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Review report on Ph.D. thesis of RNDr. Lucia Pazderová titled:
"Complexes of macrocyclic ligands with phosphonate and phosphinate pendant arms for molecular imaging"

The presented Ph.D. thesis is composed of 83 pages of text including introduction to the field, supplemented by four appendices in the form of three publications in peer-reviewed international journals and one manuscript. Overall, the thesis is well structured and written in clear and understandable English. The results are of a good scientific merit, evidenced by the three publications.

Introductory chapter provides clear overview of molecular imaging methods relevant to the topic of the thesis, and very comprehensive overview of chelators used for this purpose. It is thoroughly and extensively referenced with relevant literature.

The experimental part is logically divided into two, based on the structure of the studied compounds. The first one describes development and investigation of properties of two side-bridged bicyclic chelators, one based on triazacyclononane (TACN) and the other based on cyclen macrocycle. The design of these molecules is innovative and interesting. Unfortunately, further testing of their properties uncovered severe flaws, causing that these molecules were not suitable for the desired purpose. This first part of the thesis bears signs of an "exploratory phase" to try unorthodox ideas, where one can hardly predict the outcome. Boldness of this approach is appreciated.

The second part demonstrates that lessons were learned in the "exploratory phase". The design of the next group of chelators has been substantially improved. This part deals with three cross-bridged cyclam derivatives, and one cyclam derivative (non-cross-bridged) with a single phosphinate-bis(phosphonate) pendant arm. The intention was to accelerate kinetics of formation of metal chelates with the phosphorus-containing pendant arms, while assuring high kinetic inertness and thermodynamic stability of the chelates with the cyclam-based macrocyclic rings. The design was well thought-through in advance. The main idea to attract Cu(II) ions with geminal bisphosphinate arms and the phosphinate-bis(phosphonate) arm is interesting and innovative. But most importantly, substantial evidence is provided that the idea mostly worked as intended. The formation kinetics of Cu(II) chelates were accelerated, while dissociation kinetics were exceptionally slow, an ideal combination of properties for labeling with copper radioisotopes. Finally, the most promising chelator, with an ability to target areas with active bone metabolism, was selected for *in-vivo* tests in mice. The imaging results with

Cu-64 radiolabeled H_5**TE1PBP** are impressive, especially the absence of uptake in liver, which confirms high *in-vivo* kinetic inertness of the chelate, despite of it being the least inert of the four cyclam derivatives studied *in vitro*.

There are a few minor issues in the thesis that deserve clarification from the Ph.D. candidate. A detailed list is provided at the end of this report. I have only one general point regarding the methodology of measuring kinetic inertness of the studied metal chelates. The dissociation kinetics were studied only with acid-assisted decomplexation. Although it is the most commonly used method, it remains an open question how relevant are results obtained under conditions so far from physiological. Transchelation or transmetallation under physiologically more relevant conditions have not been tested. However, given the demonstrably high kinetic inertness of the compounds, one must admit that such experiments could require very long time to conduct and/or very sensitive analytical methods.

In conclusion, I **approve and recommend** the thesis of RNDr. Lucia Pazderová for the final defense leading to a Ph.D. degree.

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List of comments and questions:

Page 9: “However, the design of new chelating ligands must take into account the requirement of **high thermodynamic stability** to avoid exposure of the patient to the **toxicity of the contrast agent**.”

> Toxicity of the contrast agent itself, and toxicity of free Gd(III) ions that can be released from it are two different things. And thermodynamic stability is actually not the relevant factor for release and toxicity of Gd(III) ions. Kinetic inertness is.

Page 11: “The ^{64}Cu isotope is relatively readily available commercially. It can be produced in biomedical cyclotrons using **proton radiation from** an enriched ^{64}Ni isotope through ^{64}Ni(p,n)^{64}Cu **decay**.”

> “Decay” is not the right term. It is a nuclear reaction. Also, “proton irradiation of an enriched…” would be the correct way to describe the production method.

Page 14: “By far the most used radionuclide in SPECT is the ^{99m}Tc isotope. The **number of clinical examinations** using this isotope **exceeds** the **number of PET agents**.”

> I am not sure how to understand the message of this statement. It is comparing two totally incomparable things.

Page 14: “Chemistry of technetium is remarkable due to this element is able to form characteristic inorganic functional moieties.”

> I am struggling to understand the meaning of this sentence.

Page 20: “In complexation studies with copper, it showed much lower **kinetic stability** than cb-TE2A.”

> It would be good to stick to the same terminology throughout the entire text and distinguish **thermodynamic stability** and **kinetic inertness**. The incorrect term **kinetic stability** is used several times in the introduction.

Page 24: “The complexation of macrocyclic compounds does not occur sequentially as **it knows** for the linear ligands.”

> Probably a typo?

Page 29: “The comparison of efficiency and selectivity of complex formation between NOTP and NOTP^{et} showed that the latter has high selectivity to Cu(II) and is generally a more selective agent towards the **studied cations**.”

> Which studied cations?

Page 59: “The explanation is the assumption that the second ligand molecule in the $[M(L)_2]^{OC}$ intermediate helps to deprotonate the macrocycle.”

> This is very interesting observation and explanation!

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Page 59: Table 5:

1. Information about concentration is missing. This is very important information for considering kinetics of formation.

2. Was the 0.1 s result really measured, or was this the lower limit of measurable values with the method used?

3. The results are nice, but a question remains how this data translates to realistic radiolabeling conditions, which are done at much lower concentrations.

Page 61: "The **half-life of the bis(phosphinate) complex (10.7 h) is one order of magnitude shorter** than the half-life of Cu(II)-H_4cb-TE2P and is similar to the half-life of the hexacoordinated copper complex of H_4TE2P[1,8] ligand (13.4 h).[125] This, in turn, is **explained by the strong chelating ability of the bis(phosphinate)** group even in a very acidic environment."

It seems to me that these are contradictory statements.

Additional questions:

On Page 17, in chapter 3.2 Chelating agents, the first sentence says: "Metal isotopes cannot be administered to the patient in free form…"

This is incorrect. Can you name some metal isotopes that are clinically used in "free" unchelated form?

Page 42: Regarding chelator H_2**bpbtacn** and H_4**bpbcen**: "The similarity of the ligands lies in rigidity of the macrobicyclic structure, which **does not allow simultaneous coordination of the azamacrocycle amine groups and both phosphinate anions** of the pendant to the same metal ion."

Looking back at this experience, do you have any suggestion what could be changed to improve the molecular design?

Page 55: Figure 23: Distribution diagrams of (A) Cu(II)-H_5**TE1PBP** system (absorbance at 580 nm is shown as black dots) and (B) {Cu(II)-H_5**TE1PBP**}-Ca(II) ternary system.

The maxima for $[Cu(H_2L)]^-$ and $[Cu(HL)]^{2-}$ species in the distribution diagram shift to lower pH upon interaction of the bisphosphonate moiety with Ca^{2+}. Do you have any explanation for this behavior? The bisphosphonate moiety is quite far from the macrocyclic chelate and should not be affecting it.

Appendix 1 (ZAAC paper) mentions that complexation of Ga(III) was attempted.

Can you comment on the results of the Ga(III) experiments?

Appendix 2 (Polyhedron paper). It seems from the results that the two acetate arms in the side-bridged cyclen ligand are redundant.

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Would you expect the properties of the ligand to change much if the acetate pendant arms were absent?

Appendix 3 (Inorg. Chem. paper), Page 8439 of the paper: "The accelerated dissociation of Cu(II)-H_4L^2 might be explained by the relatively strong chelating ability of bis(phosphinate) pendants even in very acidic solutions, which helps the Cu(II) ion be released from the macrocyclic cavity."

Here you are trying to explain kinetic behavior with a thermodynamic effect (stability of out-of-cage chelate). I don't quite see the relationship. The causality of the events is that the metal has to leave the cage first, to be "caught" by the bis(phosphinate). But how is strong chelating ability of the pendant going to be involved in this?

Appendix 4 (manuscript)

Could you elaborate more on the advantage of using ^{64}Cu chelate for bone targeting, when the same result can be obtained with much cheaper and clinically approved [^{18}F] sodium fluoride?

Page 151: "This acceleration is even stronger in $[M_2L]^{OC}$ intermediates, whose re-arrangement is two orders of magnitude faster than that of $[ML]^{OC}$, most likely due to the weaker binding of the second Cu^{II} ion in the intermediate."

But isn't this explanation contradicting the idea mentioned earlier in the paper manuscript and this thesis, that stability of the out-of-cage complex helps to improve kinetics of formation of the in-cage complex?

Page 151: "The rate constants of the more protonated forms of the intermediates, $^{OH}k_{MxHLy}$, are one order of magnitude higher than those of the deprotonated forms, $^{OH}k_{MxLy}$. This difference should also be related to the strength of Cu^{II} binding to the bis(phosphonate) group, which is weaker in the protonated intermediate and thus enables its transfer to the macrocycle cage."

The same issue as in the previous comment.

Page 151: "Figure 4 and Table S7 show the times required for 99% complexation at a 10-fold metal ion excess for cyclam-based ligands with phosphonate and phosphinate pendant arms."

This is not a fair comparison. The previous text talks about the fact that the M_2L ternary complex has faster kinetics of formation than the other species. Here you are promoting this reactive intermediate, that the other molecules being compared probably do not have. Also, excess of metal is unrealistic under radiochemical synthesis. There, an excess of ligand is needed instead, as evidenced from the specific activity of radiolabeled compound (see below).

Page 152: "… the fastest among cyclam phosphonate/ phosphinate derivatives, presumably thanks to the appropriate stability of the out-of-cage reaction intermediate.

Again, this seems to be in contradiction with the statements above. Please explain how stability of the out-of-cage species relates to the kinetics of formation of the in-cage complex. Is it good or bad for kinetics of formation?

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Page 152: “Another reason for such a fast complexation could be the efficient proton transfer from the macrocycle nitrogen atoms to bulk water.”

> This actually seems as a much more plausible and universal explanation of the points raised above.

Page 155: “Moreover, the [^{64}Cu]Cu^{II}-te1P^{BP} tracer was obtained with a high specific activity of approximately **30 GBq/µmol of the ligand**”

> 30 GBq of Cu-64 is approximately 3.3 nmol, which means that the ligand to metal ratio was 300 : 1. That is a quite high excess of ligand. Could you comment on why such ratio was chosen or why it was needed?

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