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Peer review of the PhD dissertation "The search for new inhibitors of bacterial efflux pumps among amine derivatives of 5-arylidenehydantoin" presented by Ewa Otrębska – Machaj.

It is commonly accepted that one of the main factors affecting unprecedented increase of human life expectancy in the XX century was development of new medicines. Among them, the most influential group in this aspect were the medicines combating infectious bacterial diseases; the mankind learnt to use antibiotics in controlling the spread of various epidemics which otherwise had decimated our population for ages. But yet with the first introduction of penicillin into clinical practice, the parallel process had started in which infectious bacteria developed systems of resistance to antibiotics; soon it appeared to be an enormous problem with strains hardly controlled by any accessible medication. Currently, in spite of a significant number of various classes of antibiotics, there is a large need for new medicinal substances capable to fight infections caused by those dangerous multidrug resistant microbes.

The dissertation by Ewa Otrębska – Machaj tackles such a problem. One of the main mechanisms of drug resistance in bacteria is development of highly specialized protein systems allowing transport of xenobiotics to the outside of the bacterium cell. The systems, called efflux pumps are currently considered as very promising protein targets for new medications facilitating the action of classical antibiotics by inhibiting their bacterial export. The main goal of the thesis is medicinal chemistry development of novel hydantoin ANTI amine derivatives and assessment of how their modifications may affect inhibition of one of main efflux pumps present in clinically relevant Gram negative bacteria, AcrAB-TolC system.

More specifically the author has explored chemical modifications at position 5 of the hydantoin ring (several different arylidene aromatic substituents) and position N3 (different kinds of chain and cyclic amine moieties that has been sterically distanced from hydantoin ring by several different spacers ranging from 3 to 8 Carbon atoms). Permutation of these modifications (with additional small set of derivatives exploring two amine substituents at position 2) allowed to design and propose 44 new derivatives. All the compounds have been synthesized using combinations of Knoevenagel condensation, Mitsunobu reaction and Gabriel synthesis followed by structural analyses using ^{1}H-NMR, ^{13}C-NMR and IR spectroscopy. The substances have then been subjected to biological tests against *Enterobacter aerogenes*, a microorganism known to express the AcrAB-TolC pump in its multidrug resistance strategy. Biological assays for efflux pump inhibitors need non-trivial approaches for activity measurements; three strains of *E. aerogenes* have been used, each differed with expression level of the AcrAB-TolC system what allowed the author to obtain efflux pump inhibitory activity in a population controlled experiment. Secondly, the hydantoin derivatives had to be tested for bacteria growth inhibition alone and in combination with common antibiotics to properly assess their influence on the function of efflux pumps. These tests allowed the author to select the most promising compounds to perform scrutinized inspection of syn/anti cooperation between a ligand and an antibiotic using checkerboard method or real

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time competition with fluorescent 1,2'-dinaphtylamine efflux. The results allowed the author to present elegant structure – activity relationships analysis for each modification spot and conclude that efflux pump inhibitory activity for the group of new hydantoin derivatives mostly depends on big hydrophobic moiety at one end (modifications at position 5) and basic ionisable fragment separated by a long spacer at the other end (modifications at position N3). The observation emphasized the role of the amphiphilic nature of proposed efflux pump inhibitors.

The dissertation itself is well written and organized; its opening section gives concise but very illustrative introduction to the topic of bacterial resistance, efflux systems and efflux pump inhibitors. Defined rational of the work is precisely addressed in the results section which is written on the optimal level of complexity considering amount of work and interdisciplinary nature of presented research. The experiments are well designed and documented, observations and conclusions are clearly stated and emphasize the authors thoughtful and reflective mind as a researcher. The reviewer's impressions on the quality of the work and qualification of the PhD candidate are very positive.

This reviewer has obviously several questions, comments and suggestions to presented dissertation. One of the concerns is related to the author's assumption that 5-arylidenehydantoins always occur at the Z configuration. I wonder whether there is any structural explanation of the molecular mechanisms of the Knoevenagel condensation leading to preference of the Z but not the E configuration of N1-unsubstituted hydantoin products? The second issue is related to graphical presentation of chemical modification of hydantoin ring in section 2. (p.30-34). It might be a source of confusion for the reader since in some cases more than one connection point for R^2 substituent could be potentially considered (c.f. derivatives A2 or A4). I would recommend to use in whole dissertation the unequivocal graphic system, the author used for the same purpose at Figure 37 (using specific wave-lines to identify connection points). Also, comparing formulas at Figure 37 (p. 96) with those in Table 5 (p. 32) one can realize that A2 has two different structures; which one is true? Among minor points, this reviewer considers using "MICs" expression for plural "MIC" acceptable in colloquial language, similar to "IC_{50}s" or even "K_ds" commonly articulated during lectures, lab meetings etc. Written thesis should rather use more official "MIC values", "IC_{50} values" or "K_d values" instead. Generally, the thesis is written in good English (as long as can be judged by a non-native reviewer). I managed to identify only a few typographic or lexical errors, and would like to bring just one to the author's attention: Sir Alexander Fleming was "the discoverer", or maybe "the inventor" but rather not "the explorer" of penicillin (p. 1.).

Those comments have no effect on my very positive impression on overall value of the presented thesis. I consider it a very well presented piece of original science provided by a creative mind. Therefore, I strongly recommend the Faculty of Pharmacy, Jagiellonian University in Kraków to accept the dissertation "The search for new inhibitors of bacterial efflux pumps among amine derivatives of 5-arylidenehydantoin" and to permit Ms. Ewa Otrębska – Machaj for her public PhD defense. I also propose to consider giving a special recognition to the doctorate as provided by the State Law.

Sincerely,

Professor Krzysztof Jóźwiak