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The opinion on the PhD thesis "Synthesis and Biological Evaluation of New Donepezil Based Derivatives as Multiple Ligands Targetting Cholinestereases and Amyloid Beta" presented by Natalia Szałaj.

Polypharmacology, a fact that a drug molecule interacts with multiple targets, is on one hand a huge challenge for modern drug discovery but on the other it emerges as a very important strategy for treatment of complex conditions like cancer, depression, schizophrenia or neurodegeneration. Alzheimer's disease (AD) is a very good example of this complexity; several hypotheses have been formulated to explain etiology of the disease including: gradual loss of cholinergic transmission in the cortex, increased deposits of aggregative amyloid beta protein, hyperphosphorylation of tau protein, ischemia or oxidative stress to name the most prominent. Therefore, numerous approaches can be proposed in treatment of the disease using drug molecules targeting various identified proteins. In fact five medications have been already introduced into the clinical practice to manage AD patients; the drugs are either inhibitors of cholinesterases (tacrine, donepezil, galantamine, rivastigmine) or NMDA receptor antagonist (memantine). These medications, however, can only slow down progression of the disease and, sadly, are of moderate effectiveness. Since AD becomes a seriously growing problem for our civilization, a great number of laboratories are involved in drug discovery and development projects and polypharmacology strategies are frequently employed in those investigations.

One of the strategies are molecules of dual activities showing inhibition of amyloid beta (Aβ) aggregation in addition to inhibition of acetylcholinesterase (AChE) or butyrylcholinesterase (BuChE) enzymes. Therefore, a successful drug candidate may combine positive stimulation cholinergic transmission with blockade of deleterious effects of Aβ aggregates on weakened neurons. Very inspiring examples are hybrid molecules containing rivastigmine and donepezil scaffolds previously developed by the group of professor Barbara Malawska. The dissertation presented by Natala Szałaj describes medicinal chemistry research performed using the same strategy. The main goals of the thesis are design and synthesis of novel donepezil derivatives, verification of their dual target activities in a series of assays and finally selection of a lead compound for further development as potential anti – AD drug candidate. Combination of molecular modeling, structure – activity relationships with results of previous projects lead to proposition of new structures, donepezil based benzylamides linked to various isoindolinones and benzisothiazolones by a series of spacers. The molecules were optimized to explore both catalytic anionic site and peripheral anionic site located at the gorge entrance. The compounds were then tested for activity against AChE and BuChE using Ellman's method, and for Aβ aggregation and Aβ neuroprotection using Thioflavin T assay and SH-SY5Y cell viability tests, respectively. Blood – Brain Barrier penetration was additionally assessed for selected compounds using PAMPA assay.

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In conclusion, the author emphasizes that connections of donepezil based benzylamine scaffold with phtalimide or saccharine systems may act as dual cholinesterase and Aβ aggregation inhibitors. She precisely defines structural requirements promoting activity against AChE and BuChE; modification of both benzylamine part as well as the length and the construction of a linker towards heteroatomic part exploring the peripheral site. Unfortunately, no structure – activity relationships could be formulated for results describing effect of developed compounds on Aβ aggregation which is, however, rather typical for such activities. The work allowed to select two lead derivatives for future investigations; compound **71** (IC_{50} against *h*AChE = 33 nM; inhibition of $A\beta_{1-42}$ aggregation by 22% at 10 μM) and compound **18** (IC_{50} against *h*AChE = 268 nM; inhibition of $A\beta_{1-42}$ aggregation by 66% at 10 μM). While both show optimal BBB permeability, **18** additionally protects cells from Aβ induced cytotoxicity.

I need to underline here that the dissertation by Natalia Szałaj is very well composed and written. It is made by a collection of five research articles published mostly in 2015 accompanied by comprehensive introductory/commentary section. Three articles report original research on development of dual-target AChE plus Aβ inhibitors (*Eur. J. Med. Chem.*, 2015; *Bioorg. Med. Chem.*, 2015 and *Archiv der Pharmazie*, 2015); two others are review articles presenting the state of the art in the field of multifunctional agents as potential candidates for AD management (*Curr. Med.Chem.* 2011 and *Curr. Med.Chem.* 2015). It, therefore, makes my duty much easier since all presented material has been already peer – reviewed by respective editors. In four out of five papers Natalia Szałaj (nee Guzior) is the first author leaving no space for questioning on her leading input to the concept, experiment design and execution, interpretation or writing in each manuscript. It proves Natalia Szałaj as full grown researcher with excellent qualifications for the PhD title.

Obviously this reviewer has a significant number of specific comments, questions and suggestions listed in points below.

1. enzyme kinetic studies demonstrate noncompetitive mechanism of action of both donepezil and new derivatives. This is in agreement with most literature reports, however, there are papers suggesting a mixed type inhibition for donepezil. It seems reasonable in lights of structural studies where crystallographic model of donepezil-*Tc*AChE complex shows extended location along the binding gorge and interactions with both the peripheral anionic site and the catalytic anionic binding site (i.e. W84). Thus, one may expect that the inhibitor will affect both V_{max} and K_m of the catalytic reaction. Therefore, competitiveness of the inhibition should be verified with particular attention. In my opinion testing of non competitive mechanism by location of intersection point of all velocity plots in Lineweaver-Burk representation should be done by measurements of at least three different concentrations of an inhibitor. This is the case for data presented in Paper II and Paper III but not in Paper I, where Figure 2 shows enzyme kinetics for only 2 concentrations. Moreover, Lineweaver-Burk plot, even though it is commonly used for this purpose, has a disadvantage of compressing the data points at high concentrations into a small region, therefore a large number of data points are required to increase accuracy. Alternative solutions are offered by re-plotting the data in Dixon or Cornish-Bowden representation or direct comparison of K_m an V_{max} differences obtained straight from nonlinear regression of the Michaelis–Menten model.

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2. Can the author specify whether any organic solvent has been used to dissolve synthesised derivatives for biological assays? Small amount of DMSO or methanol may affect enzyme activity or Aβ aggregation rate.
3. Chapter 1.4. fails to provide a clear description of molecular binding mechanisms of AChE inhibitors. An illustration comparing binding mode of donepezil with those of other inhibitors would be particularly helpful to understand this complex issue which otherwise is very hard to describe in a short text.
4. The author should consider significant figures while reporting and discussing experimental data. A message in the abstract that compound **71** inhibits Aβ aggregation by 22.19% is not statistically accurate in a situation when the actual experiment has measured 22.19 ± 16.68% (p.7 paper III).
5. The quality of written English is very good, I have identified only incidental errors. The author has made a mistake in naming the saccharin derivatives in Figure 15 (p. 33) or section 5.6. (p. 39). The structure names are, however, correct at the original publication (Paper III). Another mistake is using "*Eq*AChE" term at p. 49 while the *Electrophorus electricus* (*Ee*) form of Acetylcholinesterase has been used in the experiments.

Above comments are of marginal importance and have no effect on my affirmative impression on the overall value of the thesis under review. Considering very solid medicinal chemistry project presented therein, the fact that a candidate has already published most of her research in peer – reviewed journals I strongly recommend the Faculty of Pharmacy, Jagiellonian University in Kraków to accept the dissertation "Synthesis and Biological Evaluation of New Donepezil Based Derivatives as Multiple Ligands Targetting Cholinestereases and Amyloid Beta" and to permit Ms. Natalia Szałaj for her public PhD defense. I also have a pleasure to advise granting a special recognition to the doctorate as provided by the Polish Law.

Sincerely,

Professor Krzysztof Jóźwiak