Blood-brain Barrier Permeability and AChE inhibition of ionophoric polyphenols.

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Introduction

The BBB is a semipermeable border composed of a layer of endothelial cells that separates the bloodstream from the brain and extracellular fluid in the CNS. It is an important mechanism to protect the brain from access of external toxins, fluctuations in plasma composition, and from other circulating agents in blood. This layer, however, also limits the access of therapeutic drugs into the brain. As a result, failure to cross the BBB and/or recognition by efflux BBB transporters are major impediments in the development of drugs for the treatment of AD

Acetylcholine (ACh) is an important neurotransmitter in the regulation of learning and memory processes. According to the cholinergic hypothesis, neurodegeneration in AD is manifested in cholinergic neuron loss in the brain, and therefore, an increase of ACh levels should relieve the symptoms. Acetylcholine is hydrolyzed by acetylcholinesterase (AChE), a serine hydrolase mainly found at the neuromuscular junctions and cholinergic brain synapses, and cholinesterase inhibitors enhance the level of acetylcholine. Thus, the cholinergic system has been explored as an important target for the treatment of AD. Currently, tacrine, donepezil, galantamine, and rivastigmine are FDA approved

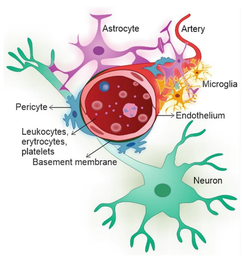

Figure 1. Blood-brain barrier representation.

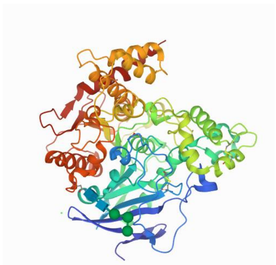

Figure 2. Acetylcholinesterase (from PDB: 6XYS).

In previous work we demonstrated that a family of multi-target polyphenols (Compounds **1-5** in Figure 3), structurally similar to natural resveratrol, show promising activity against important aspects related to AD. These compounds combine and enhance the antioxidant and antiamyloidogenic properties of natural polyphenols with the anti-AD benefits of selective metal ionophoric agents. The resulting potential drugs can inhibit the Aβ fibril aggregation up to a 96%, dissagregate pre-formed Aβ mature fibrils up to a 91%, reduce to almost a 100% the presence of hydroxyl radicals -as well as other peroxyl radicals-, chelate toxic concentrations of Cu^{2+} metal ions, and are non-toxic towards healthy eukariotic cells. in this work we present *in vitro* and *in silico* studies of BBB permeability and efflux mechanisms of our multi-target ionophoric polyphenols by means of the parallel artificial membrane permeability (PAMPA) assay and molecular models. In addition, the ability of the ionophoric polyphenols to inhibit AChE is also reported. Docking and molecular dynamics (MD) studies were also performed to determine the nature of the compound interaction with AChE, and to obtain preliminary structure-activity correlations.

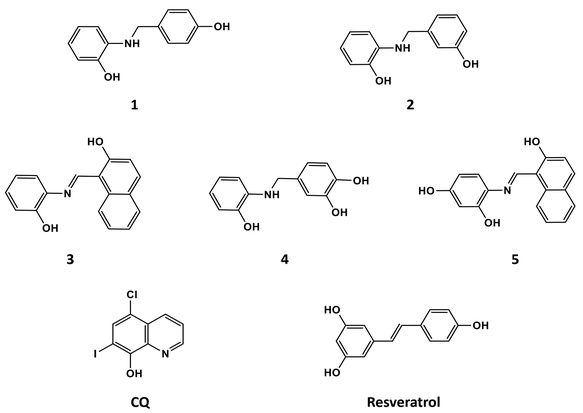

Figure 3. Compounds studied in this work: Multi-target ionophoric polyphenols (**1-5**) previously synthesized, clioquinol (CQ) and resveratrol.

References

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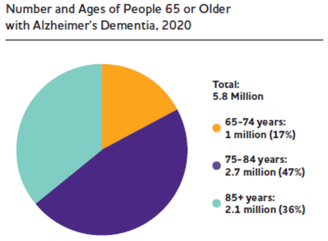
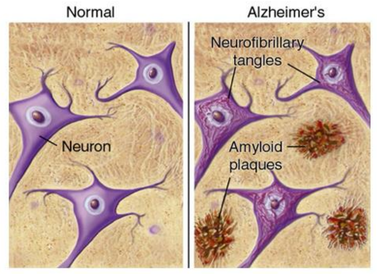
Abstract



Alzheimer's disease (AD) is the most common form of dementia that affects more than 5 million Americans and more than 40 million people around the world. The incidence is expected to rapidly increase due to the lack of any effective treatment. In previous work we synthesized a family of five ionophoric polyphenols (compounds **1-5**) that targeted important aspects related to AD. Here, in order to gain insights into their potential therapeutic value, we have tested the ability of compounds **1-5** to cross the blood brain barrier (BBB), and to inhibit acetylcholinesterase (AChE), an enzyme that is reported to be involved in the progression of the disease. We performed BBB permeability and efflux mechanisms studies by means of the *in vitro* parallel artificial membrane permeability assay (PAMPA-BBB), as well as several *in silico* methods. AChE inhibition was spectrophotometrically studied. All compounds were found permeable to the BBB and moderate inhibitors of AChE inhibitors, with the ability to interact with several residues of the active site of the enzyme, as also revealed by docking and molecular dynamics simulations. Overall, our results suggest that these compounds could effectively cross the BBB to exert their anti-AD activity, including AChE inhibition.



Results and Discussion

BBB penetration by the PAMPA assay, and in silico predicted P-glycoprotein (P-gp) net efflux ratio (NER) and substrate recognition.

- The PAMPA assay used herein estimates the passive diffusion of compounds through the BBB by measuring the effective permeability rate (P_e) through an artificial membrane impregnated with lipid extract of porcine brain.
- Compounds with log P_e >-4.5 are highly permeable, whereas compounds with log P_e <-6.3 show low permeability. Compounds with -4.5> log Pe >-6.3 are described as having uncertain permeability.
- All log P_e values at pH=7.40 (physiological) and pH=6.60 (mimicking cerebral acidosis) for the ionophoric polyphenols **1-5**, as well as all controls, are summarized in Table 1.

Table 1. Permeability (log P_e) of commercial drugs, ionophoric polyphenols **1-5**, resveratrol and clioquinol in the PAMPA-BBB assay at pH 7.40 and 6.60.

*Values from reference (Wieckowka et al. 2016)
**Calculated using Lipinski's rules (Martinez et al. 2016)

- In order to overcome the BBB, passive diffusion is needed, but the liability of efflux transporters must also be addressed.
- The P-glycoprotein is an important efflux transporter that is highly expressed at the BBB to remove harmful molecules out of the brain, and might limit the therapeutic potential of new drugs.
- The net efflux ratio (NER) by P-gp on LLC-PK1 cells was predicted *in silico* for compounds **1-5**, as well as resveratrol and clioquinol used as controls, by using DruMAP (drug metabolism and pharmacokinetics analysis platform)
- Results for NER by P-gp are displayed in Table 2 and show a low predicted efflux ratio for the ionophoric polyphenols **1-5**, as well as for clioquinol, while resveratrol is predicted to have a middle NER

Table 2. P-gp NER predicted on LLC-PK1 cells using DruMAP, and P-gp substrate recognition predicted using the server at Biozyne for compounds **1-5**, resveratrol and clioquinol.

Compound	1	2	3	4	5	Resveratrol	Clioquinol
P gp NER	Low	Low	Low	Low	Low	Middle	Low
P gp substrate recognition	Non-substrate	Non-substrate	Non-substrate	Non-substrate	Non-substrate	Non-substrate	Non-substrate

Inhibition of acetylcholinesterase (AChE) enzymatic activity and theoretical study of the compound-enzyme interaction

- Compounds **1-5,** resveratrol and clioquinol were tested in their ability to inhibit AChE by means of an acetylcholine/acetylcholinesterase assay kit, using tacrine (a potent FDA-approved AChE inhibitor) as a positive control.
- All compounds were first tested at two single concentrations, 10 mM and 50 mM. IC_{50} values were calculated only for those compounds achieving more than 60% inhibition of AChE at 10 mM concentration.
- Results are shown in Table 3 and reveal interesting inhibitory properties (low micromolar range) for structurally related polyphenols **1**, **2** and **4**. On the other hand, IC_{50} for tacrine was comparable to published data. Compounds **3** and **5**, featuring the presence of an imino group and a naphthalene structural moiety were essentially inactive. Resveratrol and, especially, clioquinol did not either display significant inhibitory properties against AChE activity.

Table 3. Inhibition of AChE by the test ionophoric polyphenols **1-5**, resveratrol and clioquinol.

Compound	% Inhibition at 10 μM	% Inhibition at 50 μM	IC_{50}(μM)
Tacrine	97.66 ± 0.27	100.00 ± 0.00	0.271 ± 0.009
Resveratrol	40.90 ± 1.38	93.78 ± 0.93	--
Clioquinol	19.11 ± 0.63	33.72 ± 1.31	--
1	60.89 ± 3.47	81.65 ± 0.59	8.95 ± 0.16
2	71.19 ± 1.90	81.65 ± 0.09	7.27 ± 0.17
3	12.66 ± 1.49	31.68 ± 1.82	--
4	68.97 ± 3.43	66.90 ± 2.47	5.44 ± 0.39
5	26.99 ± 1.99	58.53 ± 1.11	--

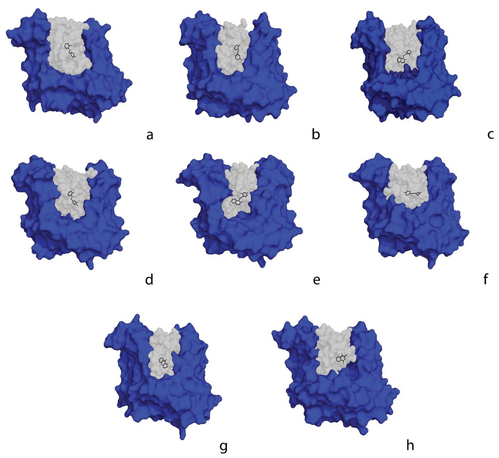


Figure 4. a-h (polyphenols **1-5**, resveratrol, tacrine, and clioquinol, respectively) depict the representative structures of each compound in the active site gorge of AChE. The following color scheme was used: compounds **1-5**, resveratrol, tacrine and clioquinol (black), AChE active site gorge (grey) and AChE receptor (blue).

Table 4. Summary of the non-covalent interactions (hydrogen bonds, hydrophobic and aromatic p-p stacking interactions) seen in the representative structure for each compound system at the active site of AChE. The average MM/GBSA binding free energy for each compound at the active site of AChE is also reported.

Receptor	Compound	Avg MM/GBSA Binding Free Energy (kcal/mol)	H-bond Interacting Residues (PAS residues, CAS-anionic residues, CAS-esteratic residues, acyl site residues, oxyanion site residues, active site residues)	Hydrophobic Interacting Residues (PAS residues, CAS-anionic residues, CAS-esteratic residues, acyl site residues, oxyanion site residues, active site residues)	Aromatic Interacting Residues (PAS residues, CAS-anionic residues, CAS-esteratic residues, acyl site residues, oxyanion site residues, active site residues)
AChE	1	-28.02±0.27	Trp84, Tyr130	Trp84, Phe330, Tyr334, Trp432	Trp84, Phe330, Trp432
	2	28.84±0.33	Trp84, Asn85, Tyr130	Trp84, Phe330, Tyr334	Trp84
	3	-17.12±0.52	-	Trp84, Phe330	Trp84
	4	-24.93±0.43	Ser124, Tyr130	Trp84, Tyr116, Phe330, Tyr334	Trp84
	5	-20.20±0.78	Asn85	Trp84, Phe330	Trp84
	Resveratrol	-26.08±0.51	Asn85, Trp432	Trp84, Phe330, Trp432	Trp84, Phe330, Trp432
	Tacrine	-35.17±0.28	His440	Trp84, Phe330, Ile439, His440, Tyr442	Trp84, Phe330, His440
	Clioquinol	-18.68±0.47	-	Val71, Trp84	Trp84

- The active site of AChE is a deep (20 Å) and narrow (5 Å) gorge composed of various domains, with the two most important sites for drug binding being the peripheral anionic site (PAS) and the catalytic active site (CAS).
- The CAS, retains two subsites: the catalytic anionic site responsible for stabilizing the binding of ACh in the CAS, and the catalytic esteratic site responsible for the hydrolyzation of ACh into acetic acid and choline
- Certain drugs inhibit AChE catalysis by binding the PAS to create a steric blockade that denies ligand passage to the active site
- Other drugs inhibit AChE catalysis by binding catalytic anionic and esteratic residues, ultimately occupying the CAS and preventing ACh binding.
- Polyphenols **1**, **2** and **4** displayed higher MM/GBSA binding free energies than **3** and **5**, which correlated with experimental results. Polyphenols **1**, **2** and **4** form a majority of their noncovalent hydrogen bonds, hydrophobic and π-π interactions with CAS anionic residues, as well as hydrophobic interactions with one PAS residue.
- Compound **1** is furthered anchored towards the top of the gorge by an upper mid gorge residue through hydrophobic and π-π interactions, whereas compound **2** is further anchored towards the top of the gorge by an upper mid gorge residue through hydrogen bonds, and compound **4** is further anchored towards the bottom of the gorge by lower mid gorge residues through hydrogen bonds and hydrophobic interactions.
- Compounds **3** and **5** only form hydrophobic and π-π interactions with CAS anionic residues, yielding no interactions with CAS esteratic or PAS residues, which can explain their low inhibition observed experimentally.

Conclusions

- **We have obtained relevant biological and pharmacokinetic information on a family of five ionophoric polyphenols that display interesting properties against AD-related factors.**
- **Our results using the *in vitro* parallel artificial membrane permeability assay (PAMPA-BBB), and *in silico* P-gp efflux models indicate that all five polyphenols can effectively penetrate the blood brain barrier without being recognized and/or expelled by the common P-gp transporter.**
- ***In vitro* experiments on inhibition of acetylcholinesterase (AChE) provide further evidence of the promising potential of the five ionophoric polyphenols, with compounds 1, 2 and 4 displaying IC_{50} values in the low micromolar range.**
- **Molecular docking and MD simulations correlate well with experimental results, and show that the polyphenols interact with residues in the catalytic active site of the enzyme in different ways as per their structural motifs, which explains their ability to inhibit the enzyme, and allows to draw preliminary structure-activity correlations.**

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