Review of PhD thesis by Ing. Lenka Vaneková, student of the study program Developmental and Cell Biology entitled Modulation of the STING signalling pathway

PhD thesis by Ing. et Ing. Lenka Vaneková was prepared in the Institute of Organic Chemistry and Biochemistry (IOCB) of the ASCR in Prague under supervision of doc. Ing. Václav Veverka, Ph.D., with consultants Ing. Andrea Brázdová Ph.D. and Mgr. Gabriel Birkuš, Ph.D. The thesis was supported by Gilead Sciences, Inc. and by the European Regional Development Fund.

The general objective of this thesis was to identify a novel STING agonist with optimal pharmacokinetic profile for chronic hepatitis B (CHB) and cancer therapy. The set of cyclic dinucleotides (CDN) was prepared by enzymatic and synthetic approach and contained modifications of nucleobase, sugar, and the phosphate groups. It was prepared at the IOCB in the "HBV Cure group" led by Gabriel Birkuš and tested *in vitro*, whereas the lead compound (CDN-I) was evaluated *in vivo* for anti-CHB effect.

The main goal of Lenka Vaneková was to develop an immunocompetent mouse model reflecting chronic hepatitis B (CHB) based on hydrodynamic injection suitable for preclinical testing of novel CHB therapeutics. Persisting expression of HBV antigens was observed in the C3H/HeN mice when using an adeno associated virus (pAAV) construct encoding genotype A HBV (published in *Physiol. Res.*). The construct contained T2308C point mutation in the START codon of the polymerase preventing formation of viral progeny, permitting thus to carry out all manipulation under Bsl2 (and not Bsl3) conditions. To further evaluate liver specific immune response after CDN-L therapy, Lenka Vaneková developed (published in *Methods and Protocols*) a low-cost, fast, and straightforward procedure for isolation of single cell suspension of mouse liver non-parenchymal cells (NPC) while still preserving antigen/epitope profiles in order to characterize multiple intrahepatic immune populations.

Because the lack of expression of the STING in hepatocytes, the target cells of HBV infection, Lenka Vaneková could not investigate the cell-autonomous defense innate immunity mechanisms directly in infected cells. Instead, she focused on the cell-dependent

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line of defense provided by immune cells expressing STING, present in the surrounding infected tissue and peripheral blood. Therefore, Lenka Vaneková tested *in vitro* therapeutic efficacy of CDNs using human PBMCs reflecting physiologically relevant *in vitro* cell-based model. TNF-α and IFN-α represent the primary and IFN-γ the late onset response to the activation of cGAS-STING signaling pathway.

Based on her previous results, Lenka Vaneková explored utility of the lead compound (CDN-L) selected on its ability to induce innate immunity via cGAS-STING pathway; multiple factors, such as the in vitro activity towards STING haplotypes, induction of cytokines in PBMC assay, human/mouse plasma stability, mouse pharmacokinetics, and *in vitro* anti-HBV activity were involved. Therapeutic potential of CDN-L was determined by means of C3H/HeN HDI murine model reflecting CHB (non-published preclinical results). Efficiency of CDN-L treatment was dependent on the agonist dose, resulted in decrease of HBcAg positivity at the terminal point of the experiment, and required signaling through IFN-α receptor for optimal effectivity.

Thesis has very well written introduction focused mostly on mechanisms of the innate immune responses as crucial first line defense against various pathogen infections, cell damage, cellular stress and/or cancer. It concerns namely with cGAS-STING pathway downstream signaling, its regulation and the role in disease control. The thesis is finished by excellent discussion, in which are summarized the results and conclusions of modulation of the STING signaling pathway and given the future perspectives. Also is attached the list of abbreviations and references.

Lenka Vaneková could clarify or discuss during defense of her thesis the following points.

1. Explain shortly mechanism of hydrodynamic-based transfection by administration of naked DNA [Why the liver (and not other organs as kidney, heart) is a primary target of DNA? What is the uptake mechanism and intracellular fate of DNA? Which types of liver cells are transfected? What is extension of hydrodynamic transfection to other laboratory animals? Is hydrodynamic delivery of DNA to the isolated liver clinically practicable and therapeutically significant method for human gene therapy?]
2. Please discuss adequacy of the HDI model with respect to the high mutation rate of the HBV reverse transcriptase in human hepatocytes. What is the consequence of introduction of T2308C mutation in the RT-START codon for stable expression of the construct and for reverse mutation of T2308C?
3. CDNs became an important medicinal chemistry tool with potential of therapeutic application in various diseases, as CHB, HIV infection, and cancer. Please compare the mechanism of potential control of CHB, HIV infection, and cancer by cGAS-STING signaling pathway with respect to the expression of STING in affected target cells.
4. Please compare potential advantages and disadvantages of the use of STING and TLR agonists in the control of CHB infection.
5. Why just HBV genotypes A and D and not other genotypes were selected for the study?

6. Page 44, the first sentence of the „1.6 HBV“ paragraph: „cGAS-STING signalization plays a crucial role in detection of viral DNA and viral clearance, such as hepatitis B (HBV).“ Please discuss with respect to the absence of STING in hepatocytes.

In conclusion, the thesis of Ing et Ing. Lenka Vaneková perfectly fulfills requirements demanded for the level of dissertation work. Lenka Vaneková is the first author of two excellent papers, and co-author of three other high-quality papers published in impacted journals. I heartily recommend submitted work for defense and depending on the outcome of defense procedure for approval of doctor degree.

Prague, January 10, 2023 Prof. Ivan Hirsch, PhD