Review of master thesis Identifying the Mode of Action for Bisphosphonium Salts – Potent Trypanosomatid Inhibitors by Bc. Jan Martínek

Formal part

The thesis by Jan Martínek has standard 6 chapters written on 47 pages. The English language – as far as I can judge – is perfect. Several typos were reported to the author for the sake of correcting them before submitting the thesis as a manuscript to a scientific journal.

The only formal flaw concerns citations, both in the text and in the reference list. However, I am convinced that the errors stem most-likely from in-compatibility of different reference editors or text processor and were not introduced intentionally. E. g. the last sentence in a paragraph 1.1.2 Trypanosoma contains three citations of which only the first one is correct: "To compensate that, DK parasites contain a mutated γ subunit of complex V (Lai et al., 2007; Schnaufer, 2010; Lun 2010)." According to the reference list, the second work was done by Schnaufer, Hashimi, Lun, Ayala and Lukeš, hence it should be cited as Schanufer et al., 2010. The same applies to the third citation that was authored by Lun, Lai, Li, Lukeš and Ayala. Moreover, in the list of references, some of the citations contain full names (e. g. page 45, Lun et al., 2010 contains full names Zhao-Rong, De-Hua Lai etc.) while others are abbreviated (e. g. page 45, Lai et al., 2008 contains abbreviations only – Lai D-H, H. Hashimi etc.). I strongly recommend polishing the references before converting the text into a manuscript.

Scientific part

I divided my comments into Major and Minor points. I would like the author to react to major points while the minor ones are usually of text polishing/correcting nature and do not require presentation in front of this audience. Nevertheless, I would like to discuss them before the defence itself.

Introduction

Major points

Chapter 1.1.2.1 Human african trypanosomiasis, page 6 the first sentence in the last paragraph states "Untreated trypanosomiasis ends fatally. Is author familiar with a work by

Jamonneau and colleagues reported in 2012 (Jamonneau et al., 2012)[1]? Could author comment on this article?

Chapter 1.3 Metabolic changes in T. brucei PF to BF, the second paragraph, the third sentence describes the fate of pyruvate in PF. Basically, the described state is the one found in text books and does not reflect the fact that pyruvate does not enter the Krebs cycle despite the presence of all Krebs cycle enzymes. Surprisingly, even the cited article does describe the following: "Early models proposed that, in most try- panosomatids, acetyl-CoA produced from glucose metabolism is converted into CO2 through the tricarboxylic acid (TCA) cycle and into acetate (Table 1). However, the recent analysis of an aconitase (step 30) knockout mutant revealed that acetyl- CoA does not fuel the TCA cycle of the procyclic trypanosomes grown in glucose-rich medium [21]. Consequently, most of acetyl-CoA (if not all) is converted into the excreted acetate." Does the author really contradict the cited article?

Minor points

I disagree with the third sentence in 1.1 Trypanosomatids, page 1: "These membrane-bounded organeles (glycosomes) play an important role mainly in the bloodstream form (BF) of *Trypanosoma brucei* (*T. brucei*)." This implies that in other stages the glycosomes are not important or even dispensable. Please rephrase or prove your statement.

Chapter 1.1.2.1 Human african trypanosomiasis, page 6 contains a list of symptoms of the late stage of the disease: "...resulting in mental disorders, musculoskeletal disorders, sensory perception and abnormal reflexes." From this sentence I understand that w/o late HAT a human does not possess sensory perception. Please rephrase.

Methods

Major points

Chapter 3.2.1 Principle of western blotting, page 11, the third sentence "The SDS coats each protein, providing an overall negative charge that denatures the protein." While it is true that SDS provides a negative charge to the protein, this charge does not seem to be the causative agent of the protein denaturation. Could you please explain a general mode of action of a detergent?

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[1] Jamonneau, V., Ilboudo, H., Kaboré, J., Kaba, D., Koffi, M., Solano, P., ... Bucheton, B. (2012). Untreated human infections by Trypanosoma brucei gambiense are not 100% fatal. *PLoS Neglected Tropical Diseases*, *6*(6), e1691. doi:10.1371/journal.pntd.0001691

Chapter 3.4.2 RNAi construct, page 15. A citation on the origin of the vector is missing as well as description of restriction enzymes used for cloning. Could you comment on this, please?

Chapter 3.4.3 Region of SDH1 (Tb927.8.6580) used for RNAi, page 15. The sequence is in total 1830 bp long, hence it does not belong to the supposed 485 bp region, neither is the region depicted. Could you comment on this, please?

Minor points

Chapter 3.1 Cell lines and cultivation, page 11. The paragraph describes using 25 C and 27 C for cultivation of T. brucei and L. donovani respectively. Is this correct or is it swapped? If the temperatures are correct, please explain the reasoning behind it.

Chapter 3.2.1 Principle of western blotting, page 11, the seventh sentence "It is then possible to identify proteins of interest by allowing a specific primary antibody to bind to the membrane." Please rephrase to emphasize that the primary antibody primarily recognizes its cognate epitope and not the membrane itself.

Chapter 3.2.2 SDH steady state levels in trypanosomatids, page 12, the sixth sentence "The organellar pellet was then resuspended in 500ul of STM and 1,5ul of 1M MgCl2 and 2,5ul of Dnase I were added." Please specify a (final) concentration of Dnase I.

Chapter 3.2.2 SDH steady state levels in trypanosomatids, page 12, the last sentence "The antibody was raised against the T. brucei SDH1 peptide..." Please provide more information on the antibody. Was it published? Was it raised by a certain company?

Chapter 3.2.3 Native assembly of complex II in trypanosomatids, page 13, the first paragraph, the last sentence "No charged dye is applied, so the native proteins separate based on their size and charge." The sentence is true by itself, however, in the third paragraph, the second sentence describes an anode buffer as containing "50 mM Tricine, 7,5 mM Imidazole pH 7,0, 0,05% deoxycholine and 0,02% dodecylmaltoside". Both deoxycholine and dodecylmaltoside are charged molecules, DDM being a well known detergent and both get into an intimate contact with the sample, hence they contradict the first sentence.

Chapter 3.3 Succinate ubiquinone:oxidoreductase activity assay, page 14, the first sentence "Similar cuvettes also contained 1mM malonate, a specific inhibitor of SDH, as a negative control." Please exclude the word similar and re-position the colon in the chapter title to succinate:ubiquinone oxidoreductase...

Chapter 3.5 Mitochondrial membrane potential, page 18. The chapter lacks a description of principle of the measurement.

Chapter 3.8 ATPase assay with purified F1-ATPase, page 22, the second paragraph, the seventh sentence "The F1-ATPase was filtered, concentrated down to 750 ul and loaded on a Superdex 200 10/300 GL." Please provide more information on filtering and concentrating the sample.

Results

Major points

Figure 17, page 28 describe a western blot upon native electrophoresis of mitochondrial/organellar proteins. This figure is compared with figure 14 in the text, however, figure 14 does not include enough information on conditions used. While figure 17 is a visualization of a western blot upon a 3-12% gradient PAGE, we have no clue what is actually shown in figure 14. Hence, one cannot exclude that the "smear" in high molecular weight area in figure 17 is not identical with the upper band in the figure 14, especially when different markers are depicted for each figure. Moreover, the text states only "...previous native electrophoresis of mitochondria." Stating that it is in fact figure 14 would be appropriate.

Chapter 4.2.1 T. brucei PF SDH1 RNAi, page 27, the second paragraph, the third sentence: „This intensity of this band is dramatically decreased over time as the SDH1 RNAi is induced, indicating that the knockdown of a core succinate dehydrogenase subunit results in the disruption of the entire respiratory complex II." Given that you were probing the complex with an antibody against an interfered subunit, I conclude that the decreasing signal reflects only disappearance of this sole subunit, not the disruption of the complex. I believe that this disruption would be manifested as lower molecular weight bands.

Minor points

Table 2, page 26, and table 3, page 28 both states in the headlines that their experiments were performed in triplicates. However, the values do show one number w/o either SEM or SD. Also, P-values are claimed to be calculated, nevertheless, it is not clear from the tables which values are of statistical significance.

Discussion

Major points

Page 38, the first paragraph, the second last sentence "The oxygen consumption rate inhibited by CD38 was partially (33%) reinstated upon addition of R-glycerophosphate, a substrate of complex I." The sentence is taken from the cited work of Luque-Ortega et al., 2010 who on this cites the article by Martin and Mukkada from 1979. I am really surprised that the author did not discuss this and hence I am obliged to ask for author's view on the described experiment by Luque-Ortega et al., 2010 with focus on complex I.

Page 40, the third paragraph, the last sentence „It would be plausible that when a cell needs to synthesize ATP, it upregulates the activity of complex II to start feeding more electrons into the oxidative phosphorylation pathway." This scenario requires overall up-regulation of metabolic activity since complex II is an integral part of Krebs cycle. Which enzymes/pathways would have to be up-regulated to keep metabolism at equilibrium?

Minor points

Page 40, the third paragraph, the third sentence „For the fun of speculation, it would be interesting if these compounds bind to the catalytic ADP/ATP pockets between the alpha and beta subunits of F1-ATPase and bind to a similar ADP/ATP binding pocket on succinate dehydrogenase." This might be my lack of feeling for the English language but is the author suggesting that the succinated dehydrogenase conatains an ATP-binding pocket?

Conclusion

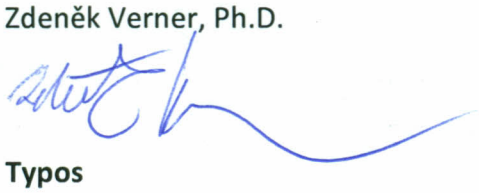
Overall, I find this master thesis very well written and I believe that it will guarantee the author the title MSc. upon defense.

Mark – B (1-).

In Prague

20/05/14

Zdeněk Verner, Ph.D.



Typos

Pg. 6, the last paragraph, the second sentence is missing full stop.

Pg. 17, chapter 3.4.6, the third sentence has a double full stop.

Pg. 39, the last paragraph, the fourth sentence – it's should be replaced with its.

I would convert comma into a dot in numbers.

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STATEMENT OF THE BACHELOR/DIPLOMA* THESIS REVIEWER

Name of the student: **Jan Martinek**

Thesis title: Identifying the Mode of Action for Bisphosphonium Salts – Potent Trypanosomatid Inhibitors

Supervisor: **Dr. Alena Zikova**

Reviewer: **Dr. Anastasios Tsaousis**

Reviewer` affiliation: **University of Kent, UK**

	Point scale[1]	Points
(1) FORMAL REQUIREMENTS		
Extent of the thesis (for bachelor theses min. 18 pages, for masters theses min. 25 pages), **balanced length of the thesis parts** (recommended length of the theoretical part is max. 1/3 of the total length), **logical structure of the thesis**	0-3	3
quality of the theoretical part (review) (number and relevancy of the references, recency of the references)	0-3	2
Accuracy in citing of the references (presence of uncited sources, uniform style of the references, use of correct journal titles and abbreviations)	0-3	3
Graphic layout of the text and of the figures/tables	0-3	3
Quality of the annotation	0-3	2.5
Language and stylistics, complying with the valid terminology	0-3	2.5
Accuracy and completeness of figures/tables legends (clarity without reading the rest of the text, explanation of the symbols and labeling, indication of the units)	0-3	2.5
Formal requirements – points in total		18.5
(2) PRACTICAL REQUIREMENTS		
Clarity and fulfillment of the aims	0-3	3
Ability to understand the results, their interpretation, and clarity of the results, discussion, and conclusions	0-3	2.5
Discussion quality – interpretation of results and their discussion with the literature (absence of discussion with the literature is not acceptable)	0-3	2
Logic in the course of the experimental work	0-3	2.5
Completeness of the description of the used techniques	0-3	2.5

* Choose one

[1] Mark as: 0-unsatisfactory, 1-satisfactory, 2-average, 3-excellent.

Experimental difficulty of the thesis, independence in experimental work	0-3	2.5
Quality of experimental data presentation	0-3	2.5
The use of up-to-date techniques	0-3	2.5
Contribution of the thesis to the knowledge in the field and possibility to publish the results (after eventual supplementary experiments)	0-3	2.5
Formal requirements – points in total		22.5
POINTS IN TOTAL (MAX/AWARDED)	48	41

Suggestions and questions, to which the student has to answer during the defense:

1. A major part of this work involved the characterization of the function of complex II or Succinate Dehydrogenase (SDH) in Trypanosomids. Despite this, you have not introduced this complex/proteins in your introduction section. As a result, someone that is not familiar with the function of complex II (e.g. consists of X number of subunits) might be a bit confused when you start presenting your results with an antibody against SDH1. Also, you will need to mention the differences between this complex versus the complex II of human mitochondria and why this could be a nice target for anti-microbial drugs.
2. Methods: Why did you use PVDF instead of Nitrocellulose and what are the differences between the two membranes?
3. Results, section 4.1.1. You have introduced a new antibody, which you have tested against purified mitochondria from *T. brucei* and *L. donovani*. How do you know that the antibody from this peptide is indeed recognizing the corresponding SDH1 from these species? What kind of experiments you could perform to confirm this?
4. Page 24, first paragraph. The whole paragraph is a discussion of the presented results and it doesn't belong in the Results section.
5. Why did you use "Student's t-test" for your statistical analysis? What are the advantages/disadvantages?
6. Having these established techniques using the two inhibitors, what other experiments you would like to do to boost your results? What are the future aspects of your work?

Eventual mistakes, which the students should avoid in the future:

- Excavata is not a kingdom, is a group or a subgroup of organisms. You might upset a lot of protistologists if you mention this.
- There are a lot of typos in the thesis, you should use an English proof-reader
- Be consistent with the numbers and units presentation (e.g. Page 4: 60 million people are at risk and 10 ~~000~~ thousand new cases), and leave a space after the number e.g. 200 mM
- uM or ug are not units of measurement. Check the whole thesis and change "u" with "μ"
- Page 3: Typo on immunocompromised
- Page 4: you are mentioning gRNA without introducing this abbreviation
- Page 4: Trypanosoma evansi is a special species
- Page 4: "Statistics say …. " Rephrase this sentence, statistics are not people.
- Page 9, Figure 6: The figure should be explained thoroughly: what are the suggested functions? There is no labeling on the individual proteins! Explain what you would like to show with this figure.
- Page 11: PVDF abbreviation is not explained.

- Page 13, line 7: we need to perform ... Rephrase.
- Page 14, Figure 8: This figure is not for the methodology section but for the introduction!
- Page 15, Section 3.4.3: This section is not informative at all. Explain or otherwise delete it.
- Page 19, line 16: Then sodium succinate 5mM and ADP 67μM were added into each well – you have been mentioning tubes and now you are mentioning wells? Please clarify.
- Page 20: Mitochondria were hypotonicaly isolated as described previously – where has this been described?
- Page 23: A gel does not run.
- Page 23, figure legend: *T. brucei* should be in italics
- Page 30: Instead of referring someone's lab, you should cite their published work.
- Page 34, line 4: typo on "mitochondrial"
- Discussion section. Most of the discussion and information on the previous work is not very well cited.

Eventual additional comments of the reviewer on the student and the thesis:

It has been my pleasure to be the external examiner and read the Master thesis of Jan Martinek. Jan has put a lot of effort and work on this project and I have really enjoyed reading his thesis. He has demonstrated that bisphophonium salts have an effect to the activity of complex II and FoF1-ATPase of *Trypanosoma brucei* and *Leishmenia donovani*, but he has also investigated the necessity of this complex to the lifestyle of *T. brucei* as well. I am looking forward to read any upcoming publications as a result of this work!

Conclusion:

In conclusion, I

recommend

the thesis for the defense and I suggest the grade "Excellent".

In **20th of May, 2014**

signature