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Re.: Evaluation of the doctoral thesis submitted by Ms Ewa Otrębska-Machaj

in her letter dated 9 February 2015, I was asked by Professor Alicja Budak, vice-dean of the Pharmaceutical Faculty of the Jagiellonian University – Medical College in Krakow, to evaluate the doctoral thesis submitted by Ms Ewa Otrębska-Machaj.

The PhD dissertation of Ewa Otrębska-Machaj entitled *The search for new inhibitors of bacterial efflux pumps among amine derivatives of 5-arylidenehydantoin,* comprising 171 pages and 241 references, was supervised by professor Katarzyna Kieć-Kononowicz from Jagiellonian University in Krakow and professor Jean-Marie Pagès from Aix-Marseille University.

In the *Introduction* section the prevalence and mechanisms of development of bacterial resistance to antibiotics and bacterial efflux pumps are presented. The structure and function of AcrAB-TolC efflux pump, one of the major efflux pumps involved in the reduction of susceptibility of MDR bacteria to a range of antibiotics is described in detail, along with strategies for reducing activity of the AcrAB-TolC efflux pump. Representative chemical groups of efflux pump inhibitors, including hydantoin derivatives recently discovered in the supervisor's laboratory at the Jagiellonian Univeristy, are discussed in detail.

The purpose of the research was modification of (*Z*)-5-(2,4-dimethoxybenzylidene)-3-(3-dimethyl-amino-2-hydroxypropyl)-imidazolidine-2,4-dione which previously showed moderate efflux pump inhibitory activity in *Enterobacter aerogenes* strain overexpressing AcrAB-TolC efflux pump at (i) 5-arylidene moiety, (ii) the linker and (iii) the amine moiety attached at position 3. In total 44 final compounds were synthesized employing Knoevenagel condensation, Mitsunobu reaction, oxirane ring opening and Gabriel synthesis, fully characterized and then subjected to microbiological studies in Gram-negative *E. aerogenes strain* Ea289 overexpressing the AcrAB-TolC pump and presenting the porin-defficient phenotype, CM-64 strain overexpressing the AcrAB-TolC pump with no change in the porins biosynthesis and the Ea294 strain lacking the AcrAB-TolC efflux pump activity. The microbiological studies included (i) determination of MIC values, (ii) studies of the effect of the prepared compounds on MICs of nalidixic acid, chloramphenicol, doxycycline and erythromycin as substrates of the AcrAB-TolC efflux pump, (iii) studies of synergism between the tested hydantoin derivatives and selected antibiotics and (iv) the real-time efflux assay using the fluorescent dye 1,2'-dinaphthylamine as a substrate of the AcrAB-TolC efflux pump. Finally, structure-activity relationship

analysis was performed to find a connection between the structures of the hree modified fragments and the efflux pump inhibitory activity. Toxicity risk of compounds and their drug-like properties were predicted using the OSIRIS Property Explorer software and the majority of compounds were predicted to be less toxic and have higher drug-score index than known efflux pump inhibitors.

Several active compounds without the intrinsic antibacterial activity increased susceptibility of strains overexpressing the AcrAB-TolC efflux pump to nalidixic acid, chloramphenicol, doxycycline and erythromycin causing a 4-fold to 256-fold decrease in their MIC values. The majority of compounds synergistically enhanced the activity of the tested antibiotics as demonstrated by calculated FIC indexes and isobolograms. In real-time efflux assay several compounds capable of increasing the potency of tested antibiotics were found to inhibit the efflux of 1,2'-dinaphthylamine in a dose dependent manner. Finally, the SAR analysis of prepared hydantoin and imidazolone derivatives pointed out that a bulky hydrophobic arylidene substituent at position 5 and a basic ionisable moiety attached at position 3 or 2, endowing the compounds with amphipathic character, are crucial for affecting the AcrAB efflux pump activity.

The candidate published as co-author 2 papers dealing with the research topic of the dissertation and the third one with the candidate as the first author has been submitted to the *Journal of Antimicrobial Chemotherapy*:

- Handzlik J, Szymańska E, Chevalier J, Otrębska E, Kieć-Kononowicz K, Pagès JM, Alibert S. Amine-alkyl derivatives of hydantoin: new tool to combat resistant bacteria. *Eur. J. Med. Chem.* **2011**, *46*, 5807-5816.

- Handzlik J, Szymańska, Alibert S, Chevalier J, Otrębska E, Pękala E, Pagès JM, Kieć-Kononowicz K. Search for new tools to combat Gram-negative resistant bacteria among amine derivatives of 5-arylidenehydantoin. *Bioorg. Med. Chem.* **2013**, *21*, 135-145.

- Otrębska-Machaj E, Chevalier J, Handzlik J, Szymańska E, Mazurkiewicz J, Boyer G, Bolla JM, Kieć-Kononowicz K, Pagès JM, Alibert S. Efflux pump blockers in Gram-negative bacteria: new hydantoin based modulators to improve antibiotic activity. *J. Antimicrob. Chemother.* submitted.

In conclusion, the doctoral thesis of Ms Ewa Otrębska-Machaj explored fighting bacterial resistance to antibiotics through inhibition of AcrAB-TolC efflux pump by a series of 5-arylidenehydantoin and 5-arylideneimidazolone derivatives, which is a highly relevant scientific topic due to threatening bacterial resistance to currently used antibacterials. Identification of AcrAB-TolC efflux pump inhibitors among prepared 5-arylideneimidazolidinedione and 5-arylideneimidazolone derivatives can serve as a good starting point for further optimization of these compounds towards better efflux pump inhibitors. The applied methods are appropriate and up-to-date and the conclusions made are based on in-depth analysis of experimental results. The dissertation is well presented, systematically structured and written in good English.

I am pleased to conclude that the doctoral dissertation of Ms Ewa Otrębska-Machaj presents an original contribution to science in the field of medicinal chemistry and I recommend it to be accepted by the Jagiellonian University in Krakow in partial fulfillment of the requirements for the doctor's degree.

Sincerely,

Professor Danijel Kikelj