BIODISTRIBUTION OF ANTITUMOR METAL COMPLEXES: SOLUTION SPECIATION AND INTERACTION WITH TRANSPORT AND TARGET MACROMOLECULES

1. Introduction

The serious side effects and resistance of anticancer chemotherapeutic drugs have motivated the development of novel metal-based compounds that combine good efficacy, selectivity and low systemic toxicity. For the more efficient drug development we have to understand better the pharmacokinetic profile, the mechanism of action. However, these processes are more complicated compared to the conventional drugs, since the metal complexes may undergo alterations via dissolution in water, interaction with biomolecules. The main aim of the project was to improve our knowledge on the solution chemistry, factors affecting pharmacokinetic and pharmacodynamic properties of antitumor metallodrugs to elucidate of the fate of the chosen metal complexes (and non-metallic anticancer compounds as well) in the biofluids, and to reveal what kinds of physico-chemical and pharmacological properties are crucial for the biological effectiveness.

In accordance with the postdoctoral project (PD 103905) plan we have performed solution equilibrium studies on various compounds with antitumor activity regarding their solution stability, stoichiometry and interaction with human serum proteins, DNA such as:

i) organorhodium, (organo)ruthenium, platinum compounds, gallium complexes;

ii) antitumor thiosemicarbazones; and pyrimidinylhydrazone derivatives having activity towards multidrug resistance cancer cells;

iii) reduced Schiff base coumarin derivatives.

2. Results

Our results related to the project were summarized in: *29 peer-reviewed papers* in international scientific journals (Σ impact factor: 107.85; independent citations: 170; the PI acted as corresponding author 21 times, among the papers 7 with D1 and 13 with Q1 journal ranking); 2 peer-reviewed articles in Hungarian scientific journals; and at 19 international and 13 national conferences. (Notably, not all of the conference participations are listed in the report since results were published in papers later).

Two PhD theses (Orsolya Dömötör (2015), Éva Sija (supervised with Dr. Tamás Kiss, 2014)) were defended successfully supervised by the PI. In addition the PI completed the *habilitation process* in 2017. Six B.Sc./M.Sc. students participated at the National Conference for Undergraduate Researchers (‘OTDK’) were supervised by the PI during this period, and one 1st (in 2017) and one 2nd (in 2015) prices were received in the Coordination Chemistry Section.

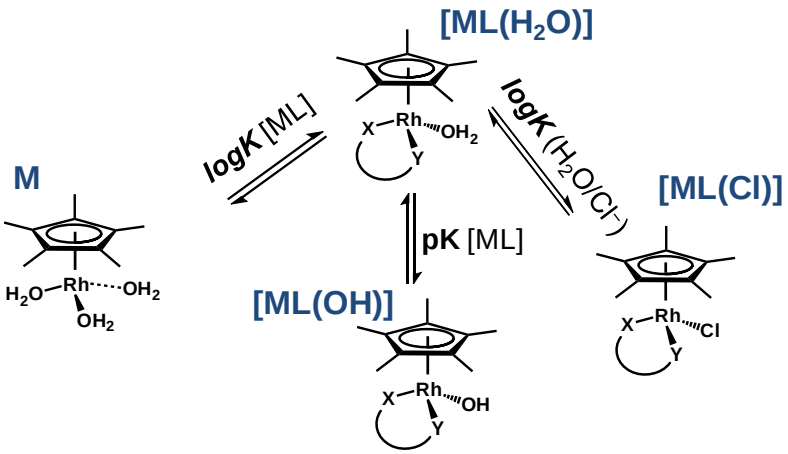
The scientific research results were publicly also promoted several times (*e.g.:* Talk at 'Everybody's Academy’ (M5 National TV Channel, July 2017): *Anticancer metallodrugs: effects and*

side effects; Talk at 'Researchers' Night in Hungary' (September 2015, Bay Zoltán Nonprofit Ltd. for Applied Research, Budapest): *Anticancer metal complexes in medicine and research*).

Notably the project was interrupted in 2014 for 23 months due to maternity leave of the PI.

2.1 Organorhodium, (organo)ruthenium and platinum compounds:

Rhodium(III) complexes: Solution stability and stoichiometry of $[Rh(III)(\eta^5\text{-}C_5Me_5)(H_2O)_3]^{2+}$ complexes formed with bidentate ligands containing various donor atom sets were characterized by the combination of different methods (^{1}H NMR spectroscopy, pH-potentiometry, UV-visible spectrophotometry) in aqueous solution in the presence and absence of chloride ions in addition to the determination of the hydrolytic processes of the organometallic cation at various chloride ions concentrations. The (O,O), (O,N), (O,S) or (N,N) donor atom containing ligands were: i) hydroxypyr(idin)one ligands (maltol, allomaltol, deferiprone), ii) 2-picolinic acid and its various derivatives (6-methylpicolinic acid, quinoline-2-carboxylic acid, 3-isoquinolinecarboxylic acid), 8-hydroxyquinoline (HQ) and its derivatives (5-sulfonate-HQ, the MDR-selective 7-(1-piperidinylmethyl-HQ)); iii) thiomaltol, iv) 2,2′-bipyridine and ethylenediamine. Formation of mononuclear, mono-ligand complexes such as $[ML(H_2O)]$ and $[ML(OH)]$ was found in almost all cases in which the ligands coordinate in a bidentate mode (Scheme 1). On the contrary thiomaltol forms bis complexes as well due to the monodentate coordination via the thione-S moiety of the second ligand. pK_a values determined for the deprotonation of the $[ML(H_2O)]$ complexes are relatively high values (8.61-10.67), which are even higher in the presence of chloride ions showing the negligible formation of the mixed hydroxido $[ML(OH)]$ species at physiological pH.



Scheme 1. Complexation and co-ligand exchange equilibrium processes for the $[Ru(\eta^5\text{-}C_5Me_5(L)(H_2O)]$ (=$[M(L)(H_2O)]$) species where L is the deprotonated form of the ligand with (X,Y) donor set

The following general stability trend was obtained for the $[ML(H_2O)]$ complexes at pH 7.4: 8-hydroxyquinolines > 2,2′-bipyridine > ethylenediamine > 2-picolinates > hydroxypyridinone ligand (deferiprone) > hydroxypyrone ligands (maltol, allomaltol). It was concluded that besides stability

constants (logK [ML(H_2O)]) also the pK_a values of these mono complexes strongly depend on the type of the coordinating donor atoms. The water/chloride exchange processes in the [ML(H_2O)] complexes were also characterized (by logK (H_2O/Cl^-)) and it was pointed out that the cytotoxicity of these metal complexes measured in different human cancer cell lines shows correlation with these exchange constants. Namely a weaker chloride ion affinity is required for the higher biological activity.

Solid phase structures of numerous Rh(η^5-C_5Me_5) complexes were determined by X-ray single crystallography (allomaltol, deferiprone, 6-methylpicolinic acid, quinoline-2-carboxylic acid, ethylenediamine (and its methylated derivatives), 8-hydroxyquinoline). It is noteworthy that a clear correlation was observed between certain crystallographic parameters and the chloride affinity of the complexes.

Interactions of these organorhodium complexes with human serum albumin, which is the most important blood transport protein, and with its amino acid side chain (His, Met, Cys) model compounds were also investigated. It was found that the strength and type of the interaction depend on the type of the coordinated ligands (dissociative binding via the cleavage of the ligand vs. complex-protein adduct formation). Based on the fluorometric and ^{1}H NMR spectroscopic results the binding of the complexes can take place at both sites I and II of albumin except the case of the high stability complexes formed with the (N,N) donor ligands, and ternary protein adducts are formed most probably by the coordination of side chain His nitrogens of albumin at the third coordination site of the organorhodium complex. *[Results were published in: O. Dömötör at al., J. Inorg. Biochem. 134 (2014) 57; E.A. Enyedy et al., J. Coord. Chem. 68 (2015) 1583; E.A. Enyedy et al., J. Inorg. Biochem. 152 (2015) 93.; C. Hackl et al., Chem. Europ. J. 22 (2016) 17269; O. Dömötör et al., Dalton Trans. 46 (2017) 4382; O. Dömötör et al., J. Organomet. Chem. 846 (2017) 4382.]*

Ruthenium(II/III) complexes: Solution equilibrium studies were also performed on half-sandwich ruthenium(II)-arene complexes being isoelectronic with rhodium(III)-arenyl complexes. [Ru(II)(η^6-*p*-cimol)(OX)(H_2O)] type complexes formed with X = O (hydroxyflavone, hydroxypyridinones, hydroxypyrones), X = S (hydroxythiopyrones), X = N (2-picolinic acid, 8-hydroxyquinoline and their various derivatives) ligands possessing proved or potential antitumor activity were involved in the project. Only mono ligand complexes are formed with the (O,O) and (O,N) donor set containing ligands. The sulfur-containing ligands form complexes of significantly higher stability compared with the hydroxypyrones; their complexes are biologically more active, and the simultaneous bi- and monodentate coordination of the ligands in their bis complexes was also demonstrated. The replacement of the aqua by the chlorido ligand in the [Ru(II)(η^6-*p*-cimol)(OX)(H_2O)] species was also monitored, which is an important activation step in the course of the mode of action of the complexes. [*A. Kurzwernhart et al., Dalton Trans. 42 (2013) 6193; E. A. Enyedy et al., J. Organomet. Chem. 734*

(2013) 38; É. A. Enyedy et al., J. Inorg. Biochem. 127 (2013) 161; É. Sija et al.; Polyhedron 67 (2014) 51; O. Dömötör et al., Dalton Trans. 46 (2017) 4382.]

The solution behavior of [Ru(II)(η^6-(2-phenoxyethanol))(L)Cl] type complexes formed with 2,2′-bipyridine and its two derivatives was also studied and significantly high stability of these complexes was found, however the slow oxidation of the Ru(II) centre takes place at pH > 6 leading to the partial loss of the arene moiety unlike the case of the analogous [Rh(III)(η^5-C_5Me_5)(L)Cl] complexes. *[G. Nogueira et al., J. Organomet. Chem. 820 (2016) 20.]*

As a continuation of our previous work on the interaction KP1339/1019 bis-indazole Ru(III) complexes [*O. Dömötör et al., J. Biol. Inorg. Chem. 18 (2013) 9.*] undergoing clinical Phase I and II trials towards human serum albumin, interaction of several cis/trans-[$RuCl_4$(1*H*-indazol)(NO)] (and some Os containing compounds as well) complexes with albumin was also studied. Albumin was found to be the main transporter for these mono nitrosyl compounds based on the measurements performed with human serum, while their interaction (regarding strength and binding sites) towards albumin was found to be very similar to that of the KP1339/1019 species. [*O. Dömötör et al., J. Inorg. Biochem. 159 (2016) 37.*]

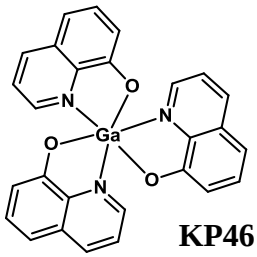
In order to characterize the interaction of some antitumor Ru(III) complexes formed with bis(aminophenolate) ligands towards DNA a novel quantitative approach was established to treat data obtained by steady-state and time-resolved fluorescence spectroscopy. [*O. Dömötör et al., J. Inorg. Biochem. 168 (2017) 27.*]

Platinum(IV) complexes: We were involved into the quantitative characterization of the interaction taking place between some promising Pt(IV) complexes and human serum albumin (HSA). These maleimide-functionalised Pt(IV) complexes showed fast and highly selective binding properties to thiol group of HSA. The albumin binding can lead either to pro-longed plasma half-life of these potential drugs and/or a more selective accumulation in the malignant tissue. [*V. Pichler et al., Chem. Comm. 49 (2013) 2249.*]

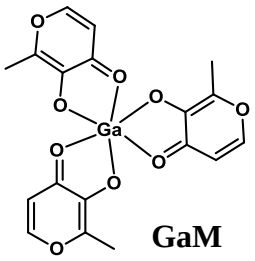
2.2 Gallium(III) complexes

The stoichiometry and stability constants of the Ga(III) complexes of 8-hydroxyquinoline (HQ) and maltol passed clinical Phase 1 trials were determined by means of pH-potentiometry, UV-Vis spectrophotometry and ^{1}H NMR spectroscopy in aqueous solution. Additionally complexation with 8-hydroxyquinoline-5-sulfonate (used as model), thiomaltol, allomaltol and thioallomaltol was also studied. Spectrofluorimetry was applied to determine the stability constants of the Ga(III)-HQ species in pure water, which is a fairly seldom approach in the literature. Ga(III) binding ability of the ligands followed the order: hydroxythiopyrones < hydroxypyrones < 8-hydroxyquinolines. As a result of the outstanding stability of tris(8-hydroxyquinolinato)gallium(III) (KP46) the decomposition of the complex is negligible at physiological pH even in the biologically relevant low concentration range.

Thus KP46 is able to preserve its original entity more considerably than other Ga(III) complexes. Moreover, intrinsic fluorescence of KP46 allows the monitoring of the cellular accumulation and distribution in human cancer cells by fluorescence microscopy. [*É. A. Enyedy et al., J. Inorg. Biochem. 117 (2012) 189*].



KP46



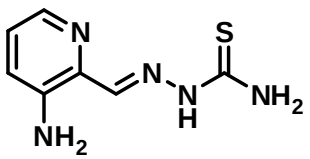
GaM

Scheme 2. Structures of the Ga(III) complexes: KP46 = tris(8-quinolinolato)Ga(III); GaM = tris(maltolato)Ga(III)

Interaction of the two clinically relevant Ga(III) complexes (tris-maltolato (GaM) and tris-8-hydroxyquinolinato (KP46), Scheme 2) with human serum albumin and transferrin was also investigated in detail in aqueous solution by the combination of various methods such as spectrofluorometry, UV–vis spectrophotometry, ^{1}H and saturation transfer difference NMR spectroscopy and ultrafiltration-UV–vis. It was found that transferrin is able to replace the ligands, especially in the case of maltol. There is no interaction between albumin and the maltolato complex, while KP46 binds to this protein relatively strongly via non-covalent bonds retaining its original entity. This finding suggests the transferrin-independent Ga(III) cellular uptake in the case of KP46. The interaction between albumin and KP46 was also confirmed by protein-complex modeling calculations. [*E.A. Enyedy et al., J. Biol. Inorg. Chem. 20 (2015) 77.*]

2.3 Thiosemicarbazones

Thiosemicarbazones (TSCs) are versatile compounds regarding their structures, metal binding abilities and pharmacological properties including anticancer activity. Triapine, the most studied representative, has already in several clinical phase I and II trials, and two other TSCs (COTI-2, DpC) have recently entered into human clinical trials. Due to the success of these compounds TSCs and their metal complexes have gained improving focus and attention. Characterization of interaction of TSCs with essential metal ions (mainly Fe(III/II) and Cu(II))) such as solution stability, redox properties is very important to understand their mechanism of action, however studies on their solution behavior are fairly rare in the literature. Our aim is to understand how the structural changes on the TSC scaffold affect the protonation processes, lipophilicity, the metal binding abilities and the redox properties revealing correlation between these



Triapine

parameters and their antiproliferative activity. In the frame of this project we have performed studies on different types of TSCs focusing on the effect of the variation of the substituents, type of the chalcogen atom (S/O exchange).

In order to reveal the effect of the S → O exchange on the stability of the metal complexes as a continuation of our previous comparative solution equilibrium studies on TSC complexes salicylaldehyde semicarbazone ligands were involved in the project. Formation of mono complexes such as [ML], [MLH_{-1}], [MLH_{-2}] was found with Cu(II), V(IV/V), while bis-ligand species of Fe(II)/(III) and Ga(III) such as [ML_2], [ML_2H_{-1}] and [ML_2H_{-2}] were also detected, in which the ligands coordinate via monoanionic ($O_{(phenolato)}^-$,N, O) or dianionic ($O_{(phenolato)}^-$,N, O^-) modes. It was concluded that the Ga(III) − salicylaldehyde semicarbazone species show unambiguously higher stability; whereas Cu(II) species have somewhat lower stability relative to the corresponding S-containing TSC analogues, however no significant decomposition of the Cu(II) complex was observed even at micromolar concentrations at physiological pH. [*E.A. Enyedy et al.; Polyhedron 67 (2014) 242.*]

Effects of methyl substituents at different positions on the 2-formylpyridine thiosemicarbazone (FTSC) core structure on various physico-chemical properties such as proton dissociation processes, aqueous solution stability, isomer distribution in different solvents, fluorescence properties, lipophilic character and Cu(II) binding ability were also investigated. We have found that methylation of FTSC on pK_a, lipophilicity and fluorescence is significant, and isomeric distribution and solution stability of the ligands strongly depend on methylation pattern. [*O. Dömötör et al., Inorg. Chim. Acta (2017) in press*] Impact of stepwise methylation of Triapine on various physicochemical and biological properties was also studied, we found that these compounds are highly synergistic with copper treatment most probably their strong Cu(II) binding ability. [*C.R. Kowol et al, J. Med. Chem. 59 (2016) 6739.*]

Speciation and antitumor activity of Cu(II) complexes of TSC-*morpholine, -piperazine conjugates* were studied revealing the formation of outstanding high stability mono complexes. In these complexes the ligands coordinate via the (S^-,N,N_{pyr},$N_{morph/pip}$) donor set increasing the metal binding ability compared to the traditional tridentate TSCs. The pH-dependence of the syn-anti isomers of the ligand precursors was also determined. *[F. Bacher et al, Dalton Trans. 44 (2015) 9071.]* The complexation behavior of a *proline-thiosemicarbazone bioconjugate* with excellent aqueous solubility with Cu(II) in an aqueous solution and in a 30% (w/w) dimethyl sulfoxide/water mixture has been studied. Predominant formation of an outstanding high stability mono complex was found in which the ligand coordinate via ($N_{pyridyl}$,N,S)(N_{Pro},COO^-) donor set. The complex displayed antiproliferative activity in CH1 ovarian carcinoma cells and inhibited Topoisomerase IIα activity. [*F. Bacher et al., Inorg. Chem. 52 (2013) 8895*.] The complexation behavior of this proline bioconjugate with Ni(II), Zn(II), Fe(II), Fe(III) and Ga(III) ions in aqueous solution has been also studied. Formation of only mono-ligand complexes were found with these ions (except to Fe(III)) in which

most probably the donor atoms of the Pro moiety are also coordinated leading to considerably high stability in the case of Ni(II) and Zn(II) ions. On the contrary low stability of the Ga(III) complexes at physiological pH was found and the low Fe(II) binding affinity of the ligand most probably contributes to its low antiproliferative activity. Between Fe(III) and the ligand, a redox reaction takes place via the oxidative cyclisation of the thiosemicarbazone. *[F. Bacher et al, Inorg. Chem. 53 (2014) 12595; F. Bacher et al., Inorg. Chim. Acta 455 (2017) 505.]*

In the literature numerous vanadium(IV/V) complexes of TSC complexes are reported without any solution characterization. In our work we studied the solution stability of V(IV/V) complexes of Triapine and two related α(N)-heterocyclic TSCs and salicylaldehyde TSC by pH-potentiometry, EPR and ^{51}V NMR spectroscopy. Dimethylation of the terminal amino group resulted in the formation of V(IV/V) complexes with considerably higher stability, while exchange of the pyridine nitrogen to a phenolate oxygen brings also stability increase. *[C.R. Kowol, et al, J. Inorg. Biochem. 152 (2015) 62.]*

Results obtained on copper complexes of various chalcogensemicarbazones were represented as a „keynote" lecture at an international bioinorganic chemistry conference. [*E.A. Enyedy et al.; XII International Symposium on Inorganic Biochemistry, Collaboration and Beyond, 2013.08.28-09.01., Wrocław, Poland*] On the other hand a Hungarian review paper was published entitled "Anticancer thiosemicarbazones and their metal complexes: relationship between stability and bioactivity" on the majority of the solution equilibrium data related to TSCs collected till now in our laboratory. [*E.A. Enyedy, Hungarian Journal of Chemistry 120 (2017) 48.*] One of the most important findings of this part of the project is that the antiproliferative activity of the studied TSCs shows a clear correlation with the stability of the Fe(II) complexes.

2.4 Chelators with pronounced activity towards MDR cells

As the appearance of resistance is a major obstacle in the treatment of cancer, there is an urgent need for novel chemotherapeutics with marked and selective antitumor activity. Numerous compounds show enhanced activity against cells with multidrug resistance (MDR) phenomena, and many of them posses chelating functionalities. In order to find relationship between the metal binding ability and selectivity we performed detailed solution equilibrium studies on some potent pyrimidinylhydrazone molecules together with the characterization of the redox properties of their Cu(II) and Fe(III) complexes. The most active compound forms highly stable complexes with Fe(III) and Cu(II) in a wide pH range with a stronger preference towards Fe(III), while the Cu(II) complex showed reversible redox cycling in biological relevant conditions. *[V.F.S. Pape et al., J. Inorg. Biochem. 144 (2015) 18.]*

2.5 Reduced Schiff base coumarin derivatives

Our studies on reduced Schiff-base coumarin ligands and albumin were used to test the fluorometric method and the possibility for computational evaluation with PSEQUAD program

instead of the various "graphical solutions" generally used in the literature. This computer program can operate on the whole recorded spectra and more emitting compounds can be taken into consideration at the same time. *[O. Dömötör et al., Bioorg. Chem. 52 (2014) 16.]*

3. Five selected papers related to the project

Orsolya Dömötör, Veronika F. S. Pape, Nora V. May, Gergely Szakács, **Eva A. Enyedy***
Comparative solution equilibrium studies of antitumor ruthenium(η^6-p-cymene) and rhodium(η^5-C_5Me_5) complexes of 8-hydroxyquinolines
DALTON TRANSACTIONS 46 (2017) 4382-4396.
IF: 4.029; ***Q1***; DOI: 10.1039/C7DT00439G

Éva A. Enyedy*, János P. Mészáros, Orsolya Dömötör, Carmen M. Hackl, Alexander Roller, Bernhard K. Keppler, Wolfgang Kandioller
Comparative solution equilibrium studies on pentamethylcyclopentadienyl rhodium complexes of 2,2'-bipyridine and ethylenediamine and their interaction with human serum albumin
JOURNAL OF INORGANIC BIOCHEMISTRY 152 (2015) 93-103.
IF: 3.205; ***Q1***; DOI: 10.1016/j.jinorgbio.2015.08.025
Independent citation: 1

Éva A. Enyedy,* Orsolya Dömötör, Krisztina Bali, Anasztázia Hetényi, Tiziano Tuccinardi, Bernhard K. Keppler
Interaction of the anticancer gallium(III) complexes of 8-hydroxyquinoline and maltol with human serum proteins
JOURNAL OF BIOLOGICAL INORGANIC CHEMISTRY 20(1) (2015) 77-88.
IF: 2.495; ***Q1***; DOI: 10.1007/s00775-014-1211-9
Independent citations: 8

Felix Bacher, Orsolya Dömötör, Maria Kaltenbrunner, Miloš Mojović, Ana Popović-Bijelić, Astrid Gräslund, Ghenadie Novitchi, Andrew Ozarowski, Lana Filipovic, Sinisa Radulović, **Éva A. Enyedy,*** Vladimir B. Arion*
Effects of Terminal Dimethylation and Metal Coordination of Proline-2-Formylpyridine Thiosemicarbazone Hybrids on Lipophilicity, Anti-proliferative Activity and hR2 RNR inhibition
INORGANIC CHEMISTRY 53(23) (2014) 12595-12609.
IF: 4.762; ***D1***; DOI: 10.1021/ic502239u
Independent citations: 7

Éva A. Enyedy*, Orsolya Dömötör, Erika Varga, Tamás Kiss, Robert Trondl, Christian G. Hartinger, Bernhard K. Keppler
Comparative solution equilibrium studies of anticancer gallium(III) complexes of 8-hydroxyquinoline and hydroxy(thio)pyrone ligands
JOURNAL OF INORGANIC BIOCHEMISTRY 117 (2012) 189-197.
IF: 3.197; Q2; DOI: 10.1016/j.jinorgbio.2012.08.005
Independent citations: 15

Szeged, 10.08.2017.