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# Review of the doctoral dissertation of mgr inż. Marek Grosicki

entitled "*Evaluation of the effect of histamine and new histamine $H_3$ and $H_4$ receptor ligands on the human eosinophils adhesion to endothelium*"

Principle Supervisor: Prof. dr hab. Katarzyna Kieć-Kononowicz (Kraków, Poland)

The review is prepared in response to the invitation received from Dr hab. Agnieszka Skowron, Prodziekan Wydziału Farmaceutycznego, Jagiellonian University Medical College (Kraków, Poland), on October 10th, 2017.

> *The PhD thesis sought to develop and optimize a new in vitro functional assay in order to investigate the adhesion of isolated human eosinophils to the endothelium. Subsequently, this work aimed to characterize the contribution of histamine and the histamine $H_1$-$H_4$ receptors on the eosinophil adhesion to the endothelial cells, as well as to use the developed assay for the evaluation of the pharmacological activity of selected $H_3$/$H_4$ receptor ligands.*

This area of research is both timely and relevant. The vital contribution of histamine in driving hypersensitivity reactions has been exploited for more than 70 years, essentially by using $H_1$-antihistamine drugs to counteract the effects of this biogenic amine in allergies. At the turn of the millennium, the identification of the newest high affinity $H_4$ receptor introduced novel concepts on the immunomodulatory role of histamine in inflammation and on the significance of ligand-biased signaling, thus fueling the revival of the global interest in histamine research. Actually, various $H_4$-targeting compounds with favorable safety profile are currently under proof-of-concert clinical testing for pruritus, atopic dermatitis, asthma, allergic rhinitis and/or psoriasis. Interestingly, eosinophils are pivotal components implicated in the pathogenesis and severity of a number of inflammatory disorders, including asthma and atopic dermatitis. Since their initial characterization by Paul Erlich in the late 19th century, these multifunctional leukocytes remain one of the most important cellular models in histamine receptor research, challenging the development of novel therapeutics for the management of unmet medical needs.

The submitted thesis consists of 124 pages comprising the following chapters: (1) Introduction; (2) Aim of the Study; (3) Experimental Procedures; (4) Results and Discussion; (5) Conclusions; (6) Future Research Prospects, and (7) a list of 334 relevant References that optimally support the theoretical background, the methodological approaches and the discussion of the results presented in the thesis. Chapters 1, 3 and 4 are divided into appropriate sections and subsections introduced by pertinent headings and subheadings, respectively. Additional material included in the thesis comprises a Preface; Acknowledgments; a concise Abstract (both in English and in Polish) that reflects all aspects of the thesis; a list of Abbreviations; and an Appendix containing copies of the 4 peer-review publications of the PhD candidate that report on work included in the thesis. The entire document is well-structured and presented in a concise and consistent manner, using clear style and format and supported by relevant figures (61) and tables (7). A recommendation would be to include in the thesis a 'Table of Figures and Tables' after the 'Table of Contents', as well as a brief *Curriculum vitae* (CV) of the PhD student. Moreover, although I am not a native English speaker, I feel that proofreading the document should be considered in order to correct superficial errors in spelling, grammar, syntax and punctuation.

- In the '*Introduction*' (Chapter 1), the reader is familiarized with the current state-of-the-art on the vital (patho)physiological role of histamine exerted through binding to the four receptor subtypes, designated as $H_1$, $H_2$, $H_3$ and $H_4$. The meticulous presentation of the molecular, biochemical and pharmacological characteristics of the histamine receptors and the detailed properties of their selective ligands -including their chemical structures- clearly shows that the PhD candidate has deeply understood the theoretical background in the field. The implication of eosinophils in a number of inflammatory pathologies is elegantly addressed in Section 1.3, focusing on the chemotactic role of histamine in mediating the pleiotropic functions of eosinophils. Likewise, the literature on the adhesion of leukocytes -including eosinophils- to the endothelium and their resulting migration to the site(s) of inflammation is appropriately reviewed in Section 1.4.

  The PhD candidate identified the knowledge gaps in this area of biomedical research and justified the need to investigate the putative role of histamine in eosinophil adhesion to the endothelium. This facilitates the dissection of the mechanisms implicated in a number of eosinophil-associated pathologies, and the identification of agents with therapeutic potential for the management of related immune-mediated inflammatory disorders.

- Following critical assessment of the available literature and considering the topicality of immunopharmacology and the variable clinical success of an expanding list of emerging therapies targeting the eosinophils, the PhD candidate formulated the *'Aim of the Study'* and addressed more detailed specific research questions in Chapter 2.

- The '*Experimental Procedures*' are described In Chapter 3, in line with the scope of the thesis and taking into consideration human variations in blood parameters, such as eosinophil count and histamine levels, as well as ethical aspects. In the first stage, three eosinophil isolation methods from human peripheral blood were developed and compared for their credibility and suitability for further testing. In the second stage, an *in vitro* assay of eosinophil adhesion to the endothelium was developed by co-culturing isolated, purified human eosinophils with EA.hy926 human endothelial cells, and washing away non-adherent cells. The assay was optimized and validated using the reference chemotactic peptide fMLP. In the third stage, the contribution of histamine to the adhesion of isolated eosinophils to the EA.hy926 endothelial cells was investigated and compared to the action of fMLP. The identification of the histamine receptor(s) implicated in the histamine-dependent adhesion process was achieved by exposing the cell co-cultures to a number of selective $H_1$-$H_4$ antagonists/inverse agonists and/or agonists with well characterized pharmacological profile, taking into account the recently described concept of biased signaling. In the final stage, a small library of $H_3$/$H_4$ ligands -synthesized at the Faculty of Pharmacy, Jagiellonian University Medical College- was tested in a pilot study aiming to assess the value of the developed assay in drug screening.
- Chapter 4 is dedicated to the '*Results and Discussion*'. Eosinophils constitute only 1-6% of circulating leukocytes in healthy humans. The difficulties in obtaining adequate numbers of highly purified, viable and functional eosinophils (98-99%) from human peripheral whole blood were overcome by optimizing a relatively fast immunomagnetic separation protocol. An extensive characterization of the isolated eosinophils demonstrated their viability and functional integrity. This approach validated the methodology and strengthened the concept of using purified human eosinophils for the development of a reliable *in vitro* assay to dissect the role of histamine in eosinophil adhesion to endothelial cells.

  This comprehensive study demonstrated that histamine dose-dependently induces eosinophil adhesion to endothelial cells, with a half maximal effective concentration ($EC_{50}$) of 1.36 μM. The histamine-dependent induction of the adhesion process was shown to be mediated via the $H_4$, but not the $H_1$, $H_2$ or $H_3$ receptors. Interestingly, at least in this experimental setting, the modulatory effects of histamine on the adhesion process were observed only in eosinophils closely interacting with endothelial cells; whereas no effect was observed when histamine-activated eosinophils or endothelial cells alone were investigated. These observations suggested the possible implication of other inflammatory mediators in the adhesion process, such as TNF-α, which are released following histamine stimulation. Finally, the small library of $H_3$/$H_4$ ligands that was tested provided preliminary evidence for the potential use of the developed *in vitro* assay in future drug testing efforts. In this Chapter, the PhD candidate suitably emphasized the significance of the findings and provided logical and realistic arguments on the circumvention of the shortcomings.
- The '*Conclusions*' presented in Chapter 5 confirm that the set objectives were successfully achieved.

- The output of this thesis raised a number of motivating scientific hypotheses and ideas that challenge the '*Future Research Prospects*' as described in Chapter 6.

In conclusion, this doctoral dissertation has original and innovative aspects stemming from the systematic demonstration that histamine induces the adhesion of eosinophils to the endothelial cells through the $H_4$ receptor. Moreover, considering the importance of the research topic, the laborious optimization of the methodological approach to isolate highly purified, viable and functional human eosinophils, and the consequent development of a reliable *in vitro* assay to study the process of eosinophil adhesion to the endothelium are important new contributions to the existing literature. Overall, the high level of the novel scientific content and the extensive methodological endeavors presented in this interdisciplinary doctoral thesis is confirmed by the resulting high-impact publications of the PhD student (3 as first author). Taken as a whole, these outcomes demonstrate the strong theoretical knowledge, the excellent technical skills and the very good independent thinking qualities of the PhD candidate.

Based on the assessment of the significant academic achievements as described above, the submitted thesis fulfills the requirements for PhD qualification. For all the reasons stated in this evaluation report, I recommend that the candidate be awarded the degree of *Doctor of Philosophy* and that the thesis be considered as a '*Distinguished dissertation*'.

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