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On the interaction of a donepezil-huprine hybrid with synthetic membrane models

Pablo Zambrano*

Alzheimer's disease is the most prevalent type of dementia today, discovered and described by Alois Alzheimer in 1907. According to the World Alzheimer Report 2021, 75% of people with dementia worldwide are undiagnosed, equivalent to 41 million people (Gauthier et al., 2021). With each passing year, the number of people affected by these diseases is increasing, and the estimates of suffering from them in the future are growing. There are currently only four drugs used against Alzheimer's disease: donepezil, rivastigmine, galantamine, and memantine. The first three, based on the cholinergic hypothesis, aim to inhibit the acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) to prevent the reduction of acetylcholine levels in the brain. Memantine, on the other hand, is based on the glutamatergic hypothesis, according to which in Alzheimer's disease there are higher than normal levels of glutamate which, through interaction with the glutamatergic N-methyl-D-aspartate receptor, lead to abnormally high levels of calcium which cause neuronal damage. Blocking N-methyl-D-aspartate receptors to reduce these calcium levels is a therapeutic target to control this disease, and this is the mechanism of action of memantine (Rubin, 2021). Recently, the drug Aducanumab has also been approved in the United States. This is a human IgG1 monoclonal antibody that primarily binds to amyloid-beta (Aβ) aggregates, soluble oligomers, and also insoluble fibrils, limiting the toxicity of these species.

Different molecular mechanisms that could be involved in the development of Alzheimer's disease have been proposed. The main ones include oxidative stress, excessive levels of glutamate, neuroinflammation, hyperphosphorylation of tau proteins, decreased levels of acetylcholine and abnormal accumulation of Aβ plaques (Lindstrom et al., 2021). Ca^{2+} dysregulation is also another important factor in Alzheimer's disease. Under normal conditions, extracellular Ca^{2+} concentration is higher than intracellular Ca^{2+} concentration. Molecular interactions of beta-amyloid molecules with neuronal membranes can significantly alter the calcium channels in the membrane, increase the influx and cause an imbalance of this ion. High intracellular calcium concentrations result in considerable toxicity and subsequently cell death. This disruption of calcium homeostasis can have other secondary consequences, such as lipid peroxidation and generation of reactive oxygen species, which can lead, in the long term, to a significant reduction in synaptic integrity (Garwood et al., 2013). Therefore, to treat this disease, we cannot only focus our strategies on targeting a single therapeutic target, but we must also address more than one for its treatment. Such a strategy is central to the development of new drugs and is now being pursued by a variety of researchers and holds great promise in the fight against Alzheimer's disease.

Understanding the molecular mechanisms of membrane protection is what motivated us to study one of the multitarget compounds whose *in vitro* and *in vivo* characteristics have been of interest (Zambrano et al., 2021). AVCRI104P4 (**Figure 1**), is a donepezil-huprine hybrid that possesses marked *in vitro* inhibitory activity of human acetylcholenesterase and human butyrylcholinesterase. In addition, AVCRI104P4 has a strong ability to cross the blood-brain barrier, which has been confirmed in several studies. In experiments in mice (APP SL) AVCRI104P4 was able to improve short-term memory (Sola et al., 2015). Furthermore, the effect of AVCRI104P4 on Aβ aggregation has been studied *in vitro* and *in vivo*. Using a thioflavin T fluorescence method (Bartolini et al., 2007), AVCRI104P4 was found to inhibit AChE-induced $Aβ_{1–40}$ aggregation *in vitro* by 41% at 100 μM and inhibited spontaneous $Aβ_{1–42}$ aggregation by 29% at a concentration of 10 μM (Viayna et al., 2010). However, our interest was focused on the study of its possible membrane-protective capacity. For this purpose, in our experiments, we used molecular models of cell membranes constructed with dimyristoyl (DM) phosphatidylcholine (PC) and DM phosphatidylethanolamine (PE) bilayers and human erythrocytes, which were exposed to different concentrations of $Aβ_{1–42}$. It has been shown by different methods that the two dominant phospholipid classes in all brain areas of patients with Alzheimer's disease and dementia are PE and PC (Söderberg et al., 1992). This approach was based on the importance attributed to Aβ-neuronal membrane interaction in the development and progression of the disease which can have important consequences at the biochemical level, such as alterations in membrane fluidity and ion channel formation (Zambrano et al., 2021). For this reason, we considered it of interest to understand the molecular mechanism of the interaction of $Aβ_{1–42}$ with cell membranes and to determine the possible protective effect of AVCRI104P4.

Our experimental results showed a protective effect of AVCRI104P4 against $Aβ_{1–42}$ peptide-induced toxicity in human erythrocytes and membrane molecular models. Using X-ray diffraction studies, we found that increasing concentrations of AVCRI104P4 neutralize the disruptive effect of $Aβ_{1–42}$ on DMPC bilayers. Furthermore, results obtained by scanning electron microscopy showed that pre-incubation of erythrocytes with AVCRI104P4 at increasing concentrations prevents $Aβ_{1–42}$-induced red blood cell morphological alterations and cell lysis.

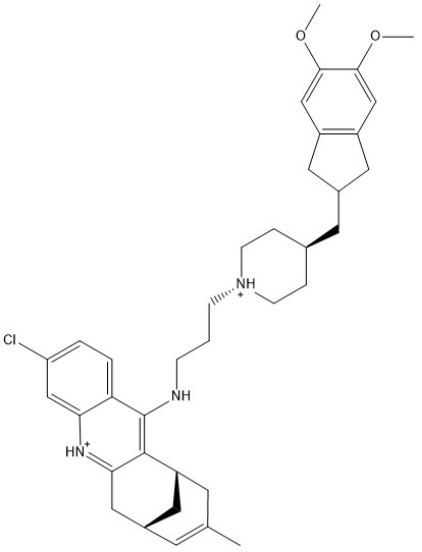

Figure 1 | **Structural representation of the molecule AVCRI104P4.**

However, the detailed mechanism of action of this phenomenon has not yet been elucidated. Considering our experimental results, it is possible to conclude that there may be different mechanisms through which AVCRI104P4 may protect the plasma membrane from the toxic effects of $Aβ_{1–42}$. One of them could be due to the direct action of AVCRI104P4 on membrane models. We found that the hybrid was able to bind to the phospholipids DMPC and DMPE, which may indicate a mechanism to protect the membrane, either by reinforcing it or by preventing the disruptive action of the amyloid peptide itself. Another possible mechanism could be attributed to the dual effect of AVCRI104P4 in binding to AChE and preventing $Aβ_{1–42}$ aggregation (AChE-induced).

A more interesting mechanism to explore is the direct interaction of free AVCRI104P4 molecules and $Aβ_{1–42}$. This approach presents itself as a much more complex intellectual challenge because many of the currently tested $Aβ_{1–42}$ anti-aggregation molecules are not completely safe to project into *in vivo* studies (Pagano et al., 2020). Undesirable effects or parallel molecular interactions of these molecules do not allow them to be considered as safe alternatives. However, elucidation of the exact three-dimensional conformations of $Aβ_{1–42}$ peptides by cryo-electromicroscopy or another structure prediction technique could lead to a more precise identification of residues interacting with neuronal membrane proteins, lipids, and sugars. These questions can be further

explored by molecular dynamics simulations. Dr. Diego Muñoz-Torrero's group has studied the theoretical behavior of huprine hybrids using these techniques (Viayna et al., 2010). Using an initial position of the compounds under study by elucidating the three-dimensional structure of X-ray crystallographic complexes with enzymes such as AChE or BuChE and adding a suitable solvent to the system, it is possible to obtain charge distributions and hypothetical reaction constants. This approach could also be applied to the interaction of AVCRI104P4 with amyloid molecules. In this way, much more specific and better targeted multi-target molecules could be designed.

From an experimental point of view, I consider that an additional experimental approach would be the optimization of model systems to test the new multitarget molecules. For example, in this specific case, it should be noted that AVCRI104P4 is a molecule that was designed for use in Alzheimer's disease, so the membrane model systems should simulate the deteriorating conditions of advanced disease states (or those whose stage is appropriate for diagnosis). This would include phospholipid composition, protein composition, chemical environment, etc.

New microfluidic techniques (Lab-on-a-chip) emerge as a novel tool to study membrane interactions with new molecules (Prasanna et al., 2021). In this technique, it is possible to use membrane models that can be visualized in real time such as giant unilamellar vesicles, which can encapsulate different types of molecules and study their interaction in detail. In addition, microfluidics could provide a miniature model of the brain (or a neuron), considering its cellular composition and a suitable chemical/physiological microenvironment. The construction of a microchip with all these features, combined with high-resolution microscopy tools, would allow real-time information to be obtained. The optimization of these model systems, we would have a much more realistic scenario in which to test new multitarget molecules and obtain realistic results.

With these general challenges still being asked, the extensive scientific data on multitarget compounds continues to grow daily, as do new families of synthetic molecules. In addition, as research into the origins of neurodegenerative diseases advances, new metabolic pathways are being discovered and new hypotheses are being tested experimentally, which will allow future research strategies and new families of molecules with potential therapeutic capabilities to be established.

Pablo Zambrano*
Max Planck Institute of Biochemistry, Cellular and Molecular Biophysics, Munich, Germany
***Correspondence to:** Pablo Zambrano, PhD, zambranolobos@biochem.mpg.de.
https://orcid.org/0000-0001-5499-6091 (Pablo Zambrano)
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