Review of diploma thesis by Sila Azak

entitled

Characterization of the immune responses against

SARS-CoV-2 virus

The work of Sila Azak Bc. performed in the laboratory of Dr. Zora Mělková, aimed to characterize the effect of Heme Arginate (HA) treatment on selected cytokines produced in response to SARS-CoV-2 infection. This study follows the clinical use of Normosang, a HA-containing compound, which improved the saturation of blood with the oxygen of COVID-19 patients, prevented the worsening of their clinical state, progression to Intensive care unit (ICU), and artificial pulmonary ventilation (Zora Mělková, unpublished).

The thesis is organized to 1. Introduction, 2. Objectives of work, 3. Literature Review, 4. Materials and Methods, 5. Results, 6. Discussion, 7. Summary, and 8. References. Bibliographic sources are correctly cited in 51 references. Following a well-written review of the SARS-CoV-2 replication cycle, the virus-induced immune response, its escape from immune surveillance, and effects of HA, Sila Azak describes in the Material and Methods section infection of Vero cell line and mice K18-hACE2 with SARS-CoV-2.

Sila Azak divided the male and female K18-hACE mice into 5 groups (3-6 animals each): (i) not infected group; (ii) infected mock-treated group; (iii) infected HA-treated mice; (iv) infected remdesivir-treated mice; and (v) infected HA and remdesivir-treated group. She determined cytokine mRNA levels in the spleen of infected mice by one-step PCR, while cytokine levels in mouse plasma by CBA (Figs. 8-11).

The major concern of the experimental part of the thesis is a lack of significant differences between control and individual groups of HA-treated K18-hACE2 mice. Thus, only trends in changes of cytokine levels and their correlation with cytokines expressed in published results in humans are discussed. Significant changes were found for IL10, IL4, and IL22 (Fig. 10 h, i, j) in the plasma of HA-treated female K18-hACE2 mice, and for TNF-α (Fig. 8 a) and IL-10 (Fig. 10 h) in the spleen and plasma, respectively, and in remdesivir-treated female of K18-hACE2 mice. In the Vero cell line, HA inhibited virus growth dose-dependently and decreased the TNFα/GAPDH ratio (significances are not shown). The effect of HA or remdesivir on virus growth in K18-hACE2 mice was not determined.

Sila Azak could clarify or discuss the following points during the defense of her thesis.

- Please explain how to conduct a trend analysis of K18-hACE2 mice data set, namely, how to compare the trends with the published data shown in the Discussion section.
- Controls whether and how remdesivir and remdesivir with HA inhibit SARS-CoV-2 growth in K18-hACE2 mice is missing (Figs. 8-11). Please explain the importance of this control.
- Concerning the sex-dependent difference in K18-hACE2 mice experiments, the effect of estrogens in females should be better discussed (Abramenko et al. IJMS 2021, 22,6551). Does estrogen level influence the expression of the ACE2 receptor?
- SARS-CoV-2 enters the African green monkey kidney-derived Vero cell line more efficiently by endosomal entry dependent on cathepsin cleavage than by TMPRSS2-dependent plasma membrane fusion. This makes infection of SARS-CoV-2 in Vero cells

sensitive to chloroquine and hydrochloroqinoline, and it caused confusion at the beginning of the COVID-19 therapy (Hofmann et al, Nature. 2020 Sep;585(7826):588-590). Are Vero cells an adequate model for the infection of human lung cells and the study of cytokine production?

- Interpretation of the histology in lungs (pages 47-49) is not clear: ..."males lung tissue in group 0 and females lungs in the HA group revealed higher inflammatory infiltration." Please explain.

<u>Minor concerns</u>:

Page 38, the 2nd and beginning of the 3rd paragraph: reference to Figures 8 and 9 would improve the readability of this part of the manuscript.

Page 26, 4.1.4. Viruses: A full description of the SARS-CoV-2 isolate S-007, i.e. SARS-CoV-2 variant B.1.1.7 with WHO label Alpha, should be provided.

Page 31: "the primers are shown in Table No 3"; correctly in Table 1

Figure legends in the figures 1-5 are missing

Fonts in the figures 1-5 should be enlarged

The work of Sila Azak represents a demonstration of the effect of HA on cytokines produced in response to SARS-CoV-2 infection in Vero cells and the K18-hACE2 mice model. Sila Azak has mastered a number of methods needed to achieve this goal. The thesis is written on a current topic and can be published after repeating some experiments and achieving statistical significance. Despite some critical comments, the diploma thesis fulfills the requirements demanded for the level of diploma work. I recommend submitting work for defense and depending on the outcome of the defense procedure for approval of the Magister degree.

Prague, September 3, 2024.   Prof. Ivan Hirsch