BIOSPECIATION OF DRUG CANDIDATES STUDIED BY THE COMBINATION OF SEPARATION AND SPECTROSCOPIC METHODS

In preclinical testing of pharmaceutical agents investigation of the pharmacokinetic behavior is as important as monitoring the pharmacodynamic properties. Drug candidates with promising *in vitro* activity often fail to enter clinical testing because of their poor pharmacokinetic performance. Extreme poor aqueous solubility, hindered ability to cross lipid membranes or short lifetime in blood circulation possibly due to low serum protein binding may diminish the *in vivo* efficacy of the tested agent. Namely, investigation of the physico-chemical properties, which essentially affect the pharmacokinetic behavior, is crucial as well. Additionally, it is important to take into account interactions with blood serum proteins, which may prolong the serum half-life of a drug preventing it from early metabolism and elimination; and they also take part in drug delivery (*e.g.* via enhanced permeability and retention effect).

The main aim of the project was to found correlations between the physico-chemical properties, serum protein binding of metal complexes (Ru(II), In(III) and Co(III) complexes) and various organic molecules (tyrosine kinase inhibitors (TKIs), antiviral and antifungal agents) and their biological efficacy using a broad spectrum of instrumentation. The main techniques used were ^{1}H NMR spectroscopy, spectrofluorometry (steady-state, time resolved and anisotropy assays), UV-visible (UV-Vis) spectrophotometry, and separation methods such as ultrafiltration and capillary electrophoresis (CE).

The obtained results were summarized in 5 peer-reviewed papers published in international scientific journals (4 Q1 and 1 Q2; summa impact factor: 22.98) and a manuscript is currently under review, the results were also presented in 1 science promotion lecture and in 2 national and 2 international conferences. Undergraduate students were also involved in the research, 2 MSc/BSc theses were prepared and 2 are ongoing under my supervision.

The main results of the project are presented below in a breakdown by the studied systems.

Ru(II)-polypyridyl complexes:

In accordance with the project plans we have investigated the solution stability and lipophilicity of Ru(II)-polypyridyl complexes, and their binding towards biomacromolecules such as HSA and DNA was assayed as well. The Ru(II)-complexes were produced by our collaborators or synthesized by the PI in Szeged.

The complexes **M1** and **B1-B3** were synthesized in the laboratory of G. Gasser (Chimie ParisTech) (Fig. 1). Complexes of the **B**-series are developed for photodynamic therapy.

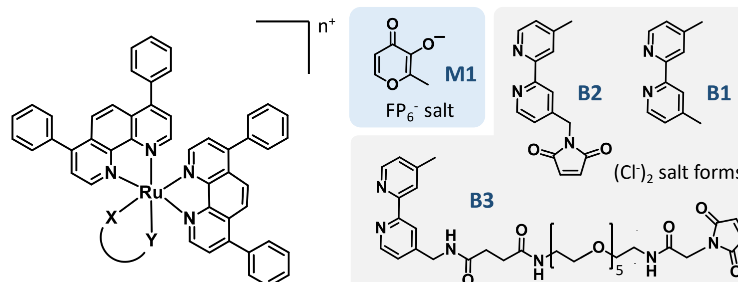


Figure 1.

According to our investigations, these complexes are stable in aqueous solution at least for 10 days, the coordination sphere remains intact, however the maleimide functional group in complexes **B2** and **B3** hydrolyzes partly at pH 7.4 (pH 7.4 corresponds to the blood pH). The aqueous solubility of the complexes becomes highly limited at high chloride ion concentration. In line with this finding, complexes **B1-B3** were more lipophilic in phosphate buffered saline (PBS) as in simple phosphate buffer, namely, the complexes show high tendency for ion pair formation with chloride ions. The complexes were highly cytotoxic in CT26 (colon carcinoma) and RPE-1 (non-cancerous retinal epithel) cells, when cells were exposed to visible 595 nm light for 2 h (IC_{50} = 0.4–8.4 μM). Complexes **B2** and **B3** were inactive on cells kept in darkness, while **B1** was still active under the same conditions (*ca.* tenfold increase in the IC_{50}

values in dark). Complex **M1** showed IC_{50} = 0.61-0.86 μM activity in the same cell lines. Cell accumulation experiments showed, that **B1** is taken up to the greatest extent in CT26 cells, **B2** and **B3** displayed 3- and 6-times lower accumulation in the same cells. The binding to HSA was significant in the case of **M1**, **B1**, **B2** and **B3**. The former two complexes bound exclusively via intermolecular bonds to the protein, and no decomposition of the complexes occurred. Complex **B2** binds to the protein in the same way, and additionally covalent binding occurs between the maleimide functional group of the complex and the only available cysteine (Cys34) of HSA (Fig. 2). The PEGylated complex (**B3**) shows weaker binding at the hydrophobic sites of albumin compared to **B1** and **B2**, and could interact with Cys34 (Fig. 2). Interestingly the binding of **B2** and **B3** to Cys34 is not quantitative, the interaction can be made stoichiometric only by denaturation of the protein. Upon denaturation of HSA, Cys34 becomes more easily available and the competing 'intermolecular sites' are also disrupted. This phenomenon refers to the occurrence of some competition, namely binding to the Cys34 either competes with the hydrolysis of maleimide and/or with the intermolecular binding at other sites of HSA. The lipophilicity and albumin binding of simple [Ru(2,2'-bipyridine)$_3$]Cl_2 and [Ru(1,10-phenantroline)$_3$]Cl_2 were also investigated for comparison.

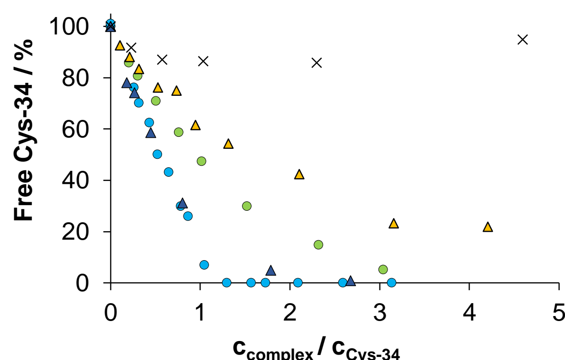


Figure 2. Free Cys-34 content of HSA at various **complex**-to-HSA ratios; symbols denote the following complexes and conditions ▲: **B2**, native protein; ▲: **B2**, denatured protein; ●: **B3**, native protein; ●: **B3**, denatured protein and ×: **B1** with native protein as negative control {pH 7.00 (100 mM phosphate buffer), assayed by 2,2'-dithiodipyridine}.

These results were published in a scientific paper [A1] and in one national conference [C1] and in an international conference [C2].

Two Ru(II) polypyridyl complexes formed with pyridyl derivatives of benzothiophene (**P1**) and benzimidazole (**P2**) pharmacophores were synthesized in cooperation with A. I. Tomaz and coworkers (University of Lisbon) (Fig. 3). Aqueous stability, lipophilicity, albumin and DNA binding properties of the two complexes were investigated in our laboratory. The complexes showed good aqueous solubility, **P1** displayed a transformation process in aqueous solution at pH 7.4 when it was exposed to sunlight, while **P2** was proven to be stable under the same conditions. **P2** is partly deprotonated at pH 7.4, it exists as +1 and +2 charged cations in 2:3 ratio. As a result of this behavior, lipophilicity of **P2** depends on the pH of the aqueous phase. In contrast, **P1** is quite hydrophilic under physiological conditions. This feature is often associated with a lower anticancer activity but that was not the case for **P1**. Both compounds were found cytotoxic on cancer cells (Colo-205, Colo-320, A2780) although to a different extent depending on the model used. In flow cytometric assays it was found that late apoptosis and necrosis are the characteristic cell death mechanisms by treatment with the two complexes. They are globally active for chemo-sensitive human cancer cells but their performance for resistant colon cells is poor. Low binding affinity of the complexes towards

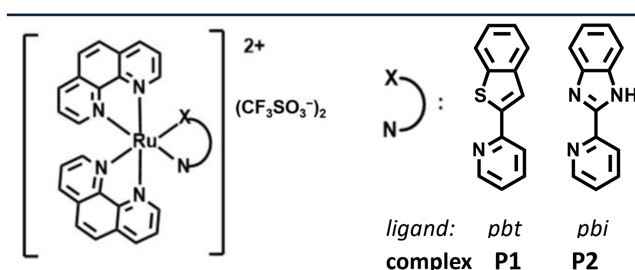


Figure 3.

HSA was found, while they are able to interact with calf thymus DNA. Most probably partial intercalation takes place. Additionally, we have pointed out that widespread fluorescent probe displacement studies can be misinterpreted easily if they are not supplemented by fluorescence lifetime measurements [A2]. Additionally, one BSc thesis (Andor Szűcs, 2020) was prepared under my supervision, comprising biospeciation studies on complexes **B1**, **B2**, **P1** and **P2**.

Numerous [Ru(II)(N,N)$_2$(O,N)]Cl type complexes were synthesized in our laboratory (Fig. 4.).

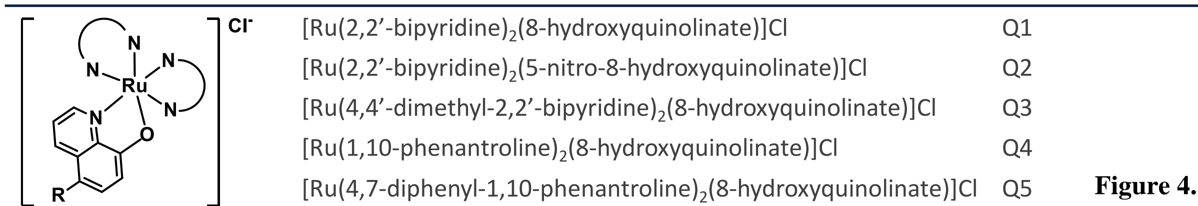


Figure 4.

The introduction of 5-chloro- or 5-nitro-7-((proline-1-yl)methyl)-8-hydroxyquinoline ligands, with good aqueous solubility and multidrug resistance reversal activity, was planned originally as well. However, purification of the synthesized complexes via the standard chromatographic and extraction approaches was not successful. Instead, a systematic study was designed to investigate the effect of the various (N,N) and (N,O) ligands on the cytotoxicity, lipophilicity and HSA and DNA binding ability of the Ru(II)-polypyridyl complexes. Started from 2,2'-bipyridine, more extended and more lipophilic (N,N) ligands were introduced, and the effect of 5-nitro substitution of the 8-hydroxyquinoline (8HQ) ligand was also studied. Cytotoxicity assays in Colo205 and MCF-7 cancer cells showed no activity of complexes **Q1** and **Q2**. **Q3** (IC_{50} = 8.9±0.3 (Colo205) 16.1±0.1 (MCF-7)) and **Q4** (IC_{50} = 53±2 (Colo205) 43±7 (MCF-7)) showed good-to-moderate activity while **Q5** could not be assayed due to solubility issues. The synthesized complexes are generally hydrophilic in phosphate buffer (except **Q5**) and they are more lipophilic in PBS. **Q5** is highly lipophilic even in water (Fig. 5). The complexes did not bind considerably to DNA in our studies, and the albumin binding falls short of what was observed for the **B1-B3** complexes (Fig. 5). The results were presented in national and international conferences [C1,C2]. An MSc thesis based on this work (Kincső Kovács, *expected date* 2023) is in preparation under my supervision.

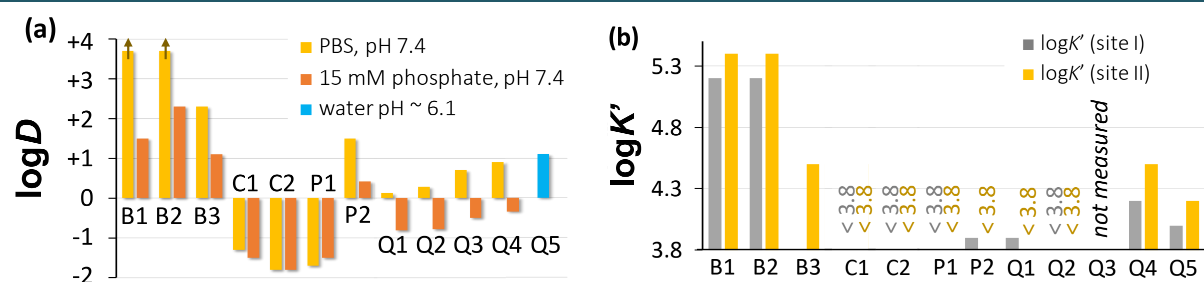


Figure 5. (a): *n*-Octanol/ water distribution coefficients (log*D*) of the Ru(II)-polypyridyl complexes determined in PBS, phosphate buffer or water; C1: [Ru(2,2'-bipyridine)$_3$]Cl$_2$, C2: [Ru(1,10-phenantroline)$_3$]Cl$_2$ {↑*: a lower limit could be estimated only, 25 °C*}. (b): Binding constants (log*K*') obtained by spectrofluorometry at the two hydrophobic sites (site I and II) of HSA {*pH 7.4 (PBS), 25 °C*}.

In the study of the above Ru(II)-complexes, some general conclusions can be drawn: (i) the high lipophilicity and/or definite tendency for ion pair formation of **B1-B3** complexes seem to give preference for the albumin binding; (ii) the lipophilicity of +1 charged **Q1-Q5** complexes is considerably lower in comparison to the +2 charged **B**-type complexes, also the ion pair formation with chloride ions is less pronounced for 8HQ complexes; (iii) the lipophilicity of the 8HQ-complexes seems to correlate in some extent with the cytotoxicity and albumin

binding affinity of the complexes; (iv) interestingly the exchange of one (N,N) ligand to 8HQ does not result in tendentious change in the lipophilicity and albumin binding ability of the complexes (see *e.g.* **B1→Q5** vs. **C2→Q4**); (v) the 8HQ complexes, having even more extended aromatic (N,N) ligands (**Q4**, **Q5**) do not bind to DNA.

Indium(III) complexes

Solution speciation and serum protein binding of selected In(III) complexes bearing (O,O) (maltol, deferiprone) and (O,N) (8HQ, 8HQ-5-sulfonate) donor sets were studied, in order to provide comparative data for In(III) and analogous Ga(III) complexes. Both Ga(III) and In(III) are group 13 metal ions; 8HQ and maltol complexes of the former ion were clinically tested against neoplasms, while ^{111}In(III) complex of 8HQ was developed for diagnostic application. Aqueous stability of the indicated ligand - In(III) complexes was characterized. The *tris*-ligand complexes of 8HQ (**InQ$_3$**) and deferiprone (**InD$_3$**) are relatively stable at pH 7.4, the *tris*-maltolato complex (**InM$_3$**) displays insufficient stability. Binding towards HSA and (apo)transferrin ((apo)Tf) of **InQ$_3$**, **InD$_3$** and **InM$_3$** complexes and the Ga(III) analogue of **InQ$_3$** (**GaQ$_3$**) together with InCl$_3$ was investigated by a panel of methods. Moderate binding of **InQ$_3$** to HSA was found. ApoTf is able to displace 8HQ, deferiprone and maltol effectively from their In(III) complexes. Spectrofluorometry was proven to be a useful method to follow the speciation of **InQ$_3$** in protein containing binary and ternary systems (Fig. 6). Studies conducted with the **InQ$_3$**/**GaQ$_3$** – HSA – Tf ternary systems revealed the more pronounced Tf binding of In(III) via ligand release, while the original **GaQ$_3$** scaffold is preferably retained upon protein interactions and significant albumin binding occurs. This behavior explains why **^{111}InQ$_3$** cannot be administered into the blood directly and a previous blood cell labelling procedure is necessary, while circulating **GaQ$_3$**, at least partly, can reach the target cells [A3].

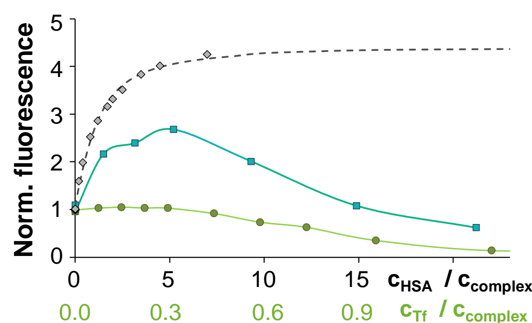


Figure 6. Changes of fluorescence intensity in the **InQ$_3$** – HSA – Tf (■), **InQ$_3$** – Tf (●) and **InQ$_3$** – HSA (♦, - - -) systems. {$c_{complex}$ = 10 µM; pH 7.40}

Tyrosine kinase inhibitors (TKI) and antiviral agents:

We have studied the stability of a series of TKI-Co(III) complexes designed as reducible prodrugs for cancer treatment (Fig. 7). We have found, that the reduced Co(II) complexes favor the release of the TKI ligand, namely the active ligand was liberated after reduction of the complex. At the same time ascorbic acid, glutathione and NADH (common reducing agents in biological systems) were not able to reduce the complexes within 24 h. Most probably proteins are responsible for reduction, but the actual reducing molecules or reductase(s) are still unknown [A4].

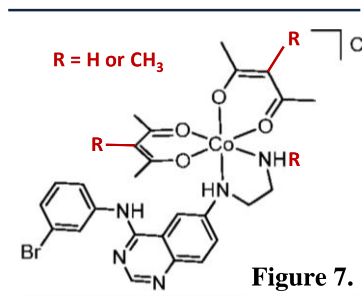


Figure 7.

Within the family of TKIs fibroblast growth factor receptor inhibitor (FGFRI) marketed drugs ponatinib, erdafitinib, nintedanib and an investigational agent MMAT081 (derivative of ponatinib) was chosen for systematic characterization. Strong serum protein binding of the marketed TKIs is well documented (but without binding constants). It is also known, that the outstanding binding of erdafitinib towards α_1-acid glycoprotein (AGP) resulted in complications in the dosing, while in the case of ponatinib and nintedanib no such clinical

issues were reported yet. We wanted to test the usefulness of our model calculations obtained from *in vitro* protein binding assays. First of all, *n*-octanol/water distribution coefficients and proton dissociation constants were determined. The compounds have poor water solubility. Ponatinib, MMAT081 and nintedanib were found to bind to HSA with moderate affinity (log*K*' = 4.5-4.6) and about one order of magnitude higher binding constants were determined for the AGP–drug adducts (log*K*' = 5.4-5.5). Erdafitinib binds to HSA and AGP with higher affinity (log*K*' = 5.0 and 7.0, respectively). Model calculations were made on the basis of the obtained binding constants and the available *in vivo* drug and protein concentrations found in the literature.

According to Fig. 8 the free concentration of nintedanib does not change considerably (3.0→2.9 nM) in acute phase, which condition is triggered by inflammation or cancer. Very similar picture was seen for MMAT081 and ponatinib. On the other hand, free erdafitinib concentration decreases significantly (4.5→2.3 nM) in acute phase. Free concentration of erdafitinib obtained in clinical studies[1] are very similar to the result of our model calculations. The results were presented in a national conference [C3] and are currently prepared for publication. A MSc thesis (Krisztina Molnár, *expected date*: 2023) is under preparation, and a lecture was also presented in the local Conference for Undergraduate Researchers (Krisztina Molnár, title: *The binding of three tyrosine kinase inhibitors to blood serum proteins: biospeciation studies, pharmaceutical section*, 2022.11.17. Szeged).

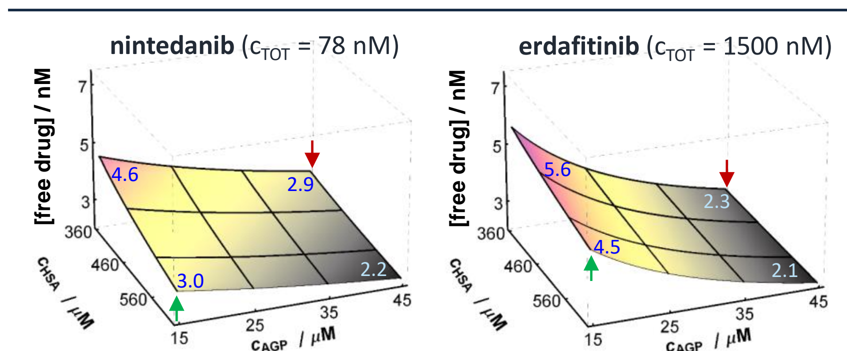


Figure 8. Model calculations on the free drug concentrations of nintedanib and erdafitinib as a function of HSA and AGP concentration; ↑: physiological protein concentrations (*ca.* 630 μM HSA, *ca.* 15 μM AGP), ↓: pathological protein concentrations in acute phase (*ca.* 360 μM HSA, *ca.* 45 μM AGP); total concentration (c_{TOT}) of the drugs reported in clinical studies were used for calculations.

Our investigations were extended to antiviral agents favipiravir, molnupiravir and imatinib, recently approved as possible therapeutic agents against COVID-19. There were a number of uncertainties in regards of plasma protein binding and blood distribution of these drugs. Therefore, thorough solution chemical characterization and investigation on their serum protein (HSA and AGP) binding were carried out. Our main conclusions are, that the only available *in vitro* serum protein binding data on favipiravir is overestimated and it is falsely referred in the literature as an *in vivo* result. The pK_a value and logD_{pH} values of favipiravir were determined by us at first and fluorescent properties of the drug were also characterized. Molnupiravir does not bind to serum proteins but gets hydrolyzed by the protein constituents of blood. Albumin and AGP binding of imatinib were characterized by various analytical methods, results were critically evaluated and compared to literature data. Model calculations were done in order to estimate the effect of albumin binding and AGP levels on the free imatinib concentration. The results show minimal albumin binding and large scale binding to AGP, that can significantly increase in cancerous condition, and consequently the serum level of free imatinib may decrease under the therapeutic window [MS1].

[1] L.Y. Li, Y. Guo, M. Gonzalez, D. Ouellet, Effect of Plasma Protein Binding on the Pharmacokinetics of Erdaftinib: Results of an Integrated Cross-Study Analysis, J. Clin. Pharmacol. 00 (2019) 1-9.

Pentamidine derivatives

Drug likeness properties of five pentamidine derivatives were studied in cooperation with the research group of T. Zolek (Medical University of Warsaw) (Fig. 9). Human serum albumin binding, proton dissociation constants and lipophilicity of the compounds were experimentally characterized in our laboratory and results were compared to computed data. Our studies indicated no considerable binding at any sites of HSA, only weak binding on the proteins surface is assumed according to the theoretical analysis. The toxicity profile of the studied pentamidines evaluated by several predictors classify them as non-toxic but some drug-likeness parameters are less promising (*e.g.* their highly hydrophilic character) [A5].

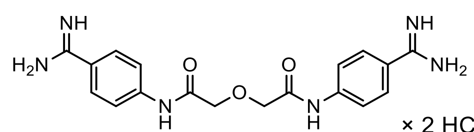
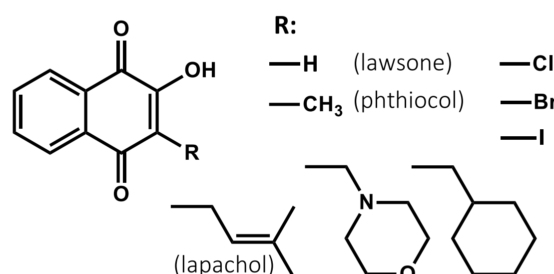
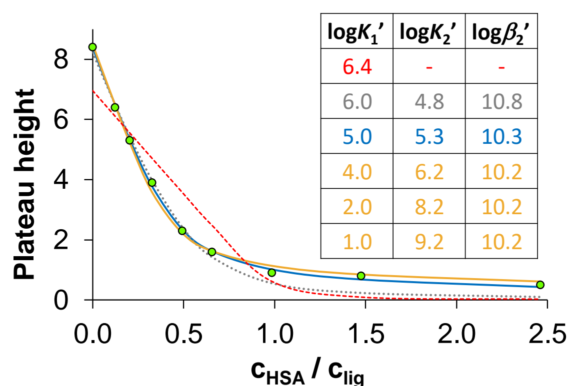


Figure 9. One example of the studied ligands.

1,4-Naphthoquinones:

The number of applicable techniques for investigation of protein binding of numerous compounds are often limited (*e.g.* in case of poor water soluble FGFRIs and InQ_3). Additionally, the reliability of equilibrium data acquired by separation techniques (ultrafiltration, CE, equilibrium dialysis *etc.*) is a subject of constant debate. We have also experienced that frontal analysis capillary electrophoresis (FACE) provided lower binding constants for the imatinib–protein systems as it was calculated in fluorometric experiments. Therefore, various 1,4-naphthoquinone compounds, lawsone and its 3-substituted derivatives (Fig. 10) were included in our investigations. Some of the compounds display valuable pharmacological effect and their albumin binding could be followed by various measurement techniques such as UV-Vis, spectrofluorometry, ultrafiltration and FACE. According to the obtained results, ultrafiltration and CE are excellent techniques to find the protein-to-drug binding stoichiometry. Only one lawsone molecule can bind to albumin, while two sites are available for the other derivatives, except the methyl-morpholine substituted compound, which did not bind to the protein. The binding constants determined by fluorometry and ultrafiltration and FACE are in good agreement, when only one ligand binds to HSA (lawsone). On the other hand, the stepwise binding constants (K_1', K_2') computed for the binding of two ligands are rather uncertain and only their product (β_2') could be well determined (see Fig. 11). A MSc thesis (Andor Szűcs, 2022) was prepared covering the results on the basic compound lawsone and three derivatives.





logK_1'	logK_2'	logβ_2'
6.4	-	-
6.0	4.8	10.8
5.0	5.3	10.3
4.0	6.2	10.2
2.0	8.2	10.2
1.0	9.2	10.2

Figure 11. Plateau height of free lapachol obtained in FACE study for the HSA-lapachol system (●) and fitted curves according to the color coding of the inserted table {c_{lig} = *50 µM, pH 7.4 (phosphate)*}.

Publications (supported by the NKFIH PD 131472 project)

Papers

[A1] A. Notaro, M. Jakubaszek, S. Koch, R. Rubbiani, **O. Dömötör**, E.A. Enyedy, M. Dotou, F. Bedioui, M. Tharaud, B. Goud, S. Ferrari, E. Alessio, G. Gasser*, *A maltol-containing ruthenium polypyridyl complex as a potential anticancer agent*, Chem. Eur. J. 26 (2020) 4997-5009.

[A2] **O. Dömötör***, R.G. Teixeira, G. Spengler, F. Avecilla, F.Marques, O.A. Lenis-Rojas, Cristina P. Matos, R.F.M. Almeida, E.A. Enyedy, A.I. Tomaz*, *Ruthenium(II) polypyridyl complexes with benzothiophene and benzimidazole derivatives: Synthesis, antitumor activity, solution studies and biospeciation*, J. Inorg. Biochem, 238 (2023) 112058.

[A3] **O. Dömötör,*** B.K. Keppler, É.A. Enyedy, *Solution speciation and human serum protein binding of indium(III) complexes of 8-hydroxyquinoline, deferiprone and maltol*, J. Biol. Inorg. Chem. 27 (2022) 315-328.

[A4] M. Mathuber, H. Schueffl, **O. Dömötör**, C. Karnthaler, É.A. Enyedy, P. Heffeter, B.K. Keppler, C.R. *Kowol, Improving the stability of EGFR inhibitor cobalt(III) prodrugs*, Inorg. Chem. 59 (2020) 17794-17810.

[A5] T. Zolek, **O. Dömötör**, M. Rezler, É.A. Enyedy, D. Maciejewska, *Deposition of pentamidine analogues in the human body – spectroscopic and computational approaches*, Eur. J. Pharm. Sci. 161 (2021) 105779.

Paper under review

[MS1] **O. Dömötör,*** É.A. Enyedy, *In vitro blood distribution and plasma protein binding of se-lected antiviral drugs (favipiravir, molnupiravir and imatinib) used against SARS-CoV-2*, paper submitted to Int. J. Mol. Sci.

Conferences

[C1] **O. Dömötör**, *Ruténium(II)-polipiridil komplexek előállítása, oldatkémiai jellemzése és kölcsönhatásuk humán szérum albuminnal és DNS-sel*, 55th Colloquium on Complex Chemistry, 2022.05.25.-27. Debrecen, Hungary (oral presentation).

[C2] **O. Dömötör**, K. Kovács, R.G. Teixeira, A.I. Tomaz, R. Vinck, G. Gasser, G. Spengler, É.A. Enyedy, *Anticancer activity and biospeciation of ruthenium(II) polypyridyl complexes*, ISMEC 2022 International Symposium on Metal Complexes, 2022.06.05.-08.Valéncia, Spain (oral presentation).

[C3] **O. Dömötör**, K. Molnár, A. Federa, É.A. Enyedy, C.R. Kowol, *Comparative solution equilibrium studies on the serum protein binding of tyrosine kinase inhibitors*, Peptide Chemistry and Chemical Biology Symposium, 2022.05.30-06.01. Balatonszemes, Hungary (poster and oral flash presentation).

Science promotion

O. Dömötör, *Some uses of fluorometry in chemistry*, Eötvös Lóránd College, Chemical Workshop (Eötvös Lóránd Kollégium, Kémia Műhely), 2022.03.07. Szeged.