# Review of PhD thesis by Prakash Shankaran

entitled

## Effects of heme arginate in HIV-1 acute infection and in latency reversal

PhD thesis by Prakash Shankaran was conceived at the Institute of Immunology and Microbiology of the First Faculty of Medicine, Charles University in Prague, supervised by MUDr. Zora Mělková, PhD. Thesis pursued highly important and timely objective of biomedical research, with the ultimate goal to purge virus reservoir in HIV-1-infected individuals. The work was financially supported by several grants, including GAČR, IGA, NPU II, BIOCEV and GAUK.

Objective of Prakash Shankaran´s thesis was to investigate effects of heme arginate (HA) in HIV-1-infected cells during acute and latent infection. This work was undertaken in the frame of larger activity of his hosting laboratory to study the "Shock and Kill" mechanism, and to characterize nouvelle agents reactivating HIV-1 from the latency. Prakash Shankaran investigated effects of HA in HIV-1-acutely infected T-cell lines, in cell lines harboring complete HIV-1 provirus or an HIV-1 ''mini-virus'' in Jurkat cell clones, and also in PBMC of HIV-1 infected individuals. He confirmed that HA inhibits HIV-1 replication during the acute infection, and showed that two heme degradation products, CO and bilirubin are responsible for decreased provirus expression, while $Fe^{2+}$ increased HIV-1 expression. In addition to the inhibitory effect, HA also reactivated HIV-1 from latency and synergized in this activity with phorbol ester or TNF-alpha. Inhibition of reactivation by N-acetyl cysteine and desferrioxamine suggests that the effects of HA were mediated by heme- and iron-induced redox stress. Importantly, the effective concentrations of HA did not induce activation of the unstimulated cells. Prakash Shankaran concludes that HA appears to possess a combination of unique properties that could help to decrease the pool of latently infected reservoir cells, and simultaneously inhibit HIV-1 replication in newly infected cells.

The thesis is classically organized to Abstract, Introduction (30 pages), Aims of the study, Material and methods, Results, Discussion, Conclusion, References, Publication and Appendix. The Appendix contains a paper published in Antiviral Research in 2011 and a manuscript submitted to Acta virologica, in both cases with Prakash Shankaran as the first author. It contains also a manuscript of a review article accepted to Folia Microbiologica. Prakash Shankaran is also a co-author of a national and an international patent application. While author could be praised for his effort to prepare one compact manuscript by synthesis of 3 papers, the result is sometimes confusing. The text starting by Materials and Methods on page 50, till the end of Discussion on page 108 is very redundant and similar but not identical with manuscripts in the Appendix. The author is not giving any help to reader

to figure out what is the "final authorized" version, which makes a critical reading of thesis, especially in the absence of electronic version quite difficult.

Comments

1. What is the efficiency of HA in comparison with other compounds frequently used in studies of reactivation of HIV-1 from latency (HDACs or DNMTs inhibitors)? Why some of those compounds were not used as controls? Some of them are reactivating HIV-1 via NFAT instead of NF-kappaB. This could be more relevant control for reactivation of HIV-1 from the latency by HA, which does not activate the T lymphocytes, than PMA, which is a known agonist of NF-kappaB. Molecular aspects of effect of redox stress on the structure of HIV-1 chromatin and provirus transcription should be exposed in more details during the defense of thesis.
2. What is the molecular basis of comparison of both inhibitory and reactivation activity of HA with some other agents showing a similar double activity effect – prostratin for example?
3. Cell line ACH-2 used in experiments shown in Figs. 4, 6, 8 (ms #1) harbors latent inducible and replication competent HIV-1. Because ACH-2 cell line does not represent a one-cycle system and because HA simultaneously reactivates latent HIV-1 and inhibits its replication, confounding effect of HA on virus replication can make interpretation of quantitative aspects of reactivation assays difficult. Please comment. Why inhibitors of HIV-1 replication were not used in these experiments to make interpretation more straightforward?
4. How the doses of Normosang used in therapy of porphyria patient correspond to concentration of HA used in presented experiments? What is the effect of Normosang on replication of HIV-1 in primary T cells, in monocyte derived macrophages and in models of primary cells?
5. What is the effect of HA on CD69 expression in primary cells (PBMCs, CD4+ T cells)?
6. Is the effect of HA synergistic (abstract) or potentiating (Fig. 4, ms #1)? Please, define. How reproducible it is? Please compare the results of HA ativity in Fig. 4B, 6C and 8B (ms #1); express it as a fold.
7. HA was used to reactivate HIV from PBMCs of HIV-1-infected aviremic donor (paper #2; Fig. 5). It is difficult to conclude that reactivation by HA really occurred without any statistics (error bars, significance tests). For how long period of time were the patients aviremic (<50 copies/ml) before the assay? What therapy was applied? Were CD8+ cells depleted? Under which ethical protocol the study was performed?

Minor points

p.23 Updated references should be used (references from 1991 are a quarter of century old).

Alternative receptors, such as galacto ceramid should be mentioned.

p. 21, 25, 26 numerous errors in punctuation are present.

p.26. Effect of methylation of the 5´LTR on HIV-1 latency is a subject of debate. References showing no effect of CpG should be used and discussed.

p. 30 Figure 4 has a poor graphical quality and insufficient text

p. 43 Figure 7 of Heme catabolism could be more adapted to the subject of thesis (see comment no. 1).

p. 170. Strangely, insufficient legend to Fig. 5 in submitted manuscript #2 is developed in more details on p. 86, legend to Fig. 25.

Despite of some critical comments, the thesis of Prakash Shankaran fulfills requirements demanded for the level of dissertation work. Prakash Shankaran is the first author of two papers, one published and another submitted for publication and co-author of further manuscript submitted for publication. His work represents the first demonstration of the stimulatory effect of heme on reactivation of the latent HIV-1 provirus. I recommend submitted work for defense, and depending on the outcome of the defense procedure for approval of the doctor degree.

Prague, August 25, 2016

RNDr. Ivan Hirsch, CSc