Review of PhD Thesis of Prakash Shankaran: Effects of heme arginate in HIV-1 acute infection and in latency reversal

The end of the long and bumpy road towards a cure for HIV-1 infection is not yet in sight. Having efficient antiretroviral drugs for minimising the viraemia, most cure strategies now aim at eradication or at least control of the so-called persistent reservoir of latently infected cells.This reservoir establishes immediately during the primary viraemia and persists life-long regardless of the subsequent suppression of HIV-1 replication. Comprising cells with replication-competent and integrated proviral HIV-DNA, this reservoir results in a quick viral rebound after interruption of ART. Several epigenetic strategies have recently been suggested and clinically tested, but we are still limited with their low efficiency and a lack of predictive markers to establish firm clinical endpoints. It is therefore very topical that Prakash Shankaran comes with a new and original approach to the HIV-1 latency reversal. Heme arginate is an approved drug for acute porphyria treatment and could be easily repurposed for the "kick and kill" strategy in fighting the persistent reservoir.

Prakash Shankaran analysed HIV-1 transcriptional activation by virtue of heme arginate in three well established *in vitro* model systems with replication-competent latent HIV-1 and EGFP-bearing replication defective vectors (mini viruses). The level of HIV-1 reactivation was measured as p24 production in cell culture supernatants or GFP positivity in cells. HIV-1 activation was observed also in PBMCs from a small cohort of ART patients. Degradation of heme leads to numerous intermediates and products with specific and often contrasting effects on the redox balance in the cell. Knowing the enzymes behind this degradation, Prakash Shankaran was able to test effects of individual factors, enzymes and specific inhibitors on HIV-1 reactivation. This represents the first step towards understanding the mechanism of heme latency reversal activity.

Thesis of Prakash Shankaran represents a reasonable piece of work and the results and experimental procedures seem sound. I have, however, also a couple of critical points to the experimental part of the thesis. In general, although the retroviral latency is an epigenetic phenomenon, Prakash Shankaran did not perform any analysis of DNA methylation or histone modifications at HIV proviruses. Neither in discussion, there is anything about the possible connections between redox ballance and the executive epigenetic control mechanisms. There are some data on the epigenetic status of ACH2, A2, and H12 cells and, for example, provirus in the H12 is hypomethylated. **Q1: What are connections between pro-oxidative factors (ROS, Fe^{2+}, etc.) and epigenetic systems?** Second, suggesting that heme arginate could be an alternative to the epidrugs like SAHA, valproic acid, azadC etc., currently under clinical examination, the author should do some comparison of reactivation efficiency. This is particularly important when heme arginate alone did not exerted any or very limited effect without cell activator PMA. **Q2: Could you critically assess the therapeutic potential of HA and compare it with vorinostat, decitabine, etc.?** Third, in the chapter 6.20, the author presents latency reversal activity of enigmatic compounds A, B, C, and inducers 1, 2, and 3. I can understand that commercially promising compounds and protocols have to be patented,

but such results should not be included into the thesis. It goes against the rules of academic openess and reproducibility of results.

Specific points: The cell and provirus activation fluctuated a bit. For example, the minivirus activation (and hence GFP positivity) in A2 cells after PMA-alone-traetment is strikingly different in Figure 14A (HA-mediated reactivation of latent minivirus) and Figure 18A (Effects of Nac and SnPP). **Q3: Please, comment.**

In Figure 15, detection of HO-2, whose expression is not inducible, would be a nice control.

Page 79 and 80, Figure 22E,F. There is some controversy with the effect of billiverdin on the PMA-mediated provirus activation. Probably a confusion billiverdin vs. billirubin.

Page 87 and Figure 26 describe latency reversal effects of TNFα and PHA. These data are not new and without HA co-stimulation do not tell nothing important to the HA topic. **Q4: Do you have any data about the synergism of HA and cell activators TNFα and PHA?**

Q5: What is the biological meaning of multiplying the EGFP-positive cells and the mean fluorescence intensity (Figure 14 and elsewhere)?

Introduction to the thesis is adequate and refers to a representative number of communications. I have just two objections: at page 25 the author writes that HIV-1 preferentially integrates into heterochromatin and quotes Jordan *et al.*, EMBO J. 20: 1726-1738, 2001. This is, however, misunderstanding, non-selected HIV proviruses are preferentially found in transcribed and gene-rich chromatin parts thanks to the LEDGF/p75 tethering. HIV-1 integration should be better discussed because the integration site and its epigenetic landscape plays an important role in transcriptional silencing and, vice versa, latency reversal. At page 26 author writes that 98% of methylation occurs at CpG islands. This is a bad mistake. CpG islands are mostly unmethylated unless localized at inactivated X chromosome in mammals or aberrantly methylated in cancer cells.

Thesis is technically satisfactory with a minimum of typesetting, word usage, and bibliographic errors. Careful ispection reveals poor quality and unreadable script in Figures 2 - 4 adopted from review papers, combination of Figures and Tables in the Results, and lack of statistical significances in several figures.

In summary, although the aforementioned errors and drawbacks limit my enthusiasm for this thesis, my overall feeling is that Prakash Shankaran´s work fulfils the usual criteria and his research represents an intersting contribution to the field. This thesis should therefore be considered as a basis for his doctoral promotion.

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