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The review of the Ph.D. thesis
of Martyna Plens-Gałąska, MsC
"Targeted inhibition of STATs in chronic inflammation and cardiovascular disease"

The dissertation of Ms. Martyna Plens-Gałąska focuses on the identification and characteristics of inhibitors of the activity of STAT transcription factors. It is part of the mainstream interest of Prof. Bluyssen's group on the role of pro-inflammatory reactions in the development of atherosclerosis and its treatment. This disease and, more broadly cardiovascular diseases, are one of the leading causes of death in developed countries. The Author of the dissertation has assumed that STAT inhibitors have therapeutic potential in this area, which justifies the search for such new compounds and the analysis of their properties.

The dissertation consists of four parts and its structure differs from the canonical form of doctoral dissertations. The first chapter serves as a general introduction to the topic, while the last one provides a general discussion of the studies. The second and third chapters describe specific sets of experiments on STAT inhibitors and the design of their database called SINBAD, respectively. Each chapter has its introduction, a description of methods used, results of studies, and their discussion. About 200 publications are cited in the text. The general Introduction is well written and indicates that cardiovascular diseases are an increasing problem in modern societies. In a synthetic and transparent way describes the mechanism of the progression of atherosclerosis, with a clear emphasis on the involvement of immune system cells in this process. To present the molecular basis of the development of atherosclerosis, the involvement of interferons, interleukin-6, and TLR4 receptor in the activation of signaling pathways inducing an inflammatory response within the forming atherosclerotic plaque is discussed in more detail. Consequently, we learn about activated transcription factors, including STATs, IRFs, and NF-κB, that control the expression of pro-inflammatory cytokines. Finally, we find the characteristics of STAT inhibitors known to date, ranging from low molecular weight synthetic inhibitors to natural products. Included schemes markedly facilitate the understanding of the discussed complex processes.

I have one comment on this part of the dissertation: when describing the activation of TLR4 receptor by bacterial LPS, it is worth remembering the role of CD14 protein in the induction of the endosomal TRIF-dependent (IFN-inducing) TLR4 signaling pathway. CD14, and more precisely its absence in endothelial cells, determines the differences in the activation of these cells *vs*. macrophages and thereby affects the range of cytokines produced by these cells.

Altogether, the first chapter provides a good introduction to the dissertation and rationalizes the aims of the study. Their goal was to identify some new and characterize more completely already known STAT inhibitors as potential therapeutic agents for atherosclerosis, and to systemize data on them in a form of a database, making them more accessible to researchers.

The following part of the dissertation presents results of experimental studies on a STAT inhibitor newly selected by the Author and called C01L_F03, and also on two previously known ones - STATTIC and STX-0019. In the introduction to the studies undertaken, the Author refers to the publication in Frontiers of Immunology (2018), where She is the first author, which describes the identification of C01, E01, F01, and also the C01L_F03 STAT inhibitors. I have the impression that considering how extensive that work is, in the dissertation, the Author rather sparingly gives some methodological details of the studies. It would be worth devoting a few sentences to the ZINC libraries of molecules. We do not find out where the compound C01L_F03 came from, or where the other two inhibitors were purchased. Are the tested compounds water-soluble? If not, what was the concentration of their solvent, and was it present in the control samples?

The method of identifying new STAT inhibitors began with *in silico* modeling of interactions of test molecules (derived from ZINC libraries) with the SH2 domain of STAT1-3 proteins vital for dimerization (hence activation) of these proteins, which was performed in prof. Bluyssen's team. The Author performed subsequently *in vitro* analysis of the influence of the tested compounds on (i) phosphorylation (activation) of STAT1-3 (immunoblotting); (ii) qPCR analysis of the influence of tested compounds on the expression of five genes whose activation is a hallmark of STAT1, 2, and 3 involvement; (iii) Chip-PCR analysis of the influence of tested compounds on the binding of STAT1, 2, and 3 to chromatin. All these studies were performed using human microvascular endothelial (HMEC) cells stimulated with INFα or IL-6. The Author then extended Her research by revealing the inhibitory effect of the tested drugs on gene expression in cells stimulated jointly with two strong inflammatory stimuli, i.e., INFγ and LPS. Importantly, She found that the drugs were also effective in conditions when cell stimulation preceded drug application. These studies were followed by elaborated RNA microarray analysis, which consisted in the selection and the functional analysis of genes whose expression was induced by the combined action of INFγ and LPS in HMEC cells and was inhibited by C01L_F03, and/or STATTIC, and/or STX-0019. There were 161 genes down-regulated by all three inhibitors, including many established STAT target genes annotated by gene ontology (GO) analysis to pro-inflammatory and pro-atherogenic responses. *In silico* analysis confirmed that the genes whose expression was inhibited by the drugs have promotors dependent on multiple STATs, IRFs, and NF-κB, and their combination. Out of curiosity, I would like to ask if the RNA microarray analyzes revealed genes that were up-regulated by the tested STAT inhibitors? If so, how does the Author interpret this result? How does

the Author interpret the result that the tested STAT inhibitors affected the expression of 53 genes with a single NFκB-binding site found *in silico* (page 58)?

Finally, to verify the influence of the tested STAT inhibitors on the functioning of the endothelium, the Author investigated the effect of these compounds on HMEC cell migration, leukocyte adhesion to HUVEC cells, and norepinephrine-induced contraction of isolated mesenteric arteries. The latter two sets of studies were performed in collaboration with Prof. Maria-Jesus Sanz from the University of Valencia. Taken together, the conducted studies form a logical sequence characterizing STAT inhibitors and their effect on the inflammatory response and endothelial function *in vitro*. They indicate that the Author mastered a wide range of experimental skills and proficiently analyzes data using available IT databases.

She also created a database named SINBAD, which is described in the third part of the thesis. The creation of this database required an exhaustive analysis of over 1500 publications in which STAT inhibitors were applied. Currently, the SINBAD database includes 175 established STAT inhibitors and provides their chemical characteristics*, in silico* modeling of their interactions with STATs, concentrations used, cells tested, and clinical trials. So far, however, none of the known STAT inhibitors has gone successfully through this stage, and inhibitors of Jak kinases acting upstream of STATs are used for the treatment of some autoimmune diseases. For my part, I would add data on solvents and solubility of the described STAT drugs. The creation and capabilities of the SINBAD database have been published in highly-rated Scientific Data published by Nature Portfolio, the first author of which is Martyna Plens-Gałąska.

Finally, chapter four of the dissertation briefly discusses STAT inhibitors and their therapeutic potential, weaknesses and strengths of inhibitors specific to one or more STAT proteins. Surprisingly, the Author also presents here results of preliminary studies on potential new STAT inhibitors selected using improved *in silico* screening and docking procedures, as well as results obtained with the combined use of two STAT inhibitors, which seems redundant to me. It is worth mentioning that Martyna Plens-Gałąska is the co-author of two reviews relevant to the topic of the dissertation published in Oncotarget (2016) and Frontiers in Immunology (2019), which together with the dissertation indicates knowledge of the subject by the Author.

Having praised the Ph.D. candidate for Her work I would like Her to address my following concerns regarding Figures 2.1 and 2.4. The blots seen in Fig. 2.1, panel b, show the inhibition of STAT1-3 phosphorylation by 50 and 25 µM C01L_F03, but at 10 and 5 µM concentrations, we see an increase in STAT1 and 3 phosphorylation compared to the control, not the partial inhibition reported by the Author (48 h, top panel in 2.1 b). Moreover, there is no densitometric analysis of blots either in Fig. 2.1 or Fig. 2.4. In addition, the results of cytotoxicity tests of the examined compounds, which the Author mentions on page 46, should be provided.

Minor point: there are several flaws in the Figures and their descriptions in chapter 2, the text would benefit from careful editing:

- the references in the text to the respective Figures and their panels are insufficient, sometimes the Figures are not cited at all yet described in the text (e.g. Figs 2.2 and 2.11). On the other hand, the results regarding the influence of inhibitors on the expression of *Vcam1* and *Icam1* from Fig. 2.11 are not described in the text. There are incorrect references to Table 2.2 (page 44, Table 2.4 is correct), to Fig. 2.2 (page 48, Fig. 2.3 is correct).
- In Figures 2.2, 2.3, 2.5, 2.7, and 2.11 there is no indication of which groups were compared to obtain the indicated *p* value (probably drug-treated *vs.* untreated). For comparison, correct marking is seen in Figs 2.12 and 2.13.
- The description of Fig. 2.8 does not reflect the course of the experiment shown (stimulation of cells before incubation with inhibitors). In Fig. 2.12 times of drug treatment seen in the Figure (most likely correct) are different from those described in the Figure legend. Fig. 2.13 shows twice the results for STX-0119 while those for C01L_F03 are missing (probably seen in panel b). Table 2.5 shows the top 25 genes, while the text mentions 30 of them.

The aforementioned shortcomings do not change my overall good assessment of the dissertation. In conclusion, in my opinion, the Ph.D. thesis of Martyna Plens-Gałąska contains original research discoveries broadening our knowledge of the properties of STAT inhibitors. The Author has demonstrated knowledge of the subject and experimental skills. The dissertation fulfills all the requirements for the Ph.D. thesis. Martyna Plens-Gałąska is worthy of the degree of Doctor (Ph.D.) and this is my recommendation for the Scientific Council of the Faculty of Biology of the Adam Mickiewicz University in Poznan. Taking into account the scientific achievements, especially resulting from the use of IT tools, I propose to award the Author of this dissertation with an appropriate distinction.

Rozprawa doktorska spełnia wszystkie warunki określone w Ustawie z dnia 20 lipca 2018 r. „Prawo o Szkolnictwie wyższym i nauce" (Dz. U. z 2018 r. poz. 1668 z późniejszymi zmianami) stawiane rozprawom doktorskim. Wobec powyższego wnoszę do Rady Naukowej Wydziału Biologii Uniwersytetu Adama Mickiewicza w Poznaniu o dopuszczenie mgr Martyny Plens-Galąski do dalszych etapów przewodu doktorskiego. Biorąc pod uwagę osiągnięcia naukowe, szczególnie te wynikające z zastosowania narzędzi informatycznych, proponuję przyznać Autorce niniejszej rozprawy stosowne wyróżnienie.