcoma cell line MG63.370 Mild cytotoxicity was also reported for [Ru([9]aneS3)(glycine)Cl] (282).368 Reduction of ruthenium(III) to ruthenium(II) occurs in the presence of AgCl2SO4 in MeCN resulting in the isolation of [Ru([9]aneS3)(MeCN)][Cl2SO4] (285).360 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.467 Complexes 277 were moderately cytotoxic against the human mammary carcinoma cell line MDA-MB-231 (IC_{50}=81 and 124 \mu M), while compounds 279 exhibited IC_{50} values >300 \mu 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, while complexes 279 basically remain intact in solution. Quite surprisingly, complexes of the type 278, resulting from the reaction of fac-[RuCl(DMSO)][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.467 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 (lev), 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 AS49 (lung)
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 \([\text{Ru}(\eta^5-p\text{-cymene})(\text{O}_2\text{O}'\text{-quinolonato})(\text{H}_2\text{O})]^+\) 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 applications\(^{373}\) and is currently being evaluated in phase I/II clinical trials for the treatment of colon cancer.\(^{374}\) Metal–curcumin complexes,\(^{375}\) 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.\(^{376,377}\)

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\(^a\)-positive noninvasive breast adenocarcinoma), and MDA-MB-231 (ER\(^a\) 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 \(\mu\text{M}\).

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 (\(\mu\text{M}\)) than those produced in activated macrophages\(^{383}\) 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 vessels\(^{384}\) 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 interest\(^{387}\) (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 \(\pm\) 0.33 and 17.75 \(\pm\) 6.82 \(\mu\text{M}\) compared to 54.73 \(\pm\) 33.30 \(\mu\text{M}\) in a nontumorigenic CHO (ovarian) cell line. In addition, inhibition of melanoma tumor growth in an in vivo assay in mice was observed.\(^{391}\)
Similarly, in vitro and in vivo anticancer activity was found for a series of complexes of the type \(291\) and \(292\). 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 \(291\) \((L = \text{isn or im})\) was reported.

Complexes \(291\) \((L = \text{inaH or ina-Tat48-60})\) were found to release NO upon one-electron reduction with the formation of trans-\([\text{Ru(NH}_3)_4(L)(\text{H}_2\text{O})])^3+\). 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 \((\text{cation})^+\text{[cis-MCl}_4(\text{NO})(\text{Hazole})]^–\) and \((\text{cation})^+\text{[trans-MCl}_4(\text{NO})(\text{Hazole})]^–\), where \(M = \text{Ru or Os}, (\text{cation}) = (\text{H}_2\text{ind})^+, (\text{H}_2\text{pz})^+, (\text{H}_2\text{bzim})^+, (\text{H}_2\text{im})^+\) or \((\text{nBu}_4\text{N})^+\), and Hazole = 1H-indazole \((\text{Hind})\), 1H-pyrazole \((\text{Hpz})\), 1H-benzimidazole \((\text{Hbzim})\), or 1H-imidazole \((\text{Him})\), on cytotoxicity and cell line selectivity have been reported. In contrast to most pairs of analogous ruthenium and osmium complexes known, ruthenium nitrosyl complexes with azole heterocycles yielded IC\(_{50}\) values in the low micromolar concentration range differing by factors \((\text{Os/Ru})\) of up to \(~110\) and \(~410\) in CH1 and SW480 cells, respectively; however, the reasons for such a striking difference are still not known.

**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.

Highly cytotoxic trinuclear organoruthenium(II)–maltol complexes have been quite recently reported. Reaction of \(\text{Ru}_3(\text{CO})_{12}\) 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 diposophine ligand in the presence of trimethylamine N-oxide dihydrate in CH\(_2\)Cl\(_2\) yielding complexes \(294–296\).

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*Figure 7* X-ray diffraction structure of ruthenium nitrosyl complex \(290\).
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 μM 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-bicyclopophosphosphate-1,2-O-isopropylidene-α-D-glucopyranoside, yielded products 297–299. 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 μM), while 299 was devoid of cytotoxicity (IC50 > 300 μM) in the first four cell lines and displayed moderate cytotoxicity (IC50 of ~60 μM) 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. 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. 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 ± 2, 52 ± 7, 22 ± 3, and 32 ± 3 μM, 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. 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), naproxen (Hnpx), or ketoprofen (Hket). 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. 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 atoms have been prepared by reaction of [(η⁶-p-cymene)Ru₂(μ-Cl)₂Cl₂] with aromatic thiols. The formation of [(η⁶-p-cymene)Ru₂(μ-SR)₃]⁺ was found to proceed through the neutral di(ruthenium–arene) complexes with two thiolato bridging ligands, [(η⁶-p-cymene)Ru₂(μ-SR)₂Cl₂]. The latter compounds were successfully used for the synthesis of dinuclear complexes with mixed thiolato bridges of the type [(η⁶-p-cymene)Ru₂(μ-SR)₂(μ-SR’)]⁺. Most of the compounds prepared are highly cytotoxic against human ovarian cancer cell lines A2780 and A2780cisR. For example, the complex [(η⁶-p-cymene)Ru₂(μ-SC₆H₄-p-tBu)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)Ru₂(μ-SePh)₃]⁺ and of [(η⁶-p-cymene)Ru₂(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. Intriguingly, Ru₆ wheels resulting from treatment of Ru₃(CO)₁₂ with 4-iPrC₆H₄SH under an inert atmosphere at elevated temperature were noncytotoxic against SW480 (colon) cancer cells.

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. The complex anion in [NEt₄][Ru₃(μ₂-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 transcriptomic and protein expression levels of the main components involved.

Attempts to optimize the conditions for using complexes 305 and 306, prepared recently, in the in vivo photodynamic therapy on mice bearing an ectopic human oral carcinoma xenograft, which is a multiparameter task, have been described. 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...
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) (IC\(_{50}\) > 100 \(\mu\)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 \(\mu\)M, respectively). The underlying mechanism of cytotoxicity presumably involves significant DNA binding affinity and the ability to inhibit the human topoisomerase I at a very low concentration (8 \(\mu\)M).

![Figure 9](image_url) X-ray diffraction structure of heterodinuclear complex 308.
Another interesting trinuclear diruthenium(III)–platinum(II) hybrid 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. The complex reduces the motility of breast and lung cancer cells more efficiently than NAMI-A.

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, hexacationic prisms, and octacationic cubes 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 recently; therefore, we will only give a short overview of the latest results reported.
In particular, a series of prismatic metalla-cages has been developed. These compounds were shown to incorporate planar complexes and organic molecules and to release them after uptake into the cancer cells. Interesting features of such cageplex systems are that (i) the physical properties of the host are retained and therefore water-insoluble guests can be delivered; (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; (iii) due to their large particle size, the enhanced permeability and retention (EPR) effect might be exploitable. 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]_{C=310} and [Pd(acac)₂]_{C=310}, respectively, while [Pt(acac)₂] and [Pd(acac)₂] were inactive because of insolubility. 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. 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. 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. Compound 312 exhibited a higher cytotoxicity than 310 (IC_{50} = 3 ± 1 μM in A2780 cell line). 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. The oxidation of GSH may in part explain the higher cytotoxicity of 312 compared to 310 and 311, which are equipotent. 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.

Pyrene can be included in metalla-cages even if it bears a functionalized side arm. This ability was exploited to develop a variety of cageplex systems as a carrier for anticancer drugs. 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 cycloplatinate 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. Incorporation of the RAPTA-derived complexes

![Figure 10](image-url) A schematic representation of a host-guest complex and the structure of metalla-cage 313.
Results

Ruthenium Compounds as Antitumor Agents: New Developments

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Cytotoxicity of host ruthena-cages, guests, and host-guest complexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>A2780 (IC$_{50}$ (µM))</td>
</tr>
<tr>
<td>310</td>
<td>23 ± 2.1</td>
</tr>
<tr>
<td>312</td>
<td>3.7 ± 1.1</td>
</tr>
<tr>
<td>313</td>
<td>4.1 ± 0.07</td>
</tr>
<tr>
<td>G1–G2</td>
<td>n.d.</td>
</tr>
<tr>
<td>G3</td>
<td>&gt; 25</td>
</tr>
<tr>
<td>G4</td>
<td>19.7 ± 6.5</td>
</tr>
<tr>
<td>G5</td>
<td>&gt; 25</td>
</tr>
<tr>
<td>G6–G14</td>
<td>n.d.</td>
</tr>
<tr>
<td>G15</td>
<td>5.9 ± 0.6</td>
</tr>
<tr>
<td>G16</td>
<td>n.d.</td>
</tr>
<tr>
<td>G17</td>
<td>8.7 ± 0.9</td>
</tr>
<tr>
<td>G1–312</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>G2–312</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>G3–312</td>
<td>5.0 ± 2.9</td>
</tr>
<tr>
<td>G4–312</td>
<td>4.7 ± 2.4</td>
</tr>
<tr>
<td>G5–312</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>G6–310</td>
<td>2.1 ± 0.14</td>
</tr>
<tr>
<td>G6–312</td>
<td>1.3 ± 0.05</td>
</tr>
<tr>
<td>G7–310</td>
<td>0.4 ± 0.03</td>
</tr>
<tr>
<td>G7–312</td>
<td>0.4 ± 0.10</td>
</tr>
<tr>
<td>G8–310</td>
<td>5.6 ± 0.9</td>
</tr>
<tr>
<td>G8–312</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>G9–310</td>
<td>0.3 ± 0.03</td>
</tr>
<tr>
<td>G9–312</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>G10–310</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>G10–312</td>
<td>1.7 ± 0.06</td>
</tr>
<tr>
<td>G11–310</td>
<td>1.2 ± 0.03</td>
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<tr>
<td>G11–312</td>
<td>1.4 ± 0.18</td>
</tr>
<tr>
<td>G12–312</td>
<td>1.2 ± 0.1</td>
</tr>
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<td>G13–310</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>G13–312</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>G13–313</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>G14–312</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>G14–313</td>
<td>1.9 ± 0.4</td>
</tr>
<tr>
<td>G15–312</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>G15–313</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>G16–312</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>G16–313</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>G17–312</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>G17–313</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.6 ± 0.6</td>
</tr>
</tbody>
</table>

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. 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.

5-Fluoro-2'-deoxuryridine (flouxuridine) is a derivative of the synthetic nucleoside 5-flourouracil and known for its high activity against metastases. Pyrenyl functionalized nucleic acids are of interest because of the fluorescence and the intercalation ability of the pyrene unit. Fluorozidine-derived careplex 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 different 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.
Dendrimers are branched molecules with a variety of properties, which were exploited as drug delivery vectors. The incorporation of pyrene-modified lipophilic dendrimers differing in size within 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. 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. 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 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.

The high cytotoxicity of thiolato-bridged dinuclear arene ruthenium complexes 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. Porphyrin-based octanuclear arene ruthenium cubes show interesting photodynamic activity, as well as many arene ruthenium-based rectangles, and demonstrate higher cytotoxicity than cisplatin.

Ruthenium-Based Dendrimers

Ruthenium-based dendrimers with a very low polydispersity. Their synthesis is well established. 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
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. 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. 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. A series of arene ruthenium(II)-based dendrimers show a clear correlation between particle size and cytotoxicity in the A2780 and the A2780/cisR (cisplatin-resistant) cancer cell lines (see Table 3). The most cytotoxic compound in this series is more active than cisplatin. The dendritic systems are more potent than their mononuclear analogs, 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.

Figure 11 Pyrene-derived dendrimeric guest molecules.
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.

Two zero-generation nitrile-functionalized DAB-derived dendrimers containing [Ru(η^5-C_5H_5)(PPh_3)_2]^+ or [RuCl(dppe)]^+ (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.

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.
Results

42 Ruthenium Compounds as Antitumor Agents: New Developments

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>A2780 (IC50 (μM))</th>
<th>A2780 (IC50 per metal center (μM))</th>
<th>A2780cisR (IC50 (μM))</th>
<th>A2780cisR (IC50 per metal center (μM))</th>
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</thead>
<tbody>
<tr>
<td>314</td>
<td>4</td>
<td>50 ± 1.4</td>
<td>200</td>
<td>32 ± 0.8</td>
<td>208</td>
</tr>
<tr>
<td>315</td>
<td>4</td>
<td>27 ± 1.3</td>
<td>108</td>
<td>25 ± 1.3</td>
<td>100</td>
</tr>
<tr>
<td>316</td>
<td>8</td>
<td>22 ± 1.2</td>
<td>176</td>
<td>15 ± 1.4</td>
<td>120</td>
</tr>
<tr>
<td>317</td>
<td>8</td>
<td>10 ± 0.3</td>
<td>80</td>
<td>9 ± 0.3</td>
<td>72</td>
</tr>
<tr>
<td>318</td>
<td>16</td>
<td>6.0 ± 1.0</td>
<td>96</td>
<td>13.2 ± 1.4</td>
<td>211</td>
</tr>
<tr>
<td>319</td>
<td>16</td>
<td>2.1 ± 0.1</td>
<td>33.6</td>
<td>2.1 ± 0.1</td>
<td>33.6</td>
</tr>
<tr>
<td>320</td>
<td>32</td>
<td>2.9 ± 0.1</td>
<td>92.8</td>
<td>9.9 ± 1.2</td>
<td>318</td>
</tr>
<tr>
<td>321</td>
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<td>51.2</td>
<td>2.1 ± 0.1</td>
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</tr>
<tr>
<td>322</td>
<td>&gt;200</td>
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<td>128</td>
<td>42 ± 5.1</td>
<td>168</td>
</tr>
<tr>
<td>323</td>
<td>8</td>
<td>23 ± 2.1</td>
<td>184</td>
<td>78 ± 9.4</td>
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<tr>
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<td>4 ± 1.3</td>
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<td>4 ± 0.3</td>
<td>32</td>
</tr>
<tr>
<td>326</td>
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<td>172</td>
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<tr>
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<td>160</td>
<td>n.d.</td>
<td>n.d.</td>
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<tr>
<td>328</td>
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<td>21 ± 5.0</td>
<td>168</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>329</td>
<td>8</td>
<td>20 ± 5.0</td>
<td>160</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>330</td>
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<td>174.0 ± 40</td>
<td>696</td>
<td>72.8 ± 1.6</td>
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</tr>
<tr>
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<td>35.6</td>
<td>25.0 ± 5.0</td>
<td>100.0</td>
</tr>
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<tr>
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<tr>
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<td>46.4</td>
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<td>337</td>
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<td>64.0</td>
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<td>35.2</td>
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<td>18.8 ± 0.6</td>
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</tr>
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<td>&gt;200</td>
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<td>&gt;200</td>
<td>&gt;200</td>
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<td>93.6 ± 7.0</td>
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<td>1.5</td>
<td>25</td>
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</tr>
</tbody>
</table>

n = 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, it is difficult to overestimate the role of the μ-chlorido-bridge splitting reaction of the dimeric precursor [Ru(arene)(μ-Cl)Cl]2, which can now be prepared more efficiently by microwave heating, 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)(μ-Cl)Cl]2 with certain dinucleating ligands, and bi-, tri-, or tetratetate building blocks, in the presence of AgCF3SO3 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 polyphenyldim 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. One in vivo animal trial showed a better tumor growth inhibition than cisplatin, a very promising result. 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.245,249 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.25,83 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.43,44,45 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 biochemical techniques, proteomics and genomics, in the investigation of underlying mechanisms of action of developing anticancer drugs increases.

Exploitation of EPR spectroscopy8,29, X-ray crystallography7,10, X-ray absorption and synchrotron-based micro-XRF spectroscopy13,14, 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),15 surface-enhanced laser desorption/ionization-time-of-flight mass spectrometry (SELDI-TOF-MS),16 ICP-MS, off-line and coupled to separation systems, for example, size exclusion chromatography (SEC) or capillary electrophoresis,44,45 advanced ion mobility mass spectrometry (IAM-MS), which can be used in combination with liquid chromatographic (LC) separations,16 and other techniques,16 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.17 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.36,47 By exploring biochemical, 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 tumor 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,37 and, in particular, thioredoxin reductase, cathepsin B and PARP,15,92,93,94 mitochondrial proteins15,93,95,96 and hormonal receptors15,93 are being considered, and recent advances in identifying new targets for metal-based drugs have been discussed.25 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 3β (GSK3β), 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 pyrido-carbazole backbone.15 In addition, nanomolar or subnanomolar inhibitors of PAK1 (MLK or MLCK) and human repair enzyme 7,8-dihydro-8-oxoguanosine triphosphatase (8-oxo-dGTPase, NUDT1/MTH1) derived from pyridylphthalimide, pyridylphthalimidamide, and 8-pyridylnaphthalimide and 8-(pyridyn-2-yl)adenine ruthenium scaffolds have quite recently been reported.17,48 The globular shape required to fill protein pockets has been tailored by occupying the remaining four coordination sites with ruthenium adducts 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.

Acknowledgments

We thank the Austrian Science Fund for financial support of the project P22339-N19 and Dr. Michael Malarek for reading the manuscript and his comments.

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PUBLICATIONS

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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