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Review
of PhD dissertation of mgr. Beata Niklas

Neurotoxic ligands interactions with insects membrane proteins

The most deadly animals in the world are not sharks, crocodiles or snakes, but mosquitoes. Mosquito-borne diseases kill several million people a year. These animals have also developed resistance against nearly all chemicals used so far. Therefore, the work devoted to new ligands acting on the membrane proteins of these insects is important not only from a scientific but also from a practical point of view.

The dissertation is based on three publications that are an integral part of it. They have been published in the following journals: *Molecules* (2021), *Molecules* (2022), and *International Journal of Molecular Sciences* (2023). All of these journals have the high impact factors, ranging from 4.9 to 6.2, and the Candidate is the first author of all these publications. As can be seen from contributions of individual authors, the Candidate made conceptualization of the scientific problem for two latest publications (together with her supervisors), and in all of them she was responsible for conducting most or even all of theoretical research, making figures, and preparing first drafts of these publication. This indicates a lot of work put into obtaining and analyzing the results.

The introduction to these publications is in English, has 29 pages, and includes description of investigated molecules (8 pages), the theoretical methods used (10 pages), aims of the work, and a reference list with 90 items. The list includes many reviews, which is good for the reader who wants to know more about particular topics, unfortunately, in many references only the first author is specified. The introduction is written clearly, in good

scientific language, but is written too briefly and some issues, not described also in publications, remain barely sketched.

The introduction is short and it contains many abbreviations, which additionally reduce its size, such as AS – allosteric site, OS – orthosteric site, IG – inactivation gate, CC – central cavity. In ASA – which is "solvent accessible surface area", the abbreviation should be SASA. Adding figures could increase volume of the Introduction and would be better for understanding of the text. For instance, there is no figure showing change of the receptor activation upon increasing concentration of the ligand. Such figure could clearly show differences between receptor ligands. Instead, there is description in the text, which is misleading since there is no mention of the receptor basal activity, also known as the constitutive activity. The reviewer would be grateful for such an explanation and what are the molecular basis of non-zero basal activity. Most of GPCRs have non-zero basal activity, and diminishing its activity is an action of certain type of drugs called inverse agonists, which are not mentioned in the text. Because of existence of inverse agonists the presented definition of efficacy is incorrect. Neutral antagonists, which do not change the basal activity, have efficacy 0, but the inverse agonists have negative efficacy. It is always necessary to use the most current definitions of presented terms, because they are also changing. Definitions of PAMs and NAMs (positive and negative allosteric modulators) are also not precise: they increase or decrease agonist affinity and/or efficacy but not activity of ligands.

The Candidate cites one sentence from abstract of ref. [25] "some ligands may be even both agonists and antagonists at different functions mediated by the same receptor". The sentence is correct but not clarified, and the reader is left with an unexplained problem. The reviewer would appreciate clarification on this issue. Another functional selectivity of GPCRs, the location bias, which adds another level to the very complex action of these receptors, is well explained. However, passing the signal from the activated receptor to the effector proteins is described in only one sentence. It would be good to explain this in more detail and possibly to show a scheme how the main types of G proteins mediate in signal transduction.

In a comparison of human and insect muscarinic receptors, it was mentioned that human M1 and M3 receptors interact with DEET repellent, however, it was not revealed that DEET is bound very weakly, several orders of magnitude weaker than in insect receptors, so in fact this is not a similarity but a difference. This is clarified in the cited reference [32] (Abd-Ella *et al.* PLoS One, 2015) but it should also be clarified in the dissertation. In another sentence it is

stated “Insects mAChRs are divided into three subfamilies (A, B, and C)”, however these so-called families consist of single members only so they are in fact subtypes.

In the Methods, in Figure 4, the Candidate shows “The zones of protein sequence alignments” and two zones are shown: the safe zone and the twilight zone. However, the twilight zone should be between safe zone and dark zone. The reviewer would appreciate showing corrected figure with all three zones and with a level of sequence identity below which any two proteins are similar. Description of the homology modeling procedure is very short and does not include for instance the multiple templates. It would be good to explain this in more detail.

The Molecular docking subchapter of Methods is much longer, which is good. For the scoring function of AutoDock Vina please explain what are “piecewise linear hydrophobic and hydrogen-bond interaction terms”. For the energy formula in the CHARMM force field there is no description of many parameters, especially *K* and those with index 0.

On page 21 there is a wrong reference to subsection 4.3.2 – it should be 4.3.4.

The last subchapter of Introduction is about Metadynamics simulations. The theoretical background is well described and illustrated with a suitable figure. However, there is too small number of citations, e.g. in a sentence “Metadynamics has been successfully applied to various biological problems, including protein folding, ligand binding, and conformational transitions in biomolecules” there are no references at all. The last sentence of the Introduction is “In this study, we use enhanced sampling techniques to find …”. There is no description what is this “enhanced sampling”. Indeed, in the latest publication of Candidate there is a detailed description of this methodology, but the simpler, schematic description would be also useful in the Introduction, especially, that this is a new feature: the path-collective variables. The reviewer would be grateful for such schematic explanation, and what is a difference between path-collective variables and the classical metadynamics and umbrella sampling.

From the Abstract: “A better understanding of the fast inactivation process and its inhibition by neurotoxins can contribute to the development of selective insecticides, as well as new analgesics.” – please explain what is a relation between insecticides and analgesics, and why analgesics are mentioned when not human but cockroach voltage-gated sodium channel was studied.

The objectives of the research are extensive and include: (a) characterization of conformational changes in the muscarinic receptor under the influence of agonist and

antagonist binding; (b) study the interactions of a novel light-switchable bitopic compound as a muscarinic receptor ligand; (c) study of the interactions of the cockroach voltage-gated sodium channel (VGSC) VSD domain with four sea anemone peptide toxins; (d) finding the entry route of the insecticide into mosquito VGSC binding site. The final objective about "insensitivity conferring resistance" is specified in an unclear way, but fortunately in the Abstract it was specified correctly as studying the role of insecticide resistance mutations.

All the above aims were completed in the three published papers of the Candidate. The papers were reviewed by independent reviewers so there is no necessity to review them again. One can only say that they are written in a very clear way, they are lengthy, full of details, and contain large number of references. They are research articles but fortunately they have rather large introductions so even the plain reader is not lost in the scientific issues. The research of the Candidate is continued in her Preludium grant on new class of insecticides and on the molecular basis of the insect sodium channel inactivation process.

In conclusion, the dissertation concerns a difficult but very important scientific problem of great practical importance. The above-mentioned shortcomings do not obscure the significant value of the dissertation and the very good scientific preparation of the Candidate. Therefore, I am fully convinced that the doctoral dissertation presented to me for evaluation meets the conditions set out in Art. 187 ust. 1 and 2 of the Act of July 20, 2018 Law on Higher Education and Science (with later changes). Therefore, I apply to the Scientific Council of the Institute of Physics of the Nicolaus Copernicus University in Toruń for admission of Beata Niklas to further stages of the procedure for awarding a doctoral degree. In addition, due to the fact that the Candidate obtained interesting results published in international journals, usage of many research techniques, and the significant contribution in each publication incorporated in the doctoral dissertation, I'm applying for its distinction.

S. Filipek

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