Development and investigations of antitumor platinum group metal and thiosemicarbazone complexes

The serious side effects and resistance of anticancer drugs used in chemotherapy still motivates the development of novel metal-based compounds that combine good efficacy, selectivity and low systemic toxicity. On the other hand, the rational drug development and optimization process strongly require information about the solution behavior, pharmacokinetics properties of the metallodrugs. In this project our main was to reveal relationship between the determined physico-chemical properties of novel anticancer metal complexes such as aqueous solubility, lipophilicity, membrane permeability, solution speciation (stability, stoichiometry), structures (in solution and in solid phase), redox reactivity and cytotoxicity. The interactions of the most promising compounds with the human blood serum proteins (albumin, transferrin) and DNA and their binding models were also investigated. These physico-chemical and biological properties, solution equilibrium processes and protein/DNA binding processes were studied by the combination of various techniques such as pH-potentiometry, spectroscopic (UV-visible, fluorometry, NMR, CD and EPR) and separation (ultrafiltration, capillary electrophoresis) methods. The cytotoxic activity of the ligands and their complexes was also assayed against chemo-naive parental and resistant cancer cell lines in addition to non-cancerous fibroblast cells. Based on our collection of comparative thermodynamic, kinetic and crystallographic data we could get in numerous cases a deeper insight into the fate of the metallodrugs studied in the biofluids, the differences and similarities in their biological effectiveness and side effects. In accordance with the project plan, mostly a) organorhodium and organoruthenium complexes of 8-hydroxyquinolines, oligopyridines, (di)picolinates, pyrithiones; copper and iron complexes of various b) thiosemicarbazones and their conjugates and c) 8-hydroxyquinolines possessing activity towards multidrug resistance (MDR) cancer cells were investigated. Additionally, solution speciation studies on other types of anticancer compounds such as d) epidermal growth factor receptor inhibitor molecules, coumarins and cobalt(III) complexes were also performed.

The obtained results were summarized in 43 peer-reviewed papers published in international scientific journals (30 Q1 and 13 Q2; summa impact factor: 186.4) during the 5-year duration of the project (2017-2021 + 1 year extension due to the pandemic situation), and the results were also presented in numerous conferences. *(Notably, only few conference participations are listed in the final report since results were published later in papers).*

One PhD thesis (János Mészáros, 2021) was defended successfully supervised by the PI based on results related to this project, and the PI has obtained the DSc title from the Hungarian Academy of Sciences (2021) in this period. The scientific research results were publicly also promoted (University of Szeged, ‘Free University’ lecture entitled *Anticancer metal compounds in medicine and research*, 27.11.2019).

a) Organorhodium, (organo)ruthenium complexes

It is well-documented in the literature that ruthenium-based complexes were identified as promising alternatives to anticancer platinum compounds since they show less severe side

effects and are less toxic in general. Thus the development of (organo)ruthenium compounds with the complexes of other platinum group metals has become one of the mainstream research areas in the field. The half-sandwich organometallic complexes represent a fairly diversified solution chemistry regarding the ligand exchange rates and thermodynamic stabilities depending on the character of the metal ion and the donor atoms. These parameters have a strong impact on the pharmacokinetic properties. In the frame of this project, we have prepared and studied numerous organometallic complexes varying the coordination modes. Namely, the solution speciation of half-sandwich complexes of $[Rh(\eta^5\text{-}C_5Me_5)(H_2O)_3]^{2+}$, $[Ru(\eta^6\text{-}p\text{-cymene})(H_2O)_3]^{2+}$ and $[Ru(\eta^6\text{-toluene})(H_2O)_3]^{2+}$ cations formed with bidentate ligands containing (N,N), (O,N), (O,O) or (N,S) donor atom sets was characterized (see the most important equilibrium processes in this type of chemical systems in Figure 1).

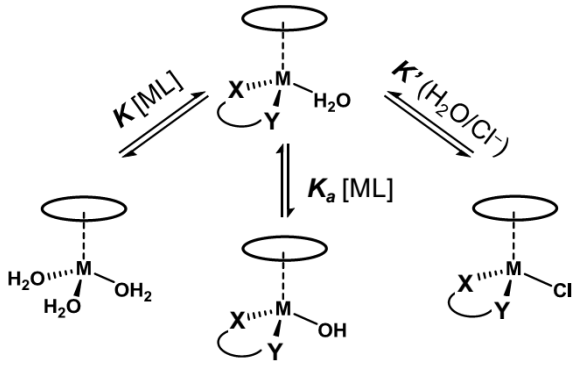


Figure 1. The most important equilibrium processes in the solution of the half-sandwich organometallic complexes.

Complexes with (O,O) donor ligand: The natural compound curcumin and its half-sandwich organometallic complexes were reported to show significant antitumor activity. Despite the large number of the reported curcumin complexes, their solution speciation was not characterized previously, most probably due to the very low aqueous solubility and photosensitivity of curcumin. In our work Ru(η^6-*p*-cymene), Ru(η^6-toluene) and Rh(η^5-C_5Me_5) complexes of curcumin and its water-soluble model ligand acetylacetone were characterized in solution and in solid phase [J.P. Mészáros, et al, J. Inorg. Biochem. 195 (2019) 91]. Our results revealed a clear trend of stability constants of the acetylacetonate complexes: Ru(η^6-*p*-cymene) > Ru(η^6-toluene) > Rh(η^5-C_5Me_5), but the highest extent of complex formation was seen for the Rh(η^5-C_5Me_5) complexes at pH 7.4. Formation constant of $[Rh(\eta^5\text{-}C_5Me_5)(H_2curcumin)(H_2O)]^+$ revealed similar solution stability to that of the acetylacetonate complex. Although curcumin showed cytotoxicity against human cancer cell lines tested (IC_{50} = 15 μM, in Colo 320 human colon cancer cells, 72 h incubation), it was not improved by the complexation with these organometallic cations, most probably as a result of the dissociation of the low stability of the complexes under physiological condition. Complexation of two (O,O) donor containing bidentate 2-hydroxy-[1,4]-naphthoquinone ligands with Ru(II)-, Os(II)- and Rh(III)-arene was also characterized systematically [J.P. Mészáros, et al, J. Organomet. Chem. 907 (2020) 121070]. The main goal was the fine-tuning of the structure of the [Ru(η^6-*p*-cymene)(lawsone)Cl] complex via changing the metal center (Ru, Rh, Os), the type of the arene (*p*-cymene, toluene, $C_5Me_5^-$), the naphthoquinone ligand

of natural origin (lawsone and phthiocol) and the halogenido co-ligand (Cl^-, Br^-, I^-) in order to obtain more cytotoxic compounds. Unfortunately, these modifications did not result in significant improvement in the cytotoxicity assayed against human cancer cell lines. The solution stability of the Rh(η^5-C_5Me_5) complexes of the studied naphthoquinone ligands was found to be lower compared to other well-known (O,O) donor ligands such as maltol, acetylacetone, or the deferiprone, the iron chelator hydroxypyridinone. Some additional alkoxycarbonylmethyl derived hydroxypyridinone compounds were also investigated to compare the stability and cytotoxicity of the complexes formed with Ru(η^6-*p*-cymene) to that of Fe(III) and Ga(III) [É.A. Enyedy, et al., Polyhedron 172 (2019) 141]. The studied (O,O) donor ligands formed significantly lower stability complexes as compared with the reference compound deferiprone in the case of all the monitored metal ions, and the alkoxycarbonylmethyl derivatization did not result in more active complexes.

Complexes with (N,N) donor ligand: Solution speciation studies and X-ray structure analysis of complexes of $[Rh(\eta^5\text{-}C_5Me_5)(H_2O)_3]^{2+}$ with (N,N) donor ligands such as N,N′-dimethylethylenediamine, N,N,N′,N′-tetramethylethylenediamine (tmeda), 2-picolylamine and 1,10-phenanthroline were also performed [J.P. Mészáros, et al., New J. Chem. 42 (2018) 11174]. Our results revealed the formation of high stability species in solution except the case of tmeda, due to the sterical hindrance between the methyl groups of the chelating ligand and the arenyl ring resulting in an increased methyl group-ring plane torsion angle. Based on the solution speciation data collected in our group till now, in this work we could reveal a strong correlation between the log*K*′(H_2O/Cl^-) and pK_a of [Rh(η^5-C_5Me_5)(L)(H_2O)] constants for a series of (O,O), (O,N) and (N,N)-chelated complexes (Figure 2.a). For the studied 12 complexes a relationship between log*K*′(H_2O/Cl^-) values and certain crystallographic parameters was found using multiple linear regression approach (Figure 2.b).

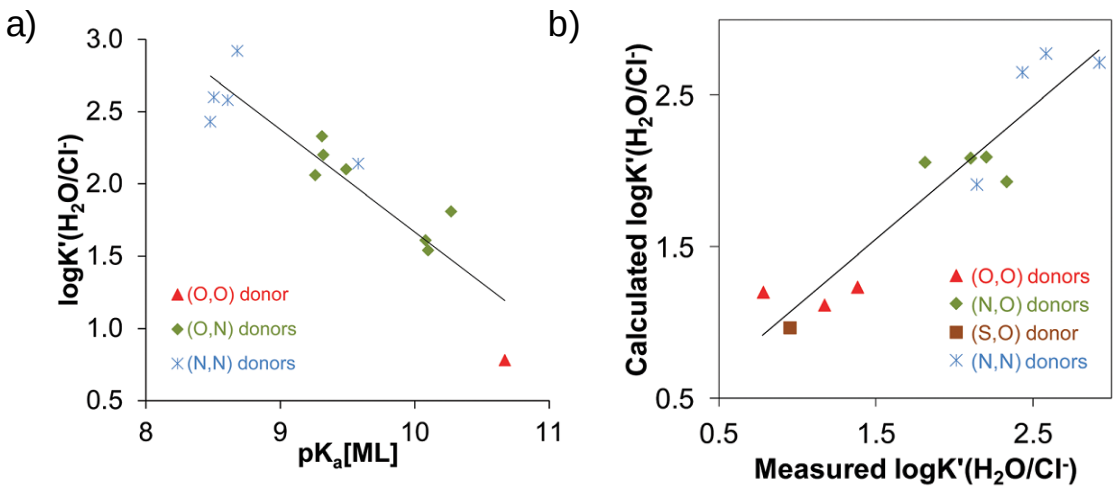


Figure 2. a) Log*K*'(H_2O/Cl^-) values *vs*. pK_a of [Rh(η^5-C_5Me_5)(L)(H_2O)] for the complexes containing various bidentate ligands with O/N/S donor atoms. b) Multilinear regression between calculated and measured log*K*'(H_2O/Cl^-) values. The calculated values were obtained using various geometrical parameters (distance(Rh–centroid), angle(X–Rh–X), torsion angle(methyl group-ring plane).

A series of half-sandwich oligopyridyl complexes was also synthesized and compared focusing on structural, cytotoxic and aqueous solution behavior [J.P. Mészáros et al., Dalton Trans. 50 (2021) 8218]. These complexes have high stability in solution, and no dissociation was found even at micromolar concentrations in a wide pH range. Based on the results, a universal model was introduced for the prediction of chloride ion capability of half-sandwich Rh and Ru complexes. The ligands and the Rh complexes showed significant cytotoxicity in A2780 and MES-SA cancer cell lines and in the cisplatin-resistant A2780cis cells, whereas the coordination to Ru(II) caused decreased toxicity most likely due to arene loss.

We aimed to develop half-sandwich [Rh(η^5-C_5Me_5)(N,N)(N)] complexes, where (N,X) (X:N or O) is a bidentate and (N) is a monodentate anticancer ligand, which are considered as prodrugs and can be activated by acidosis that is a characteristic property of the cancerous cells due to the altered metabolism. Various simple heterocyclic N donor ligands were tested to optimize the structure of the mixed-ligand complexes and imidazole nitrogen was found to be the most promising leaving ligand. 6 structures were determined by X-ray crystallography. These results were presented only at conferences, a manuscript is under preparation.

Complexes with (N,O) donor ligand: [Ru(η^6-toluene)$(H_2O)_3$]$^{2+}$ complexes of 2-picolinic acid and its four derivatives (with Br, Me, COOH substituents) were synthesized and characterized including X-ray structure analysis [J.M. Poljarevic, et al, J. Inorg. Biochem. 181 (2018) 74]. Lipophilicity, stoichiometry, solution stability and chloride affinity of the complexes were determined. Their cytotoxicity and antiproliferative effects were evaluated in sensitive and resistant human cancer cell lines. Formation of mono complexes with high stability was found in solution, and the pK_a values (8.3–8.7) of the [Ru(η^6-toluene)(L)(Z)] complexes (Z: Cl^- /H_2O) reflect the formation of low amount of mixed hydroxido species at pH 7.4. These complexes are fairly hydrophilic and show moderate chloride ion affinity and fast chloride-water exchange processes which most probably contribute to the only moderate antiproliferative effect (IC_{50} = 80 μM) on the multidrug resistant colon adenocarcinoma cell line tested.

Picolinate ligands coordinate via (N,O) donor sets to form high stability complexes and a similar binding pattern was found for dipicolinate derivatives. However, the complexation of Ru(η^6-toluene) with 2,4-pyridinedicarboxylic acid resulted in an unusual triangular complex (Figure 3) stabilized via the monodentate coordination of the second carboxylate group to the neighboring Ru(II) center [J.P. Mészáros et al., Eur. J. Inorg. Chem. 2021 (2021) 1858]. Notably, the excess of 2,4-dipic or addition of ligands picolinic acid or 1,10-phenanthroline to the Ru(η^6-*p*-cymene)-2,4-dipic complex resulted in the partial arene loss.

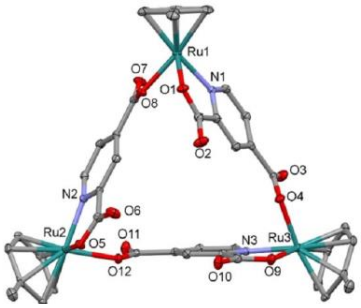


Figure 3. Structure [Ru(η^6-toluene)(2,4-dipic)]$_3$

Considerably stable and more cytotoxic half-sandwich organometallic complexes were obtained with an 5-chloro-8-hydroxyquinoline-L-proline hybrid ligand (HQCl-L-Pro), which was co-designed with our partners (I. Szatmári) to possess good water solubility and potential MDR-selectivity due to the CH_2–N subunit at position R7 [J.P. Mészáros, et al, Dalton Trans.

49 (2020) 7977]. The complex formation of HQCl-L-Pro with Rh(η^5-C_5Me_5), Ru(η^6-*p*-cymene) and Ru(η^6-toluene) was investigated by the combined use of pH-potentiometry, UV-visible spectrometry and ^{1}H NMR spectroscopy, and the complexes were isolated as well. Our results revealed the prominent solution stability of the complexes in all cases. The lipophilicity of the complexes increased with the chloride ion concentration, and the complexes showed moderate log *D* values (−0.8 to +0.4) at pH 7.4 at all tested chloride concentrations. The cytotoxicity and antiproliferative effect of HQCl-L-Pro and its complexes were assayed *in vitro*. In contrast to the structurally similar oxine, HQCl-L-Pro and its Rh(η^5-C_5Me_5) complex were somewhat more effective against drug resistant Colo 320 adenocarcinoma human cells compared to the drug sensitive Colo 205 cells. The Ru- and Rh-complexes showed a similar metal uptake level after 4 h measured with total-reflection X-ray fluorescence, while a longer incubation time resulted in higher cellular Rh concentration, which may explain the difference in the anticancer activity of the Ru and Rh complexes. On the other hand, we suspect that the possible arene loss (*p*-cymene and toluene) of the Ru complexes can also contribute to the diminished bioactivity. *Based on these results the first author of this paper has obtained the prestigious Fernando Pulidori prize from the Scientific Committee of the annual International Symposium on Metal Complexes.*

The D-proline and D-homo-proline hybrids of the 5-chloro-8-hydroxyquinoline and their Rh(η^5-C_5Me_5) and Ru(η^6-*p*-cymene) complexes were also prepared and studied [T. Pivarcsik, et al., Int. J. Mol. Sci. 22 (2021) 11281]. The Rh(η^5-C_5Me_5) complexes displayed enhanced cytotoxicity in human colon adenocarcinoma cell lines, exhibited multidrug resistance selectivity and increased selectivity to the chemosensitive cancer cells over the normal cells; meanwhile, the Ru(η^6-*p*-cymene) complexes were inactive, most likely due to arene loss. The complexes are able to bind strongly to human serum albumin (HSA) and DNA, but DNA cleavage was not observed. Changing the five-membered proline ring to the six-membered homoproline resulted in increased lipophilicity and cytotoxicity, while changing the configuration (L vs. D) rather has an impact on HSA and DNA binding.

In a comprehensive study [T. Pivarcsik et al., Pharmaceuticals 14 (2021) 518], we compared the solution chemical properties and biological activity of Ru(η^6-*p*-cymene) complexes of selected β-diketone, 8-hydroxyquinoline and pyrithione ligands. Effect of the structural variation on the biological properties and solution stability was clearly revealed. The decreased bioactivity of the β-diketone complexes can be related to their lower stability in solution. In contrast, the (O,S) pyrithione-type complexes are highly stable in solution and the complexation prevents the oxidation of the (O,S) ligands. Comparing the binding of 1,3,5-triaza-7-phosphaadamantane (PTA) and the chlorido co-ligands, it could be concluded that PTA was generally more strongly coordinated to Ru(II), which at the same time decreased the reactivity of complexes with albumin or 1-methylimidazole as well as diminished their bioactivity.

Interaction with human serum albumin: HSA has a profound effect on the pharmacokinetic properties of a drug molecule and binding to this protein can be advantageous due to the enhanced permeability and retention effect in solid tumor tissues resulting in the accumulation of protein-bound drugs close to the cancer cells. These are the reasons why we performed a detailed comparative study on the binding of various Ru(η^6-*p*-cymene) and Rh(η^5-C_5Me_5)

complexes bearing (O,O), (O,N) and (N,N) ligands to HSA by a combination of various methods such as ultrafiltration, capillary electrophoresis, ^{1}H NMR spectroscopy, fluorometry and UV–visible spectrophotometry [O. Dömötör, É.A. Enyedy, J. Biol. Inorg. Chem. 24 (2019) 703; O. Dömötör et al., Dalton Trans. 50 (2021) 11918]. Binding kinetics, strengths and sites in addition to reversibility were compared and discussed. The organometallic ions (without the bidentate ligands) were found to bind to HSA at a high extent via a coordination bond. Release of the bound metal ions was kinetically hindered and could not be induced by the denaturation of the protein. Binding of the Ru(η^6-*p*-cymene) triaqua cation was much slower (*ca*. 24 h) compared to the rhodium congener (some min), while their complexes interacted with the protein relatively fast (1–2 h). The studied complexes were bound to HSA coordinatively. The highly stable and kinetically inert Ru(η^6-*p*-cymene) complex of 2-picolinate is bound in an associative manner preserving its original entity, while lower stability complexes containing (O,O) donor atoms decomposed partly or completely upon binding to HSA (see the proposed mechanism for the binding in Figure 4). Fast, non-specific and high-affinity binding of the complexes on HSA highlights their coordinative interaction with various types of proteins possibly decreasing effective drug concentration. In the case of the complexes of 8-hydroxyquinolines and oligopyridines, it was found that the Ru(η^6-*p*-cymene) complexes of the (N,N) donor bearing oligopyridines do not bind to the protein measurably, most probably due to kinetic reasons. However, the other complexes bind significantly to albumin with fairly different kinetics. The binding affinity towards hydrophobic binding pockets shows correlation with lipophilicity along with the actual charge of the respective complexes.

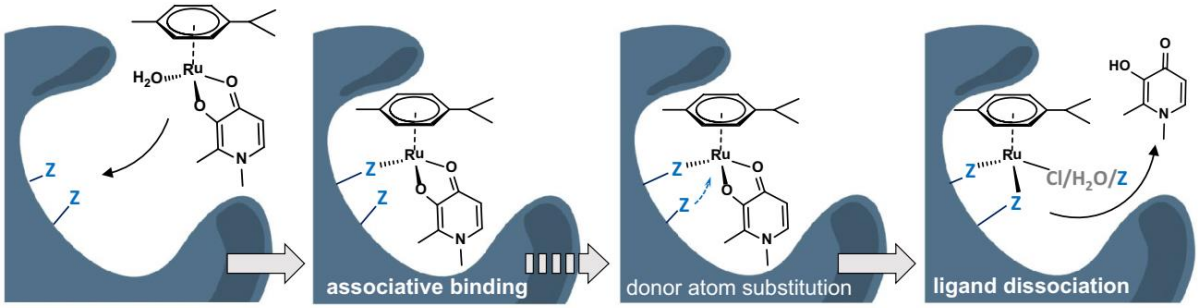


Figure 4. Presumed mechanisms of binding of the studied metal complexes with lower stability on HSA (in the case of the Ru(η^6-*p*-cymene) complex of deferiprone).

Classical ruthenium complexes: We had the opportunity to be involved in the characterization of a promising anticancer maltol-containing ruthenium polypyridyl complex [A. Notaro, et al., Chem. Eur. J. 26, 2020, 4997], which showed higher activity than cisplatin on different cell lines in 2D model and on tumour spheroids. In this work we have pointed out that the complex is stable at room temperature in DMSO over 42 h and has a half-life of 48 h in human serum, and it binds to HSA via intermolecular interactions at least at the two hydrophobic sites (I and II), preventing precipitation of the metal complex in aqueous solution. Detailed solution chemical studies on octahedral ruthenium–nitrosyl complexes containing equatorial 1*H*-indazole ligands were also performed [E. Orlowska, et al, Inorg. Chem. 57 (2018) 10702] and our results revealed foremost in the literature that the indazole

ligands in parent complexes can undergo deprotonation at physiological pH with formation of inner sphere complexes.

b) Thiosemicarbazones

Comparative studies

Based on our long-term and continuous work on the solution chemistry of versatile thiosemicarbazones (TSCs) and their metal complexes, we could built up a larger library of speciation data completed with structural characterizations. In a review article with our synthetic chemist and biologist collaboration partners we summarized and analyzed the data obtained on the interaction of α-*N*-heterocyclic thiosemicarbazones with iron ions, with the special aim of bridging the current knowledge on their mode of action from chemistry to (cell) biology [P. Heffeter, et al, Antioxid. Redox Sig. 30 (2019) 1062]. This publication turned to be a well-cited paper with 71 independent citations till now. We pointed out that *TSCs do not solely remove iron from the cells, but they should be considered as iron-interacting drugs affecting diverse biological pathways* in a complex and multi-faceted mode of action. In an other comprehensive study [É.A. Enyedy et al., Dalton Trans. 49 (2020) 16887] we investigated the influence of the type of the chalcogen atom (S: triapine, O: O-triapine, Se: Se-triapine) and the methylation of the hydrazonic NH moiety (Me-triapine) (see their structures in Figure 5) on the basic solution properties (lipophilicity, pK_a), complex formation processes with Fe(II), Fe(III) and Cu(II) ions and their cytotoxicity.

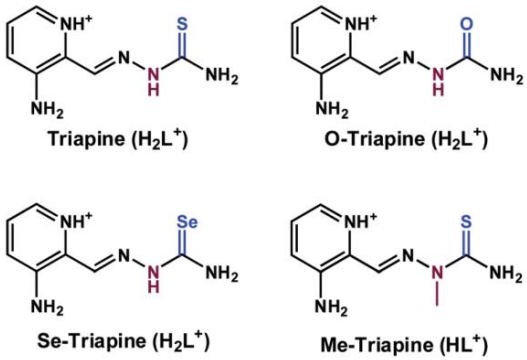


Figure 5. Chemical structure of triapine and its derivatives in their protonated forms.

Our equilibrium studies showed that Se-triapine forms Cu(II) complexes with higher, and O-triapine with lower stability as compared with triapine. Me-triapine, which is not able to coordinate via the typical (N,N,S^-) donor set, nevertheless coordinates to Cu(II) with unexpected high stability. The Cu(II) complexes of Se-triapine and Me-triapine could be relatively slowly reduced by glutathione at pH 7.4, similarly to the Cu(II)-triapine complex. Se-triapine forms high stability complexes with both Fe(II) and Fe(III) ions, while O-triapine has a much stronger preference towards Fe(III) and Me-triapine towards Fe(II). This difference in the iron preference of the ligands seems to have a strong impact on their cytotoxic effects. The Cu(II) complexes were moderately toxic, and the highest level of ROS generation was found for the Cu(II) complex of O-triapine, which is the most reducible. As a continuation of this work, we monitored how the coordination modes of (thio)semicarbazone copper(II) complexes {(N,N,S), (O,N,S), (N,N,O), (O,N,O)} can modulate the solution

chemical properties and mechanism of anticancer activity, DNA-binding, DNA cleavage ability and inhibition of the topoisomerase IIα enzyme [V. Pósa, et al., J. Inorg. Biochem. 231 (2022) 1111786]. We have found that among the Cu(II) complexes the most lipophilic species with the highest stability and membrane permeability exhibited the highest cytotoxicity. These complexes interact with DNA, and their reaction with glutathione led to heavy DNA cleavage in the case of the highly stable complexes which could be reduced in a reversible reaction with moderate rate.

In an other comparative study, the effect of the type of the additional third donor of the TSC scaffold (NNS *vs*. ONS) on the interaction of the Cu(II) complex with HSA was studied involving Asp-Ala-His-Lys and the monodentate *N*-methylimidazole as binding models [N.V. May et al, Molecules 26 (2021) 2711]. The studied TSC complexes are able to bind to albumin in a fast process, and the determined conditional constants suggest that their binding strength is only weak-to-moderate.

Studies on TSCs with (NNS) donor set

i) Effect of methylation: A series of differently methylated triapine derivatives with (N,N,S) coordination mode and their Cu(II) complexes were also investigated [S. Hager, et al., Antioxid. Redox Signal. 33 (2020) 395] regarding the stability in water, redox reactions with ascorbic acid and glutathione and anticancer activity. We have pointed out that high copper complex stability and slow reduction kinetics are key parameters for the improved cytotoxicity and paraptosis. Based on our results we could hypothesize that in the case of close triapine derivatives, intracellular reduction leads to rapid dissociation of intracellularly formed copper complexes. In contrast, *TSCs forming high stability and slowly reducible Cu(II) complexes are able to reach new intracellular targets such as the endoplasmic reticulum-resident protein disulfide isomerase*.

We have pointed out that methylation on the thioamide sulfur of triapine results in an unstable compound which decomposes relatively fast at the C=N bound, while its coordination to Cu(II) via (N,N,N) donor set is able to protect the compound against this hydrolytic decomposition process at physiological pH [K. Ohui, et al., Biomolecules 10 (2020) 1336].

ii) Effect of introduction of a phenol moiety: Complex formation of three new triapine analogues bearing a redox-active phenolic moiety at the terminal nitrogen atom with Cu(II) ions was investigated in addition to their redox properties [I. Besleaga et al., Inorg. Chem. 60 (2021) 11297]. Solution studies revealed that the ligands become air-sensitive upon deprotonation of the OH group in the basic pH range. The monocationic complexes $[CuL]^+$ are the most abundant species in aqueous solutions at pH 7.4. The attachment of a phenolic moiety undoubtedly increases the lipophilicity of ligands and Cu(II) complexes when compared to triapine analogous.

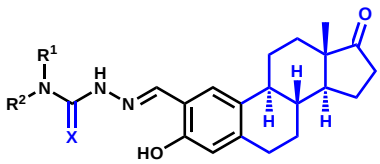
iii) Complexation of COTI-2: Stability and redox properties of Cu(II) complexes formed with COTI-2 (a novel TSC entered a clinical phase I trial in 2016) and a triapine-like nonsubstituted (COTI-NH_2) representative were investigated [J.H. Bormio, et al., J. Med. Chem. 63 (2020) 13719]. The recognition by ABCC1 efflux pump of COTI-2 could be explained by the reduction kinetics of a ternary Cu(II)-COTI-2 complex formed with glutathione. Thus, only TSCs forming stable, non-reducible copper(II)-TSC-glutathione

adducts are recognized and effluxed by ABCC1. Our finding reveals a *crucial connection between copper complex chemistry, glutathione interaction, and the resistance profile* of clinically relevant TSCs.

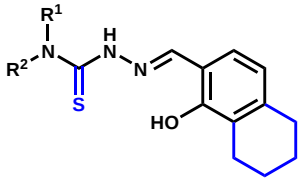
iv)Related TSCs: Additionally as a continuation of our previous works on thiosemicarbazone copper complexes, we have characterized the complexation properties of several conjugates as well such as derivatives of biotin, morpholine and methyl-imidazole [S. Kallus, et al, J. Inorg. Biochem. 190 (2019) 85; K. Ohui, et al, J. Med. Chem. 62 (2019) 512; O. Palamarciuc, New J. Chem. 43 (2019) 1340].

Studies on TSCs with (ONS) donor set.

i) Sterane-based compounds: Cu(II) complexes of an estrone-salicylaldehyde thiosemicarbazone hybrid (estrone-TSC) and its structurally related bicyclic derivative (thn-TSC) were prepared, characterized regarding their solid and solution structures, solution stability, reducibility, membrane permeability, cytotoxicity and ROS generation ability [T.V. Petrasheuskaya, et al., New J. Chem. 44 (2020) 12154]. (See the structures in Figure 6.) The complexes were found to be fairly stable and cytotoxic (IC_{50} < 0.2-2 μM); the estrone conjugation induced more lipophilic compounds with higher cytotoxicity. As a continuation, the Cu(II) complexes formed with terminal *N*-mono- and dimethylated derivatives of estrone-salicylaldehyde thiosemicarbazone hybrids [T.V. Petrasheuskaya et al, J. Biol. Inorg. Chem. 26 (2021) 775] and a (non-methylated) semicarbazone-estrone hybrid [É.A. Enyedy et al., J. Inorg. Biochem. 220 (2021) 111468] were also prepared and studied in addition to their structurally related simpler bicyclic analogues. Although, the semicarbazone complexes were found to be less stable than their TSC analogues, they displayed moderate significant cytotoxicity in cancer cells, and moderate apoptosis induction and ROS formation. While the related Cu(II)-TSC complexes represented much stronger cytotoxic activity and antibacterial effect on MRSA bacteria. The methylated estrone-TSC hybrids form high-stability complexes with Cu(II) ions, in which they coordinate in mono-anionic (O^-,N,S) or di-anionic (O^-,N,S^-) binding modes The terminal N-dimethylation results in the most stable complexes in a given ligand series. The Cu(II) complexes of *N*-mono- and dimethylated derivatives were cytotoxic in 3D spheroids of human cancer cells and caused reduced tumor growth.



estrone-TSC (X=S, R^1=H, R^2=H)
estrone-SC (X=O, R^1=H, R^2=H)
Me-estrone-TSC (X=S, R^1=H, R^2=CH_3)
Me_2-estrone-TSC (X=S, R^1=R^2=CH_3)



thn-TSC (R^1=H, R^2=H)
Me-thn-TSC (R^1=H, R^2=CH_3)
Me_2-thn-TSC (R^1= R^2=CH_3)

Figure 6. Chemical structure of the studied estrone-salicylaldehyde thiosemicarbazone hybrids and their simplified models.

ii) TSCs with increased water solubility: However, a potential drug cannot have too low water solubility; the hydrophilic-lipophilic character should be optimized. Therefore, a series of four water-soluble salicylaldehyde TSCs with a positively charged trimethylammonium moiety and their Cu(II) complexes were synthesized in a collaborative work [M.N.M. Milunovic, et al., Biomolecules 10 (2020) 1213]. The complexes were characterized by similar stability as compared to that of salicylaldehyde TSC but with higher water solubility, while the cytotoxic property remained by this modification.

Studies on TSCs with (NS) donor set

The complex formation of bidentate (N,S) donor containing pyrazole derivatives was investigated [O. Dömötör, et al., J. Inorg. Biochem. 202 (2020) 110883]. Our aim was to compare the solution speciation to that of the tridentate thiosemicarbazones and *to provide stability data on Ru(η^6-p-cymene) TSC complexes first in the literature*. Three structures were resolved by X-ray diffraction in this work. We have found the formation of high stability mononuclear Ru(η^6-*p*-cymene) complexes with (N,S) coordination mode in the acidic pH range, and increasing the pH the predominating dinuclear $[(Ru(\eta^6\text{-}p\text{-cymene}))_2(L)_2]^{2+}$ complexes with μ_2-bridging sulphur donor atoms were formed. Complexation with Cu(II) resulted in $[CuL]^+$ and $[CuL_2]$ complexes showing much higher stability compared to that of the complexes of the reference compound benzaldehyde thiosemicarbazone. The studied ligands exhibited moderate cytotoxicity against human colonic adenocarcinoma cell lines (IC_{50} = 33–76 μM), while their complexation with Ru(η^6-*p*-cymene) (IC_{50} = 11–24 μM) and especially with Cu(II) (IC_{50} = 3–6 μM) resulted in higher cytotoxicity.

TSC analogous and related ligands with antitumor activity

Tridentate salicylidene aminoguanidine Schiff bases (as analogues ligands of the TSCs with (ONS) donor set) coordinating via the phenolato O, azomethine N and the amidine N donor atoms were characterized by increased water solubility due to the positively charged aminoguanidinium moiety, while the proline conjugation increased even stronger the hydrophilic character. Moreover, the proline moiety changed the binding mode as well, namely, (O,N) donor atoms of the proline moiety were coordinated beside the phenolato O, confirmed by single crystal X-ray crystallographic analysis [O. Dömötör, et al., Molecules 27 (2022) 2044].

Complexes of the 2-aminoestradiol formed with Cu(II) and some other divalent metal ions (Zn(II), Ni(II)) were also prepared and studied [T.V. Petrasheuskaya, et al. J. Mol. Struc. 1261 (2022) 132858]. The 2-aminoestradiol displayed high cytotoxic activity on HeLa, Colo 205 and the doxorubicin-resistant Colo 320 cells, and the Ni(II) and Cu(II) complexes displayed even higher cytotoxicity in almost all tested cell lines. We also pointed out that the complexation with Cu(II) ions significantly increased the antioxidant activity of both tested aminophenols and resulted in higher percentage of apoptotic cells. Additionally, the proton dissociation processes of a series of 2-aminoestradiol hybrids of anticancer activity were studied to monitor the effect of the various substituents [F. Kovács et al., RSC Advances 11 (2021) 13885].

Studies on some (N,N,N) donor latonduine derivatives and their copper complexes characterized by nanomolar cytotoxicity were also performed [F. Bacher et al, Dalton Trans.

48 (2019) 10464; C. Wittmann, et al., J. Med. Chem. 65 (2022) 2238]. Most of the studied copper complexes showed higher anticancer activity in cancer cells than the 'gold standard' triapine. All these copper complexes were characterized by such high solution stabilities that their dissociation at μM concentrations and at pH 7.4 is negligible.

c) 8-Hydroxyquinoline derivatives with MDR-selectivity

The anticancer activity of 8-hydroxyquinolines relies on complex formation with redox active copper and iron ions. A series of 8-hydroxyquinoline derived Mannich bases was studied regarding the anticancer activity, lipophilicity and their complexation with Cu(II) and Fe(III) in our work [V.F.S. Pape et al, Dalton Trans. 47 (2018) 17032]. The solution and solid phase structures of the complexes were characterized in addition to their redox properties. We have found a correlation between the anticancer activity and the metal binding properties of the compounds, namely weaker Cu(II) and Fe(III) binding results in elevated cytotoxicity at physiological pH. In addition a linear relationship between the pK_a (OH) and IC_{50} values of the studied 8-hydroxyquinolines was found. (See these correlations in Figure 7.) One of the derivatives with CH_2–N moiety at position R7 and chlorine and fluorobenzylamino substituents was identified as a potent and selective anticancer candidate with significant toxicity in drug resistant cells. To continue this work, we have further studied the impact of the solution stability and redox activity of their Fe(III) and Cu(II) complexes on MDR-selective toxicity [V.F.S. Pape et al., Cancers 13 (2021) 154]. It was showed that the MDR-selective anticancer activity of the studied ligands is associated with the iron deprivation of MDR cells and the preferential formation of redox-active Cu(II) complexes, which undergo intracellular redox-cycling to induce oxidative stress.

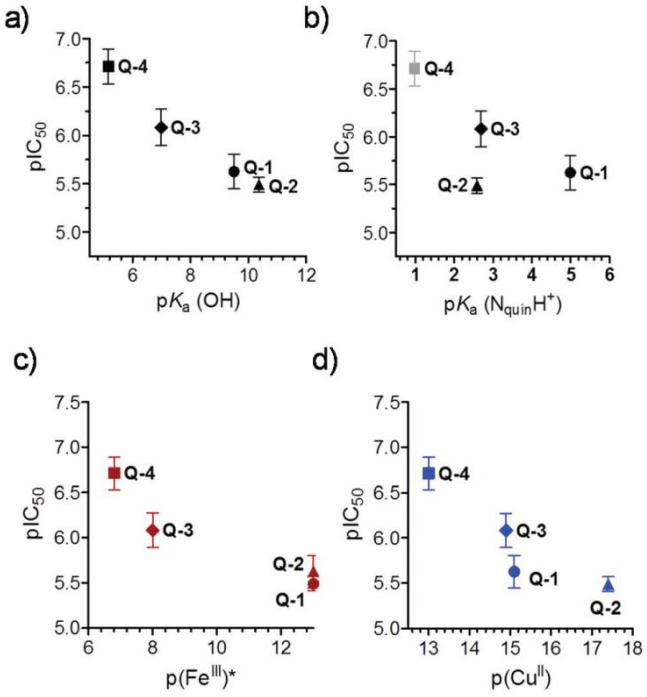


Figure 7. Correlations between pIC_{50} values measured in the MES-SA/Dx5 MDR cancer cell line and pK_a values of the studied 8-hydroxyquinoline ligands and their pFe(III) and pCu(II) values.

Our results on the complexation of the zwitterionic proline and homo-proline derivatives (presented on page 5) with Cu(II), Fe(III) and Fe(II) ions have been shown only at conferences till now, and we have pointed out the importance of the redox potential of the iron complexes in the MDR-selective toxicity.

The solution speciation of In(III) complexes of 8-hydroxyquinoline and 8-hydroxyquinoline-5-sulfonate and their interaction with HSA and transferrin was also characterized [O. Dömötör, et al., J. Biol. Inorg. Chem. 27 (2022) 315], and significant differences were seen between the behavior of the In(III) and the Ga(III) complexes. The more pronounced transferrin binding of In(III) via ligand release was seen, while the original scaffold of the Ga(III) complexes was preferably retained upon protein interactions and significant albumin binding occurred.

d) Solution speciation of related ligands and complexes of anticancer activity:

Epidermal growth factor receptor (EGFR) selective inhibitors are very efficient and promising compounds in the anticancer therapy, which is the reason why we have started to work on the characterization of their solution properties. We have performed a comparative study on the HSA binding of the clinically approved EGFR inhibitors (gefitinib, erlotinib, afatinib, osimertinib) and an investigational compound (KP2187) [O. Dömötör, et al, J. Pharm. Biomed. Anal. 154 (2018) 321]. Steady-state and time resolved spectrofluorometric measurements revealed the weak-to-moderate binding on HSA mainly in subdomain IIA, and the model calculations performed at physiological blood concentrations of HSA resulted in high (*ca*. 90%) bound fractions for the inhibitors, highlighting the importance of plasma protein binding.

Additionally, binding of six 5-hydroxy-substituted coumarins connected with the *N*-arylpiperazine substituent via propyloxy or butyloxy linkers to HSA was also characterized in details [T. Zolek el al, Eur. J. Pharm. Sci. 115 (2018) 25]. The strength of the protein binding and the proton dissociation constants of the compounds were determined not merely experimentally but computationally-determined values were also obtained showing a good agreement between them. The determined thermodynamic data were considered as important drug likeness descriptors. These studies undoubtedly improved our know-how on the characterization of protein binding processes of biologically active compounds as well.

On the other hand, HSA binding and lipophilicity of four quinizarin containing ternary Co(III) complexes, quinizarin and its sulfonated derivative (with better water-solubility) was assayed by UV-visible spectrophotometry and fluorimetry [M. Kozsup, et al., J. Inorg. Biochem. 204 (2020) 110963]. Meanwhile the ligands were able to be bound to albumin, the studied Co(III) complexes did not exhibit significant binding to this protein; thus, albumin cannot act as their carrier in the blood serum. Hypoxic prodrug activation is a promising strategy for a Co(III) complex to reduce adverse effects of anticancer therapy. Our group was involved in the characterization of promising tyrosine kinase (TK) inhibitor bearing Co(III) complexes, and based on the solution studies we pointed out that after the reduction of the Co(III) center, the forming Co(II) complex releases the respective TK inhibitor completely indeed [M. Mathuber, et al., Inorg. Chem. 59 (2020) 17794].

We were involved in the construction of a review article, in which we highlighted the importance of the knowledge of the solution speciation and distribution of metal complexes

with medicinal interest in biological milieu. Speciation data can provide essential information about the pharmacokinetic properties and can contribute to the deeper understanding of the mechanism of action. An overview on biodistribution studies for anticancer complexes in biofluids reported in the last decade was provided in the paper [T. Kiss et al, Curr. Med. Chem. 26 (2019) 580].